5-2024

# Loss of the SAC Components SldA Kinase or SldB or in vitro Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in Aspergillus fumigatus

Ashley Nywening
*University of Tennessee Health Science Center*

# Loss of the SAC Components SldA Kinase or SldB or in vitro Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in Aspergillus fumigatus


## Abstract

The opportunistic pathogen Aspergillus fumigatus represents the chief causative agent of human and animal invasive filamentous fungal infections. Triazoles, the primary therapeutic options to combat invasive aspergillosis (IA), target the fungal biosynthesis of ergosterol, a vital component of the fungal cell membrane. Unfortunately, resistance to this class of medical therapeutic has arisen globally and now threatens the future usefulness of these compounds for antifungal treatment. Infection with A. fumigatus that has acquired triazole resistance increases an already high associated mortality rate and reduces the limited arsenal of therapeutic options to combat IA. Moreover, how this specific fungal pathogen obtains resistance remains poorly understood. In this study, we show that loss of the previously uncharacterized A. fumigatus Spindle Assembly Checkpoint components SldA or SldB results in loss of susceptibility to multiple mold active medical triazoles. Furthermore, these mutants possess reduced susceptibility to antifungal compounds targeting ergosterol biosynthesis at points upstream of the triazole target Cyp51A. Loss of either component also results in signs of aneuploidy within uninucleate conidia in flow cytometry analyses for DNA content. Finally, exposure to voriconazole through laboratory experimental adaptation resulted in decreased triazole susceptibility, increased detection of sub populations of aneuploid spores in all samples of mutant as well as wild type lineages, and selection for a specific duplication in chromosome two in most lineages. Subsequent passages of adapted strains in drug-free media revealed adaptation to be unstable in most cases, with loss of adaptations typically occurring with simultaneous resolution of conidial aneuploidy, suggesting a correlation between aneuploidy and antifungal resistance in this pathogen. Together, these findings illustrate a previously unknown connection in A. fumigatus between defective function of the conserved surveillance system designed to prevent aberrant sorting of genomic material during nuclear division, and loss of susceptibility to antifungal compounds that target ergosterol biosynthesis. This knowledge helps to inform our understanding of how A. fumigatus survives and adapts to medical triazole antifungals.



## Document Type

Dissertation

## Degree Name

Doctor of Philosophy (PhD)

## Program

Biomedical Sciences

## Research Advisor

Jarrod R. Fortwendel, PhD

## Keywords

aneuploidy;antifungal susceptibility;Aspergillus fumigatus;ergosterol biosynthesis;spindle assembly checkpoint;triazole resistance

## Subject Categories

Medicine and Health Sciences

UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTORAL DISSERTATION

---

# Loss of the SAC Components SldA Kinase or SldB or *in vitro* Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in *Aspergillus fumigatus*

---

*Author:*
Ashley V. Nywening

*Advisor:*
Jarrod R. Fortwendel, PhD

*A Dissertation Presented for The Graduate Studies Council of*
*The University of Tennessee Health Science Center*
*in Partial Fulfillment of Requirements for the Doctor of Philosophy degree from*
*The University of Tennessee*

*in*

*Biomedical Sciences: Microbiology, Immunology and Biochemistry*
*College of Graduate Health Sciences*

*May 2024*

To my dogs.
I work so that you can have the best life.

# Acknowledgements

First I would like to express appreciation for my advisor, Jarrod Fortwendel, who convinced me that fungi were fascinating. I could not have found a more supportive and patient mentor. Thank you for the guidance and effort that you have put into helping me develop my research. Thank you for investing your time to help the students in your lab become better scientists.

Next, I would like to thank the members of the lab, both current and past, for contributing their expertise and for making the Fortwendel lab a creative, collaborative environment. Thank you to current lab members Adela Martin-Vicente, Xabier Guruceaga, Jinhong Xie, and Harrison I. Thorn. Also thanks goes out to past members Wenbo Ge, Qusai Al Abdallah, and Ana Camila Oliveira Souza. It has been wonderful to see how your mix of professionalism with friendship contributes to all individual members and individual research projects reaching their potential. You make the lab a great place to work.

Also, I am so grateful for the input by each of the members of my Graduate Committee, including Brian Peters, Glen Palmer, Kevin Fuller, Dave Rogers, and, of course, Jarrod. You all provided insightful thoughts and suggestions that improved my project.

I am also eternally grateful for all members of the Tennessee Fungal Pathogens Group (TFPG), and the Center for Pediatric Experimental Therapeutics (CPET). I have enjoyed participating in the bi-weekly SC@M meetings, having such great opportunities to hear about everyone's research at retreats, and generally being a part of such a welcoming and collaborative group of scientists. Thank you all.

Finally, I am grateful to have friends and family who are supportive and endure my eccentricities and occasional overenthusiastic and oft long-winded explanations of my work without zoning out completely. I appreciate it.

The work described herein was supported by the CPET and funding by the National Institutes of Health RO1Al158442, RO1Al143197, RO1Al139388, RO1Al142509, and RO73650198.

# Preface

The body chapters of this dissertation are organized in a way that first introduces readers to the topic and provides context for this project by providing a review of the literature and necessary background information that supports our rationale for choosing the research topic, objectives, and hypotheses. Next, the materials and methods used to interrogate specific questions are described, leading to a presentation of the experimental results. Analysis of these outcomes transitions to conclusions made based on our findings. Finally, the body ends with a discussion relating all research elements back to our final thoughts about the results and their significance.

For readers to have immediate access to the full presentation of our previously published literature review article, the final pre-print author submission of the article is reproduced in **Appendix A**. This mode of presentation allows for Chapter 2, which uses it as the basis, to incorporate a review of the literature and and expand further to provide background information relevant to the dissertation's larger goals and objectives. References in the chapters to relevant sections, tables, or figures in this appendix look like the following example. The chapter 2 callout to **Figure A.1** refers to **Figure 1** in **Appendix A**.

**NOTE ON PDF NAVIGATION:** Document navigation is greatly facilitated by using Adobe Acrobat "Previous view" and "Next view" functions. For "Previous view," use quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For "Next view," use Alt/Ctrl+Right Arrow on PC or Command+Right Arrow on Mac. Using these quick keys in tandem allows the reader to toggle between document locations. Since every scroll represents a new view, depending on how much scrolling is done for a specific view destination, more than one press of the back or forward arrows may be needed. For additional navigational tips, click View at the top of the PDF, then Page Navigation. These Adobe Acrobat utilities may not be functional for other PDF readers or for PDFs opened in web browsers.

# Abstract

Ashley V. Nywening

## *Loss of the SAC Components SldA Kinase or SldB or in vitro Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in Aspergillus fumigatus*

The opportunistic pathogen *Aspergillus fumigatus* represents the chief causative agent of human and animal invasive filamentous fungal infections. Triazoles, the primary therapeutic options to combat invasive aspergillosis (IA), target the fungal biosynthesis of ergosterol, a vital component of the fungal cell membrane. Unfortunately, resistance to this class of medical therapeutic has arisen globally and now threatens the future usefulness of these compounds for antifungal treatment. Infection with *A. fumigatus* that has acquired triazole resistance increases an already high associated mortality rate and reduces the already limited arsenal of therapeutic options to combat IA. Moreover, how this specific fungal pathogen obtains resistance remains poorly understood. In this study, we show that loss of the previously uncharacterized *A. fumigatus* Spindle Assembly Checkpoint components SldA or SldB results in loss of susceptibility to multiple mold active medical triazoles. Furthermore, these mutants possess reduced susceptibility to antifungal compounds targeting ergosterol biosynthesis at points upstream of the triazole target Cyp51A. Loss of either component also results in signs of aneuploidy within uninucleate conidia in flow cytometry analyses for DNA content. Finally, exposure to voriconazole through laboratory experimental adaptation resulted in decreased triazole susceptibility, increased detection of sub populations of aneuploid spores in all samples of mutant as well as *wild type* lineages, and selection for a specific duplication in chromosome two in most lineages. Subsequent passages of adapted strains in drug-free media revealed adaptation to be unstable in most cases, with loss of adaptations typically occurring with simultaneous resolution of conidial aneuploidy, suggesting a correlation between aneuploidy and antifungal resistance in this pathogen. Together, these findings illustrate a previously unknown connection in *A. fumigatus* between defective function of the conserved surveillance system designed to prevent aberrant sorting of genomic material during nuclear division, and loss of susceptibility to

antifungal compounds that target ergosterol biosynthesis. This knowledge helps to inform our understanding of how *A. fumigatus* survives and adapts to medical triazole antifungals.

# Contents

# List of Figures

# List of Abbreviations

| | |
|---|---|
| AAS | *Aspergillus* Sinusitis |
| ABPA | Allergic Bronchopulmonary Aspergillosis |
| AIA | *Aspergillus*-Induced Asthma |
| APC/C | Anaphase Promoting Complex/Cyclosome |
| CAR | Contractile Actomyosin Ring |
| CAS | Caspofungin |
| CENP-A | Centromeric Protein A |
| CFDA | 5,(6)-Carboxyfluorescein Diacetate |
| CFW | Calcofluor White |
| Ch. 5 | Chromosome 5 |
| Ch. 5L | Chromosome Five Left Arm |
| CIN | Chromosomal Instability |
| CLSI | Clinical and Laboratory Standards Institute |
| CN | Copy Number |
| CNA | Chronic Necrotizing Aspergillosis |
| CNPA | Chronic Necrotizing Pulmonary Aspergillosis |
| CNV | Copy Number Variation |
| CPA | Chronic Pulmonary Aspergillosis |
| CPC | Chromosomal Passenger Complex |
| CR | Congo Red |
| crRNAs | CRISPR RNAs |
| DDR | Dna Damage Response |
| DEG | Differently Expressed Genes |
| DI | DNA Index |
| DSB | Double Strand Break |
| EBI | Ergosterol Biosynthesis Inhibitors |
| EuPaGDT | Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool |
| FK | Fungal Keratitis |
| GMM | Glucose Minimal Medium |
| GMS | Gomori Methenamine Silver |
| GO | Gene Ontology |
| gRNA | Guide RNA |
| HygR | Hygromycin Resistance Cassette |

| | |
|---|---|
| IA | Invasive Aspergillosis |
| IFI | Invasive Fungal Infections |
| IPA | Invasive Pulmonary Aspergillosis |
| Ipl1 | Increased Ploidy Level 1 |
| i(5L) | Isochromosome 5 Left Arm |
| KT-MT | Kinetochore Microtubule |
| MCC | Mitotic Checkpoint Complex |
| MEC | Minimum Effective Concentration |
| MEN | Mitotic Exit Network |
| MFG | Micafungin |
| MIC | Minimum Inhibitory Concentration |
| MOI | Multiplicity Of Infection |
| MOPS | Morpholinepropanesulfonic Acid |
| MVA | Mosaic Variegated Aneuploidy |
| NDC1 | Nuclear Division Cycle 1 |
| ORF | Open Reading Frame |
| PAM | Protospacer Adjacent Motif |
| PCA | Primary Cutaneous Aspergillosis |
| PEG 3350 | Polyethylene Glycol 3350 |
| PhleoR | Phleomycin Resistance Cassette |
| PI | Propidium Iodide |
| PMA | Phorbol 12-Myristate 13-Acetate |
| PMF | Progressive Massive Fibrosis |
| qPCR | Quantitative PCR |
| RNP | Ribonucleoprotein |
| ROS | Reactive Oxygen Species |
| RPMI-1640 | Roswell Park Memorial Institute-1640 |
| RT | Room Temperature |
| RZZ | Rod/Zw10/Zwilch complex |
| SAC | Spindle Assembly Checkpoint |
| SIN | Septation Initiation Network |
| SMM | Sorbose Minimal Medium |
| SNC | Supernumerary Chromosome |
| SPB | Spindle Pole Body |
| SSB | Single Strand Break |
| TA | Triamcinolone Acetonide |
| WGS | Whole Genome Sequencing |
| WHO FPPL | World Health Organization Fungal Priority Pathogens List |
| YPD | Yeast Peptone Dextrose |

# Chapter 1

# Introduction

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Ctrl/Alt+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command+Right Arrow, respectively. See the Preface for further details.

## 1.1 *Aspergillus fumigatus* Is a Ubiquitous Fungus and an Opportunistic Pathogen

*Aspergillus fumigatus* is a saprophytic fungus and an important human and animal pathogen. This prevalent species of filamentous fungus, also referred to as a mold, represents a common environmental organism. It can be found often growing on surfaces as roughly circular colonies, blue-green in color with a white or cream border. However, within materials like soil or decomposing wood, *A. fumigatus* grows as often imperceptible networks of mycelia distributed throughout the organic matrix (Morelli, Kerkaert, and Cramer, 2021). Microscopic analysis reveals these colonies to be composed of branching filaments known as hyphae, which form as the result of repeated cycles of cell division that are not followed by total separation of daughter cells. Instead, each new cellular compartment remains attached to the previous growth, thus forming long tubular filaments of compartments. Each compartment is partly divided from the next by a structure called a septum. This structure contains pores through which cytoplasm and even organelles may pass, but which can become sealed when necessary to protect sections of healthy compartments by preventing the perpetuation of damage from compromised sections (J P Latge and Chamilos, 2019; Thorn et al., 2024). *A. fumigatus* cells are also characterized by the presence of multiple nuclei within each compartment (Morelli, Kerkaert, and Cramer, 2021). Like other *Aspergillus* species, *A. fumigatus* primarily reproduces and propagates by producing millions of microscopic spores called conidia, each of which typically are packaged with the genomic content of a single nucleus (Adams, Wieser, and J. H. Yu, 1998; Deak et al., 2011). These structures are minuscule (often only 2-3$\mu$m), highly stress tolerant, and exhibit long-term viability (J. P. Latge, 1999; Lamarre et al., 2008). *Aspergillus* species conidia are perfectly

suited to detaching from conidiophores when disturbed and dispersing through air currents (Levetin, 2004). Conidia or hyphal material can also be spread by natural forces or as a result of human and animal activity that dislocate contaminated water, soil, vegetation, or other materials (J. P. Latge, 1999; Dagenais and Keller, 2009). When these structures settle, they can remain dormant or germinate dependent on the external conditions. *A. fumigatus* can tolerate a wide range of temperatures and utilize a variety of organic materials for food (Andersen et al., 2011; Paulussen et al., 2017; Ren et al., 2017; J. Zhang et al., 2017). Within the environment, *A. fumigatus* plays an important role for recycling carbon and nitrogen (J. P. Latge, 1999). In addition to absorbing nutrients available through the natural decomposition of materials like vegetation or deceased animals, the mold can secrete digestive enzymes when necessary to further break down organic debris, thus releasing nutrients for its own use, but also returning essential materials back into the soil (De Vroey, 1979; J P Latge and Chamilos, 2019; Horta et al., 2022). These factors combine to make *A. fumigatus* a ubiquitous organism, often found within both indoor and outdoor spaces and on every continent worldwide except Antarctica (Andersen et al., 2011; Paulussen et al., 2017).

Due to the prevalence of *A. fumigatus*, the average person will breathe in about 300 spores of this mold species alone every day (J. P. Latge, 1999; Escobar et al., 2016; J P Latge and Chamilos, 2019). Owing to their minuscule size, these conidia can not only adhere to the lung epithelium, but can become lodged within the alveoli of the mammalian lung (D. W. Denning, Park, et al., 2011; Richard et al., 2018). Fortunately, this common low-level exposure poses low risk to most healthy human and animal populations. Immunocompetent hosts can clear the spores through phagocytosis by resident alveolar innate immune cells or expel the spores encased in mucus by coughing/sneezing (J. P. Latge, 1999; Dagenais and Keller, 2009; Paulussen et al., 2017; Richard et al., 2018). Unfortunately, spores which are not cleared from the lung tissue due to host immune dysfunction, defects in mucociliary clearance, or myriad other causes may become problematic and can lead to disease (Pihet et al., 2009; Warkentien et al., 2012; Webb et al., 2018; Mead et al., 2023; Sardeshmukh et al., 2023). Aspergillosis is the the disease state which can result from *Aspergillus* species invasion into a susceptible patient's system.

Aspergillosis is a condition characterized by a spectrum of severity ranging from relatively minor allergic symptoms to life-threatening disseminated infections. Many conditions may predispose an individual to some form of aspergillosis. However, some forms of aspergillosis, (often more minor involvements such as such as skin and toenail infections), can occur without apparent predisposing conditions, especially following a breakdown of protective barriers (Avkan-Oguz et al., 2020; Bowyer, Bromley, and D W Denning, 2020; M. E. Brown et al., 2021). In this example, *A. fumigatus* persists within a specific niche of an immunocompetent individual, evading clearance by immune cells and subsisting primarily from the breakdown of keratin. Forms of aspergillosis including occurrences of primary cutaneous aspergillosis (PCA) and fungal keratitis can also occur in immunocompetent individuals usually following major or minor injuries or surgery

(Avkan-Oguz et al., 2020; Bowyer, Bromley, and D W Denning, 2020; Meng et al., 2024). In these instances, *Aspergillus* causes an infection in an area such as skin or eyes after some abrasion or cut creates a breach through which fungal elements may pass. *A. fumigatus* represents one of the most common causative agents of fungal keratitis (FK), a disease which impacts one to two million individuals annually and unfortunately contributes to occurrences of patient blindness when the site of infection impacts the eye (L. Brown et al., 2021; Kamath et al., 2023; Meng et al., 2024). FK is most common in agricultural settings where individuals experience simultaneous abrasion and fungal inoculation from plant matter, but can occur associated with any pastime or incident that results in eye injuries such as from flying mud, metal particles, or sawdust which become embedded in an insufficiently guarded eye (Rosa, Miller, and Alfonso, 1994; Bharathi et al., 2007). FK can also occur in diabetics and those using topical or systemic corticosteroids (Rosa, Miller, and Alfonso, 1994). FK even occurs resulting simply from contact lens wear, especially due to use of incorrectly formulated contact lens solution (Rosa, Miller, and Alfonso, 1994; Cheung, Nagra, and Hammersmith, 2016; M. E. Brown et al., 2021; Kamath et al., 2023). FK commonly causes symptoms like ocular pain, photosensitivity, and vision loss due to physical damage to the cornea and surrounding structures. This damage can be caused by penetrating fungal growth and release of collagen-hydrolyzing enzymes, but is also partly due to immune-mediated inflammation which can itself cause further tissue necrosis, scarring, and resulting opacity of the cornea and the area surrounding the cornea (Ansari, Miller, and Galor, 2013; M. E. Brown et al., 2021). Around 40% of FK occurrences result in the need for replacement of the damaged cornea. However, about 10% of FK infections result in damage which requires the entire eye to be removed, resulting in the loss of between 84,000 and 115,000 eyes every year globally (L. Brown et al., 2021; Kamath et al., 2023). As with severe invasive forms of disease, FK is difficult to successfully treat and causes a burden to public health reducing quality of life and costing millions of dollars every year in the U.S. alone (Collier et al., 2014; M. E. Brown et al., 2021; Ting et al., 2021; Kamath et al., 2023).

Other forms of aspergillosis relating to hypersensitivity/sensitization (atopy) to *Aspergillus* antigens can also occur in patients who have a functional immune system, especially in conjunction with conditions such as asthma or cystic fibrosis. While colonizing the lung or airways,*A. fumigatus* can release dozens of allergenic compounds which can cause continual issues and worsen symptoms for asthma and CF patients (Amitani et al., 1995; J. P. Latge, 2001; M. J. Seidler, Salvenmoser, and Muller, 2008; Bowyer, Bromley, and D W Denning, 2020; Oda et al., 2021). These may develop conditions like allergic bronchopulmonary aspergillosis (ABPA), *Aspergillus* sinusitis (AAS), *Aspergillus* bronchitis, or *Aspergillus*-induced asthma (AIA) (Zmeili and Soubani, 2007; A. Shah and Panjabi, 2014; Kosmidis and D W Denning, 2015; Goh et al., 2017). Occurrences of chronic or allergic forms of aspergillosis, such as chronic pulmonary aspergillosis (CPA), are generally less immediately life-threatening than invasive disease, but occur more frequently and can complicate or exacerbate preexisting issues. When tested, around 28% of all asthma patients

test positive for hypersensitivity to *Aspergillus* antigens, and around 13% of asthma patients develop ABPA (Agarwal, 2009; A. Shah and Panjabi, 2014). Studies have found that ABPA occurs in up to 6% of the CF patient population (Zmeili and Soubani, 2007). Individuals with mild to moderate immune dysfunction or who have damaged or dysfunctional lungs may also be subject to persistent and/or recurring *Aspergillus* infections like chronic necrotizing aspergillosis/chronic necrotizing pulmonary aspergillosis (CNA or CNPA) (Zmeili and Soubani, 2007; Blanco et al., 2011; Barac et al., 2017). For example, progressive massive fibrosis (PMF), a state of lung damage which can result from factors like prolonged and repeated exposures to silica dust (resulting in a condition known as silicosis), predisposes patients to CNPA (Blanco et al., 2011; Barac et al., 2017).

While the incidence of allergic or chronic forms of aspergillosis is higher and certainly poses a risk to patients, invasive infections are generally more problematic and life-threatening (Kosmidis and D W Denning, 2015; Richard et al., 2018). *A. fumigatus* is the leading cause globally of invasive filamentous fungal infections within susceptible human and animal populations (Seyedmousavi et al., 2015; Badali et al., 2022). Invasive aspergillosis (IA) is generally considered the most severe form of the disease and is associated with an unacceptably high mortality rate. Approximately 14,000 cases of IA occur each year in the United States alone, costing somewhere between 600 million and 1.2 billion dollars in patient care annually (Benedict et al., 2019; Zilberberg et al., 2019; Bradley et al., 2022). The incidence of IA has increased in recent history due in part to advances in medical care which have extended the lifetimes of susceptible patient populations, such as those with innate or acquired immune suppression (Groll, P. M. Shah, et al., 1996; J. P. Latge, 1999; Zmeili and Soubani, 2007; Zilberberg et al., 2019). For example, in the U.S., diagnosed cases of IA rose from about 36,000 in 2004 to North of 51,000 in 2013 with the associated costs for patient care rising to over 592 million dollars in 2013 (Zilberberg et al., 2019).

Patient groups which have been identified as at increased risk for IA include those combating hematological malignancies, leukemia or other forms of cancer, those receiving immunosuppressive therapy to prevent rejection following bone marrow, stem cell, tissue, or whole organ transplant, and patients with HIV/AIDS. Additionally, patients can become susceptible to invasive skin, subcutaneous, and pulmonary infections due to steroid use, uncontrolled diabetes, prior tuberculosis or sarcoidosis, or where there is other prior damage to tissues (J. P. Latge, 1999; Patterson, Kirkpatrick, et al., 2000; Dagenais and Keller, 2009; G. D. Brown et al., 2012; Kosmidis and D W Denning, 2015; J M Rybak, J R Fortwendel, and Rogers, 2019; Giacobbe et al., 2020; Cadena, Thompson 3rd, and Patterson, 2021; Badali et al., 2022). In one study, IA occurred in 30% of patients with acute myelogenous leukemia–myelodysplastic syndrome (AML-MDS) (Slobbe et al., 2008). IA occurs in between 26% and 45% of all patients with chronic granulomatous disease (CGD) (King, Henriet, and Warris, 2016). As indicated, patients with CF are also at increased risk of disease due to defects in mucociliary clearance of inhaled conidia (Pihet et al., 2009; Muller, M. Seidler, and Beauvais, 2011). (Moreover, *Aspergilli* may produce toxins and

other compounds which further impede the activity of cilia, as well as causing further damage or otherwise impeding the removal of spores from the lungs) (Amitani et al., 1995; J. P. Latge, 2001; M. J. Seidler, Salvenmoser, and Muller, 2008). Cases of IA also occur in patients with bacterial or viral respiratory tract infections, including the flu and COVID-19. Viral infections can weaken immunity and allow for secondary bacterial or fungal infections to take hold (Verweij, Bruggemann, et al., 2020; Koehler et al., 2021; Oda et al., 2021; Alshrefy et al., 2022; Badali et al., 2022; Mead et al., 2023). Moreover, the widespread use of mechanical ventilation and catheterization of patients admitted to the ICU with COVID-19 was shown to increase the likelihood for such bacterial or fungal secondary infections to occur, and in the case of IA, increased the associated mortality (Alshrefy et al., 2022; Boyd et al., 2022; Rouze et al., 2022). Use of steroids and other medications in patient treatment also likely exacerbated IA involvements (Boyd et al., 2022). Estimated rates of pneumonia associated with ventilator use in patients with COVID-19 ranged from around 7.6% of intubated patients to reaching 87%, with an average incidence of around 30 to 45% (Ippolito et al., 2021; Boyd et al., 2022; Fumagalli et al., 2022). Among this group, up to 30% develop IA (Boyd et al., 2022). The base mortality rate of patients with COVID-19 who developed ventilator-associated pneumonia was estimated at 42.7% (Ippolito et al., 2021; Boyd et al., 2022). While it is difficult to ascertain the mortality when IA was involved, several studies illustrate that rates of observed mortality rose significantly in these patients in step with rates of IA occurrences (Boyd et al., 2022; Fumagalli et al., 2022).

Even otherwise healthy individuals can succumb to severe infection without a clear predisposing incident, though this likely results from abnormally high or constant exposures to fungal spores (J. P. Latge, 1999; Kosmidis and D W Denning, 2015). Of course, regardless of an individual's prior health status, invasive *Aspergillus* infections can result in essentially any region of the body exposed as a result of traumatic injury. Like other invasive fungal infections (IFI), invasive aspergillosis can impact previously healthy individuals following forms of penetrating trauma like punctures, fractures, and burns sustained from common accidents such as automotive accidents, occurring among soldiers as a result of combat, or affecting both first responders and members of the general populace following natural (or man-made) disasters (Kowacs et al., 2004; M. Zhang et al., 2010; Warkentien et al., 2012; Giacobbe et al., 2020; Sardeshmukh et al., 2023). IA can occur following other forms of trauma, as IA has even occurred in (and ultimately contributed to the death of) individuals who managed to barely survive drowning (Kowacs et al., 2004; Yamawaki et al., 2016; Koide et al., 2020).

IA represents the most severe form of infection and is associated with an unacceptably high rate of mortality even when diagnosed in a timely manner. The associated mortality falls between 30 and 95% fatality dependent on the patient population in question (even with recommended application of antifungal therapeutics) (Osawa et al., 2007; Dagenais and Keller, 2009; D. W. Denning, Park, et al., 2011; Mayr and Lass-Florl, 2011; Maertens et al., 2016; Pianalto and Alspaugh, 2016; Benedict et al., 2019). Without treatment, IA is

typically fatal, with mortality in untreated individuals ranging from 80% to nearly 100% for certain patient groups (Maertens et al., 2016; Bowyer, Bromley, and D W Denning, 2020; A. V. Nywening et al., 2021). Approximately 200,000 cases of IA occur each year worldwide, of which *A. fumigatus* is the causative agent in over 60% of cases (Steinbach et al., 2012; J M Rybak, J R Fortwendel, and Rogers, 2019). IA often begins within the lung where conidia germinate, invade the lung epithelium, and expand as hyphal filaments through the lung tissue obtaining nutrients released from damaged cells. The mold growth destroys arterioles along the way and causes localized lack of blood flow (ischemia), areas of blood coagulation/clotting (thrombosis), and tissue death (necrosis) (Richard et al., 2018). Studies find that the cause of death related to IA is often respiratory failure due to loss of lung function (Garcia-Vidal et al., 2015). However, *A. fumigatus* can spread (disseminate) from the initial site of invasion to other regions of the body including the liver, kidneys, spleen or central nervous system by invasion through tissues and bone or through the vasculature (Groll and Walsh, 2001; Linden, Warris, et al., 2009; Richard et al., 2018). Unfortunately, partly because symptoms of aspergillosis can be mistaken for other diseases and physicians are often uninformed and unaware of the potential for this disease to occur among susceptible patients, this is also an under-diagnosed disease (Blanco et al., 2011; Oda et al., 2021). IA has been found as one of the chief causes which likely contributed to patient death among susceptible patients, but was not discovered and diagnosed until before the patient died; often only revealed during autopsy (Winters et al., 2012; Howard, Pasqualotto, et al., 2013; Tejerina et al., 2019; Mudrakola et al., 2022). Moreover, even where a physician is aware of the threat of aspergillosis, clear diagnosis of a patient infection can be difficult, leading to wide variations in the timing and application of patient therapies (X. Liu et al., 2011; Tejerina et al., 2019; Mudrakola et al., 2022).

## 1.2 Therapy Options to Combat Aspergillosis

Of the limited number of approved therapeutics, the triazoles remain the primary option recommended to combat *Aspergillus* infections, with voriconazole representing the primary drug of choice for invasive disease (Patterson, Thompson 3rd, et al., 2016). Only two other classes of antifungal are currently available as alternative or combinatorial treatments. From the polyene class, only the drug Amphotericin B is approved for treating invasive disease but is often avoided due to known nephrotoxicity and other intravenous infusion-related drug toxicity resulting from its use (Laniado-Laborin and Cabrales-Vargas, 2009). The only other antifungal class available are the echinocandins. However, while tolerated much better than Amphotericin B, these compounds have fungistatic rather than fungicidal activity against species of *Aspergilli* and failure of echinocandin prophylaxis occurs frequently with some reports of breakthrough infection rates as high as 28% (Bowman et al., 2002; Lionakis, Lewis, and Kontoyiannis, 2018; Aruanno, Glampedakis, and Lamoth, 2019). The triazole antifungals however are fungicidal against *A. fumigatus*, can be administered orally rather than intravenously and can be given for extended periods with lower treatment-associated

adverse events (Ullmann et al., 2007; Patterson, Thompson 3rd, et al., 2016; J M Rybak, J R Fortwendel, and Rogers, 2019). In fact, extended triazole therapy, either for prophylaxis to prevent infection or in response to chronic involvements, occurs often (Cornely et al., 2007; Ullmann et al., 2007; Blanco et al., 2011). Unfortunately, extended periods of patient treatment with triazole antifungals do come with one grave concern: the *in vivo* adaptation of *A. fumigatus* due to repeated triazole exposure and consequent development of azole resistance (Hodiamont et al., 2009; Burgel et al., 2012).

## 1.3 The Problem of Triazole Antifungal Resistance

The unacceptable cost of IA to public health is increased by the involvement of a triazole-resistant strain. Whether acquired from an environmental source or developed within a patient's own system as a consequence of therapy, infection with *A. fumigatus* which has adapted to be resistant to triazole therapy increases the expected risk of patient mortality (Qiao, W. Liu, and R. Li, 2008; Lestrade et al., 2019; Bradley et al., 2022). Unfortunately, since the first recorded incidence of resistant infection in 1997, encounters with triazole-resistant *Aspergillus fumigatus* have increased to an alarming level (Paassen et al., 2016; Rivero-Menendez et al., 2016). Recently, in an effort to bring much-needed attention to an underprioritized and understudied category of human pathogens, the WHO released the fungal priority pathogens list (WHO FPPL), a report on the 19 fungi that represent the greatest threats to public health (Fisher and D W Denning, 2023). *A. fumigatus* was one of only four species placed in the Critical category, the highest priority tier of the list, alongside *Cryptococcus neoformans*, *Candida auris*, and *Candida albicans* (Parums, 2022). This report highlights the increasing risk that invasive *Aspergillus fumigatus* infections, especially those exhibiting antifungal resistance, pose to susceptible populations worldwide in an effort to promote clinician awareness, endorse the development of new treatment options, and to stimulate research in the area of antifungal resistance.

While efforts continue to bring light to this problem, the strategies and mechanisms which enable triazole resistance in *A. fumigatus* remain poorly understood. In many cases, none of the usual confirmed or suspected underlying factors are detected within an apparently azole-resistant *A. fumigatus* isolate and the mechanism(s) which are providing resistance remains mysterious. This work will summarize the content of three articles related to my student research project. The first article, a review of triazole resistance according to the literature available at the time of its release, provides a reasonably detailed summary of the issue of triazole resistance in this species, as well as the prominent mechanisms that have been confirmed or suspected to support triazole resistance. The second work provides detailed methods and results from our construction and screening of a collection of *A. fumigatus* mutants genetically altered by CRISPR/Cas9 gene editing to lack the function of individual predicted kinase enzymes. This article revealed how the loss of previously unstudied *A. fumigatus* protein kinases or their regulators impacted

susceptibility to the echinocandin class of medical antifungals. The third manuscript is a reproduction of the most current draft of a prepared-for-publication article which contains the bulk of the aims, methods, experimental strategies, results, and conclusions from my own student research project.

# Chapter 2

# Mechanisms of Triazole Resistance in *Aspergillus fumigatus*[1]

**NOTE:** This chapter refers frequently to content in **Appendix A**. When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See Preface for further details.

## 2.1 Introduction

This chapter provides a brief summary of the information collected within the review article "Mechanisms of Triazole Resistance in *Aspergillus fumigatus*" (**Appendix A**) (Nywening et al., 2020). This previously published article provides an overview of the issue of triazole resistance in this species and provides context for the objectives and hypotheses which comprised my own student research project. In addition to outlining the main points from this article, this chapter will also include updated information on the issue of triazole resistant *A. fumigatus*. The chapter will also touch on known and suspected contributors to the appearance of resistance to triazole antifungals in other fungal pathogens. Many such strategies may be similarly employed by *A. fumigatus* to subvert triazole activity which have simply not yet been clearly documented and confirmed to impact antifungal efficacy in this species. In this brief overview, we will primarily focus on resistance that is relevant to our hypotheses, the interpretation of our results, and discussion of the understudied phenomenon of antifungal resistance in *Aspergillus fumigatus* in the following chapters.

[1]Published article used with open access permission. A. V. Nywening et al. (Dec. 2020). "Mechanisms of triazole resistance in Aspergillus fumigatus". In: *Environ Microbiol* 22.12, pp. 4934–4952. ISSN: 1462-2912 (Print) 1462-2912. DOI: 10.1111/1462-2920.15274. (**Appendix A**).

## 2.2 Article Summary

*Aspergillus fumigatus* is a common environmental saprophytic mold, but is also an opportunistic human pathogen and the primary causative agent of invasive human and animal filamentous fungal infections. This species spreads in environments primarily through the aerial dissemination of minuscule asexual spores known as conidia. These reproductive structures can travel widely through air currents or through water, soil, or contaminated materials. Once deposited, the conidia can germinate in a wide range of conditions and subsist on various forms of organic matter. As *A. fumigatus* is prevalent in both outdoor and indoor spaces, (often referred to as a ubiquitous organism), human exposures are commonplace and cannot be easily avoided. The average human is exposed to hundreds of *A. fumigatus* spores every day. However, healthy individuals typically have low risk for developing severe disease states following such typical low-level exposures to *A. fumigatus* spores, which are usually cleared by the innate immune system. Unfortunately, if these spores remain in the mammalian lung, they may germinate, grow, and solidify an infection, or result in an allergic/hypersensitivity-related reaction. Involvement with an *Aspergillus* species can lead to aspergillosis, a disease state which can appear with a spectrum of severity ranging from milder allergic symptoms to severe invasive infections, especially when disseminated to multiple organs or tissues. Individuals may be at risk for potentially life-threatening forms of aspergillosis if they possess any of a wide variety of predisposing conditions ranging from severe immunodeficiency syndromes to cancer. The global rise of aspergillosis cases over the last few decades is largely due to medical advances which improved the lifespan of susceptible patient populations. Conditions which can predispose individuals to infection include acquired or inherited immunodeficiency, lung diseases (including viral infections such as the flu or COVID-19), asthma, cystic fibrosis, extended use of steroids, following surgery, and many other conditions. Even patients with no apparent predisposing condition may become diseased with continual or sufficiently high exposure to conidia. However, even low-level exposures can be problematic for individuals with preexisting lung conditions such as asthma.

Chronic involvements involving the lung are more common than severe invasive infection and often require prolonged antifungal therapy applications, sometimes lasting years. Invasive aspergillosis (IA) is less common than allergic or chronic involvements, but represents the most serious form of the disease and unfortunately is associated with high costs of care and high mortality (Slobbe et al., 2008). IA can progress rapidly in certain patient types, in some cases becoming problematic within weeks or days of the initial exposure or predisposing incident (Benet et al., 2013; Min et al., 2017; Sardeshmukh et al., 2023). Severity varies based on individual patient-specific and strain-specific factors, including the underlying disease or condition of the patient, the speed of diagnosis and type of antifungal therapy applied, and characteristics unique to the strain. Depending on these factors, IA carries an expected mortality ranging from about 30 to 90%, even with application of current antifungal therapy. Without therapeutic intervention, IA is typically

fatal. Aspergillosis is also not a reportable disease in the United States currently. Partly due to the lack of information and understanding of the risks of aspergillosis, combined with often vague and non-specific presentations of disease across patients, this disease has a high rate of missed diagnosis and accurate approximations of the prevalence of infections and the burden on public health remain elusive. One study of autopsy records found that aspergillosis was one of the four most common missed diagnoses which likely caused or contributed to the death of the patient, but remained undiagnosed until after the patient's demise (Winters et al., 2012; Webb et al., 2018). However, based on retrospective studies, the incidence of IA is likely around 46 cases per million individuals. (More current estimates place the annual rate at around 14,000 cases in the United States each year, with an associated cost of care between 600 million and 1.2 billion dollars (Zilberberg et al., 2019; Bradley et al., 2022)). Moreover, while many cases appear sporadically in individual patients, outbreaks of disease have occurred, usually associated with circumstances where a population of predisposed individuals are collected together, such as in a hospital or nursing facility, juxtaposed with some event or circumstance which causes dispersal of spores. Nosocomial infections have been linked to contaminated airflow in indoor ventilation or construction near the facility.

Therapeutic options to combat invasive infections are limited, with the triazole class of antifungals representing the frontline options for antifungal therapy. Triazoles are fungicidal against *Aspergillus* species. The primary antifungal action of these compounds involves binding to and inhibiting a key enzyme in the pathway of synthesis of ergosterol, analogous to the cholesterol found in mammals. Inhibition of this enzyme, known as 14-$\alpha$ sterol demethylase and encoded by the genes *cyp51A* and *cyp51B* in *A. fumigatus*, (also known as ERG11 in many species of fungi), prevents the correct production of ergosterol, the key membrane sterol of fungi (Rivero-Menendez et al., 2016; Basenko et al., 2018). This results in cell membrane dynamics becoming destabilized and fungal cell lysis. In other fungal species the action of triazoles is also suspected to contribute to cell death by causing a backup in the biosynthesis pathway resulting in production and accumulation of alternative toxic sterol compounds (J. M. Rybak et al., 2017). The triazoles are currently preferred for primary therapeutics due to comparable efficacy but lessened associated risks of negative events (D. W. Denning, Radford, et al., 1997; Verweij, Chowdhary, et al., 2016). Only two alternative classes of antifungal are currently approved to treat aspergillosis: the polyenes and echinocandins. From the polyene class, only the drug amphotericin B is currently approved for use. Lipid formulations of the drug are better tolerated and reduce the associated toxicity. The echinocandins, including caspofungin, are another option. (However, failure of echinocandins to prevent the advancement of aspergillosis occurs frequently, with estimates of breakthrough infection in up to to 28% of cases (Bowman et al., 2002; Lionakis, Lewis, and Kontoyiannis, 2018; Aruanno, Glampedakis, and Lamoth, 2019)). Combination therapy with several antifungals simultaneously is also utilized and certain involvements may require surgery to remove infected tissues (Walsh et al., 2008; Hodiamont et al., 2009; Bellete et al., 2010; Patterson, Thompson 3rd, et al., 2016).

Moreover, certain patients require extended treatment or prophylactic antifungal therapy, for which the primary drugs of choice are also the triazoles (D. W. Denning, Park, et al., 2011). Posaconazole is often applied for at-risk individuals to prevent fungal infections and can be given for months or even years successfully with low occurrence of toxicity (D. W. Denning, Riniotis, et al., 2003; Hodiamont et al., 2009). Unfortunately, triazole antifungal therapy, especially when applied for extended times, carries the risk of promoting antifungal resistant infection to emerge. The rise of antifungal resistance now threatens the future usefulness of the limited arsenal of currently available antifungals, as well as increasing associated costs of care and, most importantly, placing patients at higher risk of mortality (Howard, Cerar, et al., 2009; Falcone et al., 2011; Linden, S. M. Camps, et al., 2013; Lestrade et al., 2019).

The global insurgence of *A. fumigatus* which displays resistance to the medical triazole antifungals is regrettable. While the true impact of triazole resistance on treatment outcomes is not fully appreciated, it is known that involvement of a resistant strain can complicate patient treatment and increase the associated mortality for IA (Falcone et al., 2011; Lestrade et al., 2019). Triazole resistance in this species is now recognized as a global problem. However, development of triazole resistance in *A. fumigatus* remains poorly understood. Of the mechanisms which are currently known or suspected to contribute to triazole resistance in *A. fumigatus*, the majority involve alterations to the expression of gene transcript for the enzymes targeted by these antifungals, (encoded by the genes *cyp51A* and *cyp51B* in this species), or a mutation which affects the ability of the compounds to associate with the enzyme, both of which reduce the effectiveness of the drug (Chowdhary, Sharma, Boom, et al., 2014; Lazzarini et al., 2016; Badali et al., 2022). Alterations which impact the enzyme Cyp51A are predominantly blamed for resistance, with the influence of transcriptional or sequential Cyp51B changes less certain (Lazzarini et al., 2016; J M Rybak, J R Fortwendel, and Rogers, 2019). Resistance development is generally known to develop through two distinct routes. Because triazoles are utilized for control of fungal growth in agriculture and industrial applications, *A. fumigatus* can become cross-resistant to medical antifungals following exposures in an environmental setting. A predisposed individual may then encounter such a strain and develop an infection which is already non-susceptible to one or more medical triazoles. This route is generally accepted as the most prominent source for triazole resistant patient infections. The other route of resistance development is known as *de novo* resistance development, in which a patient infection becomes resistant following exposure to medical antifungals within a patient (Snelders, Huis In 't Veld, et al., 2009; Verweij, Kema, et al., 2013; Hagiwara, Takahashi, et al., 2014; Verweij, Chowdhary, et al., 2016). *Aspergillus* which is not killed in the course of treatment can adapt to become resilient to future triazole encounters, eventually developing into a truly resistant strain (Bellete et al., 2010). Clinical cases of triazole resistant aspergillosis, both *de novo* and environmentally acquired, remain a threat to multiple patient populations, with incidence of recorded resistance in certain patient groups rising to 26% of confirmed IA cases and associated mortality of 100% (Paassen et al., 2016). Overall estimates place

the incidence of triazole resistance among cases of IA currently at around 7 to 8% (Lestrade et al., 2019; Resendiz-Sharpe, Merckx, et al., 2021; Resendiz-Sharpe, Dewaele, et al., 2021). Unfortunately, just as the incidence of aspergillosis is likely underestimated, the frequency of triazole-resistant aspergillosis is also very likely underappreciated, especially in the United States where in addition to the lack of requirement for physicians to report suspected cases of aspergillosis, there is a lack of regular testing of patient isolates to determine antifungal susceptibility (Krishnan Natesan et al., 2012). Moreover, many cases of suspected IA produce only negative patient cultures, precluding efforts to detect drug susceptibility, and many infections occur in regions that cannot be easily sampled (such as cerebral aspergilloma), though they can be detected and diagnosed through radiological (CT) scans or PCR-based or antibody assay from samples of blood. (Slobbe et al., 2008; D. W. Denning, Park, et al., 2011; Barac et al., 2017; Bongomin et al., 2020).

The current literature maintains that triazole resistant *A. fumigatus* infections are primarily acquired through exposures to environmental strains which have become resistant by adapting to agricultural fungicides (Linden, S. M. Camps, et al., 2013). Due to the similar chemical nature and mode of action, agricultural and industrially applied fungicides can drive acquisition of cross-resistance to medically utilized triazoles (Groll and Walsh, 2001; Snelders, Huis In 't Veld, et al., 2009; Ren et al., 2017; J. Zhang et al., 2017; Buil et al., 2019). *A. fumigatus* is prevalent in agricultural settings subsisting on dying and dead vegetation or other decomposing organic materials. When demethylase-inhibiting antifungal compounds are applied to crops or naturally-derived construction materials, *A. fumigatus* which happens to be there becomes exposed to these fungicides. Like other environmental saprobes, *A. fumigatus* posesses an impressive ability to adapt to various forms of stress, such as from fluctuating temperatures, weather conditions, and the competition for nutrients and secretion of stress-inducing compounds from other microorganisms. Such stimuli can drive both temporary and permanent changes to the intracellular events and even the genome to allow for adaptation (Groll and Walsh, 2001; Sniegowski and Gerrish, 2010; Yona, Frumkin, and Pilpel, 2015; Buil et al., 2019). Many cases of triazole-resistant infection instead develop within the patient as a result of repeated therapeutic application of triazole antifungal (Howard, Cerar, et al., 2009; S M Camps et al., 2012). *De novo* resistance can occur rather quickly, beginning within months of the first triazole application (D. W. Denning, Riniotis, et al., 2003; S M Camps et al., 2012). The current list of mechanisms which are known or suspected to contribute to triazole resistance in *A. fumigatus* develop similarly regardless of the route of acquisition and largely involve changes to the triazole target which reduce the effectiveness of the drug or alterations that remove the drug from the fungal cells (Howard, Webster, et al., 2006; Linden, S. M. Camps, et al., 2013; Chowdhary, Sharma, Kathuria, et al., 2015; Wiederhold et al., 2016) (**Table A.1**).

Changes which are known to allow *A. fumigatus* to tolerate the activity of both agricultural and medical demethylase inhibitors usually include either transient or genomically-fixed, mutation-based changes that impact the expression of *cyp51A*, mutations to *cyp51A*

that result in an enzyme with lessened capacity for the drug to bind and inhibit the enzyme, changes to the pathway of ergosterol biosynthesis at a point upstream of the Cyp51A and -B enzymes (Hmg1), and changes to the expression of protein pumps which actively remove the drugs from within the fungal cell. Among changes which impact expression of *cyp51A*, one which has been identified as a clinically-relevant resistance mechanism is a mutation to the CCAAT-DNA binding complex (CBC) subunit HapE, resulting in a substitution mutation at the amino acid P88. When the proline at position 88 is replaced by leucine, the HAP complex posesses a reduced ability to suppress the expression of the *cyp51A* gene. Consequently, the antagonistic transcription factor SrbA is able to drive expression of the gene (Fabio Gsaller et al., 2016) (**Figure A.1C**). Another example of *cyp51A* overexpression occurs when a portion of the promoter region upstream of *cyp51A* is duplicated. Promoter region changes are detected commonly in both environmental and clinical triazole resistant isolates and usually occur in one of two forms: TR34, or TR46 (**Figure A.1B**). The TR34 alteration is nearly invariably accompanied by a mutation of L98H. The leucine at position 98 of the *cyp51A* sequence resides near the first substrate recognition site of the enzyme. This change results in a structural alteration that reduces the capacity for triazoles to bind. TR34/L98H is known to confer pan-azole resistance; reducing susceptibility to voriconazole, itraconazole, posaconazole, and experimental triazoles like ravuconazole, as well as to various demethylase-inhibiting agricultural/industrial fungicide compounds. The TR34/L98H mutation is one of the mechanisms most commonly identified in resistant patient infections, (suggested to be the most common mechanism that develops in both environmental and clinical isolates globally), and has been associated with treatment failure and increased mortality (Mellado, Garcia-Effron, Alcazar-Fuoli, Melchers, et al., 2007; Verweij, Mellado, and Melchers, 2007; Snelders, Karawajczyk, et al., 2010; Lockhart et al., 2011; Chowdhary, Kathuria, et al., 2012; Bader et al., 2013; Pham et al., 2014; Abdolrasouli et al., 2015; Chowdhary, Sharma, Kathuria, et al., 2015; Fuhren et al., 2015; Fabio Gsaller et al., 2016; F. Gsaller et al., 2016; Wiederhold et al., 2016). Moreover, TR34/L98H mutations do not appear to come with any defect in fitness or virulence, (meaning these are not as likely to be selected against and removed from the population even once the stress of the drug subsides) (Mavridou et al., 2013; H. C. Wang et al., 2018). The TR46 is invariably joined by several other mutations; a substitution Y121F and T289A. The tyrosine amino acid at position 121 is expected to hydrogen-bond with the heme group within the Cyp51A enzyme. Researchers expect the Y121F influences triazole binding affinity more than the T289A mutation (Snelders, S. M. Camps, et al., 2015) (**Figure A.2A**). Patients with this mutation-based mechanism also suffer high rates of treatment failure and mortality (Linden, S. M. Camps, et al., 2013). This mechanism is also one of the most common to develop in the environment, and also occurs in the context of *in vitro* adaptation to agricultural triazoles (Linden, S. M. Camps, et al., 2013; Ren et al., 2017). This mutation reduces susceptibility to voriconazole, itraconazole, and posaconazole (Linden, S. M. Camps, et al., 2013).

Another commonly detected resistance-conferring mutation occurs at G54 of the Cyp51A sequence. This mutation alone is sufficient to impact susceptibility to itraconazole

and posaconazole (Diaz-Guerra et al., 2003; Mann et al., 2003; Mellado, Garcia-Effron, Alcazar-Fuoli, Melchers, et al., 2007; Pfaller et al., 2008; Snelders, Karawajczyk, et al., 2010; D. W. Denning, Park, et al., 2011; Krishnan Natesan et al., 2012; Bader et al., 2013; Chowdhary, Sharma, Kathuria, et al., 2015). The Glycine at position 54 appears to directly interact with the triazole antifungal compounds, situated within the substrate access channel (Diaz-Guerra et al., 2003; Xiao et al., 2004; Snelders, Karawajczyk, et al., 2010; J M Rybak, J R Fortwendel, and Rogers, 2019) (**Figure A.2B**). Several mutations of G54 have been identified to date, including substitutions with arginine (R) or glutamate (E), both of which have been reported to increase posaconazole MIC values when introduced into a previously susceptible strain to 30-fold the original level, while replacement with a tryptophan (W) results in a 250-fold increase in MIC to posaconazole. These mutations also impact itraconazole susceptibility, but apparrently not voriconazole susceptibility (Manavathu et al., 2000; Diaz-Guerra et al., 2003; Mann et al., 2003; Nascimento et al., 2003; Xiao et al., 2004; Pfaller et al., 2008; Tashiro and Izumikawa, 2016).

A Cyp51A G138 mutation has also been reported in clinically resistant *A. fumigatus* (Manavathu et al., 2000; Xiao et al., 2004; Howard, Webster, et al., 2006; Snelders, Karawajczyk, et al., 2010; D. W. Denning, Park, et al., 2011). Isolates with this mutation tend to be resistant to voriconazole and itraconazole, but not to posaconazole (Manavathu et al., 2000; Xiao et al., 2004; J M Rybak, J R Fortwendel, and Rogers, 2019). The amino acid G138 is also situated within the ligand access channel of Cyp51A and substitutions with arginine (R) likely disrupt the binding of triazoles like voriconazole with the enzyme, while the side chains of posaconazole might allow for better associations (Xiao et al., 2004; J M Rybak, J R Fortwendel, and Rogers, 2019). Another common mutation, M220, is associated with resistance to itraconazole, as well as lessened voriconazole and posaconazole susceptibility (Diaz-Guerra et al., 2003; Mellado, Garcia-Effron, Alcazar-Fuoli, Cuenca-Estrella, et al., 2004; J. Chen et al., 2005; Mellado, Garcia-Effron, Alcazar-Fuoli, Melchers, et al., 2007; Pfaller et al., 2008; Rodriguez-Tudela et al., 2008; Snelders, Karawajczyk, et al., 2010). Again, this mutation appears to develop *in vivo* during extended patient therapy, with the specific mutations M220T and M220K occurring in patient infection (Howard, Pasqualotto, et al., 2013). This mechanism causes reduced itraconazole, voriconazole, and posaconazole susceptibility.

The last Cyp51A mutation-based mechanism which was discussed in **Appendix A** is G448S. This mutation confers voriconazole and itraconazole resistance, but does not provide resistance to posaconazole (Manavathu et al., 2000; Mellado, Garcia-Effron, Alcazar-Fuoli, Cuenca-Estrella, et al., 2004; Xiao et al., 2004; Pfaller et al., 2008; Howard, Cerar, et al., 2009; Bellete et al., 2010; Krishnan Natesan et al., 2012; Fuhren et al., 2015). Again, G448 is situated near the heme cofactor in the Cyp51A enzyme. This mutation also develops *in vivo* during extended patient triazole antifungal therapy (Manavathu et al., 2000; Xiao et al., 2004; Howard, Cerar, et al., 2009; Bellete et al., 2010; Krishnan Natesan et al., 2012).

Other changes besides these have been suggested as potential sources of altered triazole susceptibility (Hokken et al., 2023). Often, apparrently triazole resistant isolates do

not possess any alterations in the Cyp51A enzyme (Fraczek et al., 2013; Verweij, Chowdhary, et al., 2016; Wiederhold et al., 2016; Buil et al., 2019). For many isolates, the cause of resistance remains unknown. However, multiple non-Cyp51A-related mechanisms have been identified as contributing to triazole resistance. These usually involve a combination of changes to gene expression such as overexpression of drug efflux pumps, or enhanced adaptability due to biochemical or genome changes (Bellete et al., 2010; S M Camps et al., 2012; Fabio Gsaller et al., 2016; F. Gsaller et al., 2016). While the **Appendix A** article did not attempt a comprehensive list of all changes which were confirmed or strongly suspected to influence triazole susceptibility, a few key suspects were reviewed.

Mutations which impact the function of Hmg-CoA reductase, the rate-limiting enzyme in the pathway of ergosterol biosynthesis upstream of Cyp51A activity, are also found in triazole-resistant isolates (Hagiwara, Arai, et al., 2018; Buil et al., 2019; J M Rybak, J R Fortwendel, and Rogers, 2019). Mutations which result in changes to amino acid at residue 269 of Hmg1 in *A. fumigatus*, such as S269F, might impact the function of the sterol-sensing domain and are found among patient isolates (Hagiwara, Arai, et al., 2018). Other mutations that affect the sterol sensing domain are also found which when introduced into a previously susceptible isolate increase the voriconazole MIC by at least four fold, including the substitution mutations S305P and I412S or deletion of F262 (J M Rybak, J R Fortwendel, and Rogers, 2019).

Increased export of triazole compounds from the cell also allows fungi like *A. fumigatus* to withstand exposures to triazole antifungals (D. W. Denning, Radford, et al., 1997; Slaven et al., 2002; Nascimento et al., 2003; Ferreira et al., 2005; Fraczek et al., 2013). Overexpression of drug efflux pumps that actively remove the drug from fungal cells is blamed for reducing susceptibility to triazole antifungals (S M Camps et al., 2012; Fabio Gsaller et al., 2016; Dudakova et al., 2017; J M Rybak, Ge, et al., 2019; Hokken et al., 2023). *A. fumigatus* often upregulates putative efflux transporter genes in triazole contexts, including those from the multidrug resistance (MDR) major facilitator superfamily (MFS) and ATP-binding cassette (ABC) varieties. However, very few of these have yet been convincingly shown to impart triazole resistance in a clinical setting (Ferreira et al., 2005; Fraczek et al., 2013; Paul, Diekema, and Moye-Rowley, 2013). Overexpression of the cdr1B/abcC gene has been detected in clinical isolates that showed resistance to voriconazole, itraconazole, and/or posaconazole (Fraczek et al., 2013). Deletion of *cdr1B* causes an isolate to show increased triazole susceptibility, indicating that this efflux mechanism does regulate triazole susceptibility to some degree (Fraczek et al., 2013; Paul, Diekema, and Moye-Rowley, 2013; Hagiwara, A. Watanabe, et al., 2016). Other studies have shown that isolates constitutively overexpressing the ABC transporter encoding gene *atrF* posses resistance (Slaven et al., 2002; Nascimento et al., 2003; Fraczek et al., 2013; H. C. Wang et al., 2018). Several predicted MDR transporters are also implicated, including orthologs of Mdr1 and Mdr2 in *A. fumigatus*. Exposure to itraconazole was found to result in increased expression of *mdr1* by about fourteen-fold, while deletion of *mdr1* in a *wild type* background results in increased

itraconazole susceptibility (Fraczek et al., 2013). Changes in expression of *mdr3* and *mdr4* are also suggested to influence triazole susceptibility, though evidence supporting their importance for resistance in either laboratory mutants or clinical isolates is currently lacking (Nascimento et al., 2003).

Further cited causes which may influence the susceptibility of *A. fumigatus* isolates to the azoles include such strategies as an increased capacity for formation of a biofilm, a hydrophobic extracellular matrix which could prevent the drug from reaching areas of fungal growth(Jabra-Rizk, Falkler, and Meiller, 2004; Muller, M. Seidler, and Beauvais, 2011; Long et al., 2018; Morelli, Kerkaert, and Cramer, 2021). Like other species of fungi, *A. fumigatus* often secretes components to form a thick, protective extracellular matrix (ECM) (Muller, M. Seidler, and Beauvais, 2011). Enhanced biofilm formation is associated with increased fungal survival in the context of triazole exposure, especially including *Candida* species like *C. albicans* and *C. dubliniensis* (Ramage et al., 2001; Jabra-Rizk, Falkler, and Meiller, 2004). *In vitro* growth of *A. fumigatus* shows that samples which form biofilms are less susceptible to various antifungals than samples which have not (Muller, M. Seidler, and Beauvais, 2011). Only indirect evidence has shown biofilm formation to influence the progression of disease or the success of antifungal therapy within the context of *A. fumigatus* patient infections (M. J. Seidler, Salvenmoser, and Muller, 2008; Muller, M. Seidler, and Beauvais, 2011). ECM formation is associated with persistent and chronic aspergillosis involvements, especially in patients with asthma or cystic fibrosis (M. J. Seidler, Salvenmoser, and Muller, 2008; Muller, M. Seidler, and Beauvais, 2011).

A link between calcium signalling and mitochondrial function with triazole resistance is also described (Bowyer, Bromley, and D W Denning, 2020). Reports of calcineurin-mediated triazole resistance in other species are relatively common (Edlind et al., 2002; X. M. Jia et al., 2009; S. Liu et al., 2015). Fungal mutants which lack any of a number of components which support intracellular calcium signalling can result in heightened susceptibility to triazoles (S. Liu et al., 2015). Studies have shown that intracellular signalling involving the second messenger calcium, and specifically involving the Ca2+/calmodulin (CaM) regulated serine/threonine protein phosphatase calcineurin (also known as protein phosphatase 2B (PP2B)), support triazole stress responses in multiple fungal species (Juvvadi, F. Lamoth, and Steinbach, 2014; S. Liu et al., 2015). The activity of calcineurin/PP2B is required for growth and survival within a mammalian host, adaptation to varied forms of stress, and is required for resistance to triazoles (Juvvadi, F. Lamoth, and Steinbach, 2014; S. Liu et al., 2015; Juvvadi, S. C. Lee, et al., 2017). Calcineurin's primary target for dephosphorylation is the transcription factors PRZ1 and CRZ1 orthologs. Calcineurin-mediated PRZ1/CRZ1 signaling then induces the expression of target genes (S. Liu et al., 2015). Compounds which target calcineurin may show promise as novel antifungal therapy.

Often none of these suspected mechanisms are detected in a strain which appears phenotypically resistant to triazole. In such cases, the mechanism behind the change in drug susceptibility is yet unknown. Cases of resistance for which the causative mechanism

of resistance remains uncertain point to the need for future research to uncover strategies whereby this important human pathogen can circumvent triazole activity. Until resistance in *A. fumigatus* is better understood, the future usefulness of the triazole class of medical antifungals to combat aspergillosis is in jeopardy.

Several fascinating developments in the field of mycology were which shed more light on the issue of triazole resistance in *A. fumigatus* were published after the release of this article. In recent years, studies on triazole resistance in other pathogenic fungi such as *Candida* and *Cryptococcus* species, points to changes in the content of chromosomes as a clinically-relevant mechanism of triazole resistance acquisition (Selmecki, Forche, and Berman, 2006; A. M. Selmecki et al., 2009; Brimacombe et al., 2019; Handelman and Osherov, 2022). Aneuploidy, a form of genetic instability often resulting from chromosomal instability, is a state of abnormal genetic content packaged within a nucleus that consists of either the loss or gain of additional whole chromosomes or of large segments of chromosomes (Selmecki, Forche, and Berman, 2010; Bolanos-Garcia and Blundell, 2011; Garribba and Santaguida, 2022). Chromosomal instability (CIN) is defined as a continual increased predisposition to/occurrence of chromosomal missegregation that often results in the gains or losses of whole or partial chromosomes in daughter nuclei (Thompson, Bakhoum, and Compton, 2010). Aneuploidy provides copy number variation (CNV), which in turn impacts the dosage and thus expression of the affected genes, which consequently impacts the prevalence of certain gene products (Anderson et al., 2015; X. Fan et al., 2023). Depending on the chromosome(s) involved, aneuploidy can provide resistance to echinocandins, triazoles, or forms of genotoxic stress (Kline-Smith, Sandall, and Desai, 2005; G. Chen et al., 2012).

Until relatively recently, the literature maintained that unlike pathogenic yeast species, filamentous fungi including *A. fumigatus* do not appear to employ aneuploidy as a strategy for stress adaptation. However, aneuploidies have now been associated with clinically relevant loss of triazole susceptibility in *A. fumigatus*, and with resistance developed in *A. flavus* (Barda et al., 2023; Khateb et al., 2023).

In 2023, an article confirmed that aneuploidy was present within clinical isolates of *A. fumigatus* which were resistant to triazole antifungals (Khateb et al., 2023). The study evaluated sixteen triazole resistant isolates collected from patients with aspergillosis, but for which the collected isolates did not have mutations in the *cyp51A* gene. Aneuploidy was identified within samples from two patients with chronic pulmonary aspergillosis (CPA) and from one patient with a cerebral aspergilloma. Though no single aneuploidy was held in common between the separate patients, the aneuploidies all involved gains of chromosomal material through duplications in certain regions. The researchers confirmed that one isolate possessing an aneuploidy that involved the region of chromosome four that contains the *cyp51A* gene produces biologically relevant overexpression of *cyp51A*.

The capacity for filamentous fungal pathogens such as *A. fumigatus* to utilize aneuploidy as a strategy to withstand antifungal stress remains poorly studied. Moreover,

as in yeast, whatever mechanisms allow *A. fumigatus* to produce chromosomal instability during triazole stress remain unclear (Polakova et al., 2009; Selmecki, Forche, and Berman, 2010). Researchers propose that that fungal pathogens likely have a directed strategy to promote the generation of aneuploidies in response to certain kinds of stress as a means of adaptation. Some have suggested that inducible changes to cell cycle pathway regulation may be a source for chromosomal imbalance (Kline-Smith, Sandall, and Desai, 2005; Selmecki, Forche, and Berman, 2010; Hill et al., 2013; Harrison et al., 2014; Brimacombe et al., 2019). For example, altered expression or phosphoregulation of cell cycle regulating proteins can result in the accumulation of resistance-conferring aneuploidies (Stemmann et al., 2002; Davies and Kaplan, 2010; G. Chen et al., 2012; Pennisi, Ascenzi, and Masi, 2015; Brimacombe et al., 2019).

## 2.3 Conclusion

A key factor limiting our understanding of how *A. fumigatus* responds to antifungals remains the insufficient study of the *A. fumigatus* genome. Many assumptions regarding intracellular pathways are based on studies of yeast-form fungi or of model species of filamentous fungi. *A. fumigatus* is predicted to encode many yet uncharacterized regulators of intracellular pathways such as kinases and phosphatases, some of which may play crucial roles to support survival in the context of antifungal stress. Unfortunately, many genes in *A. fumigatus* remain completely unstudied and the contribution of such yet uncharacterized fungal proteins to biological processes such as antifungal stress responses remains unknown. Studies to characterize *A. fumigatus* genes and identify antifungal stress phenotypes may uncover promising targets for future standalone or combinatorial therapies.

# Chapter 3

# Loss of Septation Initiation Network (SIN) Kinases Blocks Tissue Invasion and Unlocks Echinocandin Cidal Activity Against *Aspergillus fumigatus*[1]

**NOTE:** This chapter refers frequently to content in **Appendix B**. When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See Preface for further details.

## 3.1 Introduction

This chapter provides a brief summary of information collected within the article "Loss of Septation Initiation Network (SIN) kinases blocks tissue invasion and unlocks echinocandin cidal activity against *Aspergillus fumigatus*" (**Appendix B**) (A. V. Nywening et al., 2021). This previously published article provides context for the work presented in later chapters of this ETD. The publication included descriptions of experimental protocols and methods which mirror many of the same approaches employed in the current work. The rationale and detailed accounts of the materials and methods required have been made clear and are already accessible to the public. Moreover, this published article describes in detail the construction of a collection of single-gene disruption strains made in the *wild type A. fumigatus* strain CEA10/A1163. This library of kinase-disruption strains was the same collection which contained the *sldA* gene disruption strain, which was later identified as having a reduced triazole susceptibility phenotype. This discovery prompted further analysis which is described later in **Chapter 4**. Therefore, this previous study reported the discoveries and experiments which both overlapped with and prepared the way for my

[1] Published article used with open access permission. A. C. O. Souza* A. Martin-Vicente* (*Co-first authors) A. V. Nywening et al. (2021). "Loss of Septation Initiation Network (SIN) kinases blocks tissue invasion and unlocks echinocandin cidal activity against Aspergillus fumigatus". In: *PLoS Pathog* 17.8, e1009806. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1009806. URL: https://www.ncbi.nlm.nih.gov/pubmed/34370772. (**Appendix B**).

own student research project. In this brief summary, I will primarily focus on information and results from the publication which are relevant to my own student research including hypotheses, the interpretation of results, and discussion of the understudied phenomenon of antifungal resistance in *Aspergillus fumigatus* contained within the following chapters.

## 3.2 Article Summary

The options available to treat invasive aspergillosis infections are restricted currently to three classes of antifungal. The echinocandins are an option. These include caspofungin, micafungin and anidulafungin, which are generally effective and well tolerated. Echinocandins inhibit the activity of the β-1,3-glucan synthase enzyme, encoded by the gene*fksA* in *A. fumigatus*. This enzyme is vital for producing cell wall component glucan. The echinocandins are fungicidal toward *Candida* species, but against *A. fumigatus*, echinocandins produce only fungistatic activity; acting to inhibit the emergence and progression of fungal growth (Bowman et al., 2002; Aruanno, Glampedakis, and Lamoth, 2019). The action of echinocandins causes compartments in hyphae to lyse, especially at the apical tip cells. However, the presence of septa in *A. fumigatus*, which can be plugged to prevent the spread of damage to neighboring compartments, protects regions of filaments within the fungal growth from being killed (J P Latge and Chamilos, 2019; Thorn et al., 2024).

Echinocandins are often used as salvage therapy for invasive aspergillosis, where the primary antifungal therapy is failing. These are also used for prophylaxis to prevent the emergence of infections in predisposed individuals. However, their use is limited due to their fungistatic rather than fungicidal activity against this mold pathogen. Moreover, *A. fumigatus* has been seen to adapt to echinocandin stress. Some accounts have reported the incidence of breakthrough infections during echinocandin therapy as high as 28% (Lionakis, Lewis, and Kontoyiannis, 2018). Moreover, some research reports that breakthrough infections occurred more frequently when echinocandins were used for prophylaxis instead of triazoles (Gomes et al., 2014).

Recent work indicates that changes to phosphoregulation which regulate intracellular activities occur in *A. fumigatus* in response to echinocandin exposure. This indicates that in *A. fumigatus*, the physiological changes that allow for survival in the context of echinocandin stress depend on the actions of protein kinases and phosphatases for coordination of responses (Juvvadi, Cole, et al., 2018; Manfiolli et al., 2019; Chelius et al., 2020; Mattos, Palmisano, and Goldman, 2020; Mattos, L P Silva, et al., 2020; L. P. Silva et al., 2020). The processes which enable adaptation to echinocandin also appear to involve changes through kinase and phosphatase activity. Therefore, novel antifungals might be made which alter the pathways of phosphoregulation to disrupt the capacity for fungi to overcome echinocandin stress (Dos Reis et al., 2023). However, at the onset of this article, the majority of genes in the *A. fumigatus* genome predicted to encode protein kinases remained uncharacterized. We set out to create a collection of mutants in a *wild type* background strain of *A. fumigatus* which

each have an individual gene disruption which is designed to eliminate the function of the encoded putative kinase protein. Genes predicted to encode orthologs of known protein kinases of *A. nidulans* were first identified via BLAST search of the A1163 (CEA10) genome using the database FingiDB (De Souza et al., 2013; Basenko et al., 2018). Our search yielded 148 putative protein kinase encoding genes. We designed homology repair templates and CRISPR RNA sequences specific to each gene to cause a disruptive insertion within the coding sequence, and ensured low homology to other regions of the genome to reduce the possibility for off-target DSB. Using a miniaturized version of our modified *in vitro* CRISPR-Cas9 system, which allows for smaller reaction volumes and can be performed in a 96-well plate, we attempted a minimum of three individual transformation experiments for each gene (Al Abdallah, W. Ge, and J. R. Fortwendel, 2017; Abdallah et al., 2018) (**Figure B.1**). We succeeded in producing a library of 118 mutants for which loss of the gene function produced a viable strain and proceeded to phenotypically characterize each one in terms of growth on media and susceptibility to various forms of cell wall stress. We were especially interested to discover whether any of our collection exhibited altered susceptibility to various antifungal compounds.

Seven mutants exhibited a defect in capacity for vegetative growth on agarose media, showing growth reduced by 50% or more (**Figure B.2A**). Among these was the mitogen-activated kinase MAPK, encoded by the gene *mpkA* (AFUB_070630), and a kinase that works upstream of it, MAPKK, encoded by *mkkA* (AFUB_006190). These both operate in a pathway which regulates cell wall integrity, and loss of either of these has previously been shown to cause compact colony growth. Deletion of another gene encoding the kinase MAPKKK, *bck1* (AFUB_038060), produced a milder reduction of colony growth than the two aforementioned kinases (**Figure B.2B**). Other disruption mutants which exhibited reduced growth included a disruption in the catalytic subunit of Protein Kinase A, encoded by *pkaC1* (AFUB_027890), and disruption of the PAK kinase encoded by *cla4* (AFUB_053440) (**Figure B.2A**). This result was not suprising, as both of these had been shown previously to be important for vegetative growth in *A. fumigatus*. However, this led credence to the usefulness of our library for correctly identifying phenotypes associated with loss of protein kinase function. Other mutants that showed distinct growth defects included an ortholog of a kinase that regulates the actin cytoskeleton encoded by *prk1* (AFUB_006320) and an ortholog of the eukaryotic LAMMER kinase encoded by *lkh1* (AFUB_016170), which were previously uncharacterized. Eighteen additional mutants exhibited vegetative growth that was decreased mildly or moderately (between 10% and 50% (**Figure B.2B**). Thirteen mutants showed a defect in conidiation indicative of impaired asexual differentiation (**Figure B.2C**). Out of these, several had been previously studied and shown to be required for conidiation.

Screening for alterations in susceptibility to forms of cell wall stress using the agents calcofluor white and congo red revealed seven mutants which exhibited increased susceptibility to both CFW and CR. These two compounds inhibit cell wall synthesis by interacting with chitin chains and preventing their linkage to glucan. An additional seven mutants

were hypersusceptible only to CR (**Figure B.3A and B**). Among these were, again, the MAP kinase genes *mpkA* and *mkkA*. However, again, *bck1* did not show a clear impact on CFW or CR susceptibility. This result was unexpected as it conflicts with prior indications that loss of the Bck1 kinase should likely impact vegetative growth and susceptibility to cell wall stressors. We concluded that the possibility existed that our *bck1* mutant was able to produce a partly functional product.

Analysis for mutants with altered susceptibility to CFW or CR also revealed protein kinases which exhibited resistance upon loss of kinase function, rather than hypersusceptibility. Among these were *pkaC1*, which showed reduced CFW susceptibility, and the gene mutants of *kfsA*, *cmkA*, and *stk22*, which showed reduced susceptibility to CR (**Figure B.3C**).

To evaluate whether any library mutants showed altered susceptibility to cell wall damage from echinocandin stress, we assayed each mutant to determine its susceptibility to caspofungin by conventional broth microdilution MEC assays. Our assays revealed forty four mutants which exhibited a reduction in MEC by one dilution, indicating an increase in caspofungin susceptibility by 2-fold upon loss of the kinase function. Screening revealed five mutants for which the minimum effective concentration (MEC) was decreased by at least two dilutions, indicating a four-fold increase in the susceptibility level of the mutants to the echinocandin. The *mpkA* and *mkk2* mutants were both hypersusceptible to caspofungin, along with *pkaC1*, each of which, again, support cell wall integrity. The *mpkA* and *pkaC1* genes are previously known to be necessary for stress responses to echinocandin antifungal in *A. fumigatus*. Among the other mutants which show heightened susceptibility to caspofungin, two had predicted involvement as members of a three-kinase signaling cascade in the previously uncharacterized *A. fumigatus* Septation Initiation Network (SIN) (**Figure B.4A**). The SIN pathway was previously uncharacterized in *A. fumigatus*, but in other species is known to regulate the formation of septa, which, again, are important for protecting hyphal filaments from potentiation of cell wall damage during echinocandin stress. These two mutants possessed a disruption of *sidB* (AFUB_095460) or of *sepL* (AFUB_050750). (Note that here I have fixed an error in the original article, which reported the gene ID for *sepL* as "AFUB_05070". The actual, full gene ID in A1163/CEA10 according to FungiDB is "AFUB_050750") (Basenko et al., 2018). The SidB and SepL mutants showed heightened susceptibility to CFW, CR, and caspofungin. For another mutant for which the putative gene product is also involved in this pathway, *sepH*, the initial results appeared to indicate that the strain did not share the phenotypes of the SepL and SidB mutants. SepH is one of the two SteK-class proteins regulating the SIN pathway as a tripartite signalling cascade, along with SepL. (SidB is instead an AGC-class kinase). This was suprising, as SepH is expected to act directly upstream in the cascade from SepL and SidB (**Figure B.4A**). However, further analysis of the mutant revealed that disruption attempts had produced a mutant which could express a partly functioning SepH protein. In order to study the impact loss of this kinase has in *A. fumigatus*, A whole-gene deletion strain was constructed and utilized for this study. Indeed, the resulting deletion strain, which we referred to as

Δ*sepH*, phenocopied the SepL and SidB mutants in regard to both colony size defect and sensitivity to cell wall stressors (**Figure B.4B and C**).

In the fungus *N. crassa*, loss of the ortholog two of these proteins results in the formation of aseptate hyphae. Analysis of our *A. fumigatus sepH* deletion, *sepL*-1, and *sidB*-1 mutants revealed that loss of any one of these genes completely eliminated formation of septa (**Figure B.4D**). However, the morphology and positioning of nuclei within the hyphae appeared grossly normal, indicating that loss of these genes (and loss of septation) does not impact the positioning and production of nuclei. This is not suprising, as in *A. fumigatus*, nuclear division and cytokinesis, (which in this species is not followed by complete separation of daughter cells, but instead cells remain attached and are simply separated by a septum), are linked, but are not directly coupled. Each cellular compartment within *A. fumigatus* hyphae is typically multinucleate. Nuclear division can occur successfully multiple times before the cell partitions off into separate compartments.

Complementation of the *wild type sepL* sequence by re-integration into the native locus repaired the SIN pathway defect and restored septa formation (**Figure B.4D**). Simultaneously, the *wild type* growth and cell wall susceptibility phenotype was also restored in the sepL complement (**Figure B.4B** and **C**).

In the study described in **Appendix B**, echinocandin stress was found to result in a loss of viability of the hyphae for all three mutants (**Figure B.5**) and (**Figure B.6**). Rather than the typical fungistatic activity which echinocandins normally elicit in *A. fumigatus*, for these mutants, the echinocandins had become fungicidal, producing a zone of clearance in both caspofungin and micafungin E-test assays (**Figure B.5A** and **B**). (For the Δ*sepH* mutant, a few microcolonies were seen growing within this zone of clearance, which will be discussed more momentarily). To quantify the viability of these SIN kinase mutants in the presence of echinocandin, we used a fluorescence-based assay that stains only metabolically active (and thus alive) hyphal compartments. Samples of each strain were grown with or without o.5 μg/ml micafungin for 12 hours. Then, CFDA staining was performed followed by microscopy and counts of viable vs. inviable hyphae. Results showed a significant increase in death (CFDA negativity) of SIN kinase mutant hyphae/germlings compared to the *wild type* (**Figure B.5E**). Moreover, while the *wild type* exhibited colonies in which certain sections were damaged/lysed (**Figure B.5E** black dotted lines), but other sections remained viable, for the CFDA negative SIN mutants, an "all or nothing" phenotype existed. Either the entirety of the hyphal filaments for the germling were alive, or the entire filament and each of its branches were dead (**Figure B.5E**) black arrow). When the SIN mutants were incubated with micafungin for 24 hours instead of 12, the cultures were devoid of CFDA positivity while the CEA10 parental strain remained largely viable (**Figure B.5D**).

To test for differences in echinocandin susceptibility of mature hyphae (rather than using conidium as inoculum and allowing to grow in the presence of drug from the beginning), we cultured conidia from each strain to maturity before adding micafungin

(again, 0.5 µg/ml) to the medium. Hyphal damage was then visualized by using propidium iodide (PI), which only enters cells which have become permeable due to damage. Following micafungin dosage, each SIN kinase mutant exhibited a higher uptake of PI into the hyphae than was observed for the *wild type* (**Figure B.6B**). Again, the regions of the *wild type* colonies which did show stain uptake were often separated from branches of hyphae within the same colony which continued to exclude the stain (**Figure B.6B** white arrowheads), whereas the SIN mutants tended to show an "all or nothing" staining pattern.

The increased susceptibility of the strains to forms of cell wall stress was likely due to loss of the septa. These barriers normally allow hyphal compartments to be sealed off from one another when necessary by plugging the pores with woronin bodies (J P Latge and Chamilos, 2019; Thorn et al., 2024). (In fact, later work performed in our lab as part of another study again found that abrogation of septa formation through loss of SIN regulators causes the damage of echinocandins to spread throughout the filament and cause rampant cell damage and lysis) (Thorn et al., 2024).

To determine whether intact SIN kinase signalling/septation is required to allow for *A. fumigatus* to establish an infection, experiments evaluating the capacity of these mutants to cause tissue invasion into a host lung, (which is often a characteristic of the beginning stages of invasive pulmonary aspergillosis) were performed. Two separate murine models of immunosupression were used; dosage with triamcinolone acetonide alone (the corticosteroid model), or with both cyclophosphamide and triamcinolone acetonide (the chemotherapeutic model). In both models, mice were intranasally inoculated with $10^5$ total conidia of a specified strain, or received only saline control inocula. The mutants were found to lack the capacity for meaningful invasion into host tissues (**Figure B.7**) and correspondingly show a profound reduction in virulence in a mouse model of infection (**Figure B.8**). While the *wild type* began to exhibit mortality at day +4 of the experiment, with 100% mortality by around day +7 in the chemotherapeutic model and 60% by day +15 in the corticosteroid model (**Figure B.7A** and **B**), the *sepH* deletion and *sidB*-1 mutants exhibited avirulence in both models of infection, and the *sepL* mutant exhibited only 50% mortality, and only in the chemotherapeutic model. (The *sepL* complimented strain exhibited virulence similar to the *wild type*). Moreover, in a mouse model of infection with micafungin therapy, loss of the *sepL* gene resulted in improved survival of echinocandin-treated mice (**Figure B.10**). These results indicated that in *A. fumigatus*, an intact SIN kinase cascade, and intact septa formation, is required for survival in the context of echinocandin exposure, and is also required for *A. fumigatus* invasion into the lung epithelium of a mammalian host. To examine the impact loss of these genes has on the capacity of *A. fumigatus* to invade host lung epithelium, we analyzed silver-stained tissue samples from each group. By day +4, the *wild type* was seen forming deeply invasive hyphae within the tissue (**Figure B.8**). In contrast, the SIN kinase mutants only formed small masses of hyphae within the open airways, at the surface of the epithelium but lacking meaningful invasion into the tissue. Together, these results indicated that the *A. fumigatus* SIN pathway is essential for virulence

and invasive growth within the lung. To quantify the degree of fitness of each strain within the murine lungs, qPCR-based assays for fungal burden were performed. These assays revealed that the Δ*sepH* mutant and the *sidB*-1 mutant show significantly reduced levels of fungal-specific DNA compared to the *wild type* (**Figure B.9A**). The *sepL*-1 mutant showed burden which was reduced, but to a level that did not reach significance in the experiment. Moreover, an ELISA-based assay to measure levels of the pro-inflammatory cytokines IL-1β and TNFα within the lung tissues revealed that mice inoculated with conidia from any of the SIN kinase mutants exhibited significantly lower levels of inflammatory cytokine compared to the *wild type* (**Figure B.9B** and **C**). Tests to determine whether this difference were actually due to a lessened fitness of the mutants indicated that, when incubated for 16 hours with THP-1 cells, each mutant caused less IL-1β release than CEA10 (as measured through ELISA) (**Figure B.9D**). These findings suggest that SIN kinase signaling is required for *A. fumigatus* to cause host inflammatory response.

To determine whether the fungicidal phenotype shown *in vitro* upon loss of function of any of these SIN kinases translates *in vivo* to allow for clearance of fungi from the lung in response to echinocandin therapy, we employed our murine model of invasive aspergillosis with the addition of echinocandin therapy. As the Δ*sepH* and the *sidB*-1 mutants were completely avirulent in prior assays, we only utilized our *sepL*-1 mutant for this experiment, and only employed the chemotherapeutic model of immunosupression (in which *sepL*-1 produced 50% mortality). In the *wild type* group without echinocandin treatment, 100% mortality was seen by day +14, while by day +14 the *sepL*-1 infected groups exhibited 75% mortality (**Figure B.10A**). With addition of micafungin therapy at a dose of 1.0 mg/kg per day, mortality dropped in the *sepL*-1 infected groups, but not to a level which was deemed significantly different from the untreated *sepL*-1 infected group, but was significantly less than CEA10 treatment or control. (This lack of significance was likely due partly to the already low virulence of the *sepL*-1 strain). In an assay to determine whether loss of *sepL* function plus the addition of echinocandin treatment results in further reduction of fungal burden within the lung, all samples of both CEA10 and *sepL*-1 were positive for fungal DNA at day +4. However, 75% of lung cultures from CEA10-infection group with micafungin treatment remained positive for fungal DNA at day +4 (**Figure B.10B**), while explants from the micafungin treated *sepL*-1 group did not produce positive cultures. This data indicates that loss of septation may enable echinocandin therapy to cause clearance of *A. fumigatus* from lung tissues within a mammalian host. These findings were exciting, as they implied that development of compounds that inhibit the SIN/septa formation show potential for allowing echinocandin therapy to clear rather than only limit *Aspergillus* infections. Moreover, echinocandin prophylaxis combined with an SIN kinase inhibitor might improve the success of prophylaxis.

To further explore previously unstudied effectors of the SIN signalling pathway, we also produced mutants which lacked the function of putative regulators of the SIN kinase cascade. These mutants included the gene predicted to encode *A. fumigatus* ortholog of

alpha actinin, encoded by *acnA*, which is essential for septation in *A. nidulans*. In *A. nidulans*, *acnA* results in a complete loss of the contractile ring assembly. We also produced a mutant lacking function of the predicted MLC1 ortholog of *S. cerevisiae*. This encodes a myosin light chain that regulates the activity of myosin heavy chain interactions supporting the assembly of the contractile ring. Loss of MLC1 function in *S. cerevisiae* or the ortholog in *S. pombe* (encoded by *cdc4*) is lethal for both species, resulting in an inability to complete cytokinesis. However, in *A. fumigatus*, they have conserved roles for septa formation. BLAST search of the A1163/CEA10 genome revealed two orthologs of Mlc1p, encoded by AFUB_091530, which showed 42% identity with the MLC1 of *S. cerevisiae*, and a gene which appeared to be an ortholog of calmodulin, AFUB_067160, which showed only 34% identity. We chose the gene with higher % identity to produce a gene mutant for study and dubbed this gene *mlcA*. However, BLAST search did not reveal an ortholog of the *A. nidulans* AcnA protein within the genome of CEA10. However, searches using the sequence of the *ain1* from *S. pombe* resulted in one hit, a gene encoded by AFUB_055850, which possessed 49% identity to this query. We dubbed this gene *ainA*. Because loss of septa is not immediately lethal to *A. fumigatus*, we expected loss of either gene might abrogate septation, but not result in lethality. Indeed, we were able to obtain viable mutants for both of these genes.

Characterization of their phenotypes revealed that both Δ*mlcA* and Δ*ainA* phenocopy the SIN kinase mutants in terms of their lack of septa (**Figure B.11A**), their fungicidal rather than fungistatic phenotypic susceptibility to echinocandins (**Figure B.11B**), their lack of virulence in a corticosteroid immunosuppression murine model of IA (**Figure B.12A** and **C**), and their lack of meaningful invasion into host lung tissue (**Figure B.12B** and **D**).

Taken together, these results indicated that loss of septum formation due to deletion of the kinases SepH, SepL, or SidB, or of their regulators MlcA or AinA, results in strains which show reduced capacity for host tissue invasion and virulence, and a switch from simply fungistatic to truly fungicidal outcomes in the presence of echinocandins, all resulting from the inability of the strains to form septa within the fungal hyphae. Future work may reveal key kinases or regulatory components of *A. fumigatus* intracellular networks like the SIN which show potential as drug targets for novel standalone or combinatorial antifungal therapies. These may improve the survival of patients with life-threatening *Aspergillus* infections.

## 3.3 Conclusion

In *A. fumigatus*, the processes that form septa in hyphae involve a modified process of cytokinesis which does not result in a separation of daughter cells, but instead the two cellular compartments remain attached. The repeated cycle of hyphal extension and septal formation normally produces long filaments with individual compartments containing usually several nuclei, separated by septa through which nutrients and materials may pass. It has long been known that septa play a role for protecting the hyphae from stress.

When required, the septa can become impermeable and form a barrier between structurally compromised compartments to protect yet intact compartments. (Envision the function of so called "blast doors" in space ships in every sci-fi space opera popularized in modern history). Loss of septation likely results in the switch from fungistatic to actual cidal activity of echinocandins by removing these buffers and allowing hyphal damage to radiate through the whole hyphae.

As mentioned, the Δ*sepH* mutant was generally hypersusceptible to both caspofungin and micafungin, but produced some small colonies of growth within the zone of clearance in echinocandin E-test assays. Later studies performed by another student in our lab revealed that deletion of other previously uncharacterized regulators of the SIN pathway produced a similar result. Deletion of the *A. fumigatus* ortholog of the kinase activators SepM or MobA, essentially phenocopy the *sepL* and *sidB* mutants from the present study in regard to lack of septa formation, hypersusceptibility to CFW and CR, fungicidal rather than fungistatic susceptibility to echinocandin, and reduced virulence in a murine model of IA (Thorn et al., 2024). Moreover, during an echinocandin E-test assay, the Δ*sepM* mutant produced minuscule colonies within the zone of clearance (**Figure B.4**, inset images within the referenced publication) (Thorn et al., 2024). When these microcolonies were cored and transferred to drug-free medium, they formed viable colonies on the agar, indicating that echinocandin had acted in a fungicidal manner for the majority of Δ*sepM*, but failed to cause fungicidal action against these microcolonies. Efforts to determine what was different about these colonies revealed that each had somehow overcome the loss of the *sepM* gene, and had begun to form septa again (Figure 7D within the referenced publication). The adaptations which allowed these Δ*sepM* microcolonies to reinstate septa formation appears to have enabled these colonies to withstand the activity of the echinocandins. Hyperseptation appears to be a natural response to echinocandin stress, as samples of stained *wild type* CEA10 collected from near the zone of clearance on drug strip diffusion assays reveals a hyperseptate phenotype, which had also been reported previously (Moreno-Velasquez et al., 2017). Therefore, during caspofungin exposure, *A. fumigatus* appears to induce septation in a manner which might rely on signalling that is independent of SepM function, potentially via activity of its putative SIN kinase binding partner SepL that does not require SepM.

This study revealed that protein kinases mediate survival in the presence of echinocandin antifungal stress. However, the study focused on mutants which were differently susceptible to echinocandin. None of the mentioned mutants showed a difference in susceptibility to the currently preferred, frontline antifungal options, the triazoles. Thus, we sought to assay the collection of disruption mutants for altered susceptibility to the mold-active medical triazole voriconazole. As in this study, we predicted that loss of certain protein kinases encoded in the *A. fumigatus* genome would reveal multiple mutants for which the susceptibility was shifted by more than a single dilution. Furthermore, we expected that these mutants might generally exhibit heightened susceptibility to this class of antifungal. This screen, and the study of the singular resulting uncharacterized hit, constituted the bulk

of my own student research project, the main results of which are summarized in the next chapter.

# Chapter 4

# Loss of the SAC Components SldA kinase or SldB or *in vitro* Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in *Aspergillus fumigatus*[1]

**NOTE:** This chapter refers frequently to content in **Appendix C**. When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See Preface for further details.

## 4.1 Introduction

This chapter provides a brief summary of information collected within the prepared-for-publication article "Loss of the SAC components SldA kinase or SldB or *in vitro* adaptation to triazole produces aneuploidy associated with triazole heteroresistance in *Aspergillus fumigatus*" (**Appendix C**). This article includes the bulk of the key aims, hypothesis, experimental approaches and results from my student research project. This manuscript describes the construction and characterization of whole-gene deletion and complementation strains made in the *wild type A. fumigatus* strain CEA10/A1163 for the previously uncharacterized *A. fumigatus* SAC components SldA and SldB. The article covers the process of analyzing the important phenotypes resulting when the function of these crucial components of the mitotic checkpoint are not present, with special emphasis on the impact that loss of their function has on the phenotypic susceptibility of *A. fumigatus* to antifungal drugs. Moreover, this article reports the finding that *wild type A. fumigatus* responds to triazole exposures by accumulating aneuploid conidia. Finally, *wild type* selected for a specific chromosomal

[1]Prepared for publication manuscript reproduced with permission from all contributing authors: A. V. Nywening, H. Thorn, J. Xie, A. Martin-Vicente, X. Guruceaga Sierra, W. Ge, J. G. Gibbons, and J.R. Fortwendel (2024). Currently titled "Loss of the SAC Components SldA Kinase or SldB or *in vitro* Adaptation to Triazole Produces Aneuploidy Associated with Triazole Heteroresistance in *Aspergillus fumigatus*" (**Appendix C**).

aneuploidy during *in vitro* adaptation very similar to our SAC deficient SldA mutant, implying that *A. fumigatus* increases chromosomal instability in response to triazoles, potentially through directed SAC failure. This brief summary provides a short outline of the work. The most recent manuscript draft of the prepared-for-publication work summarized in this chapter has been reproduced in **Appendix C**, which readers are encouraged to access to obtain a complete view of the content.

## 4.2 Article Summary

The issue of triazole antifungal resistance in the human pathogen *Aspergillus fumigatus* remains a significant problem worldwide but has been insufficiently studied. Resistance to this class of medical antifungals, which currently remain the primary therapeutic option used clinically to treat patient infections, threatens the continued usefulness to combat life threatening *Aspergillus* disease. Unfortunately, the strategies whereby this species adapts to become triazole resistant are especially mysterious. For years, species of pathogenic yeast have been known to utilize specific aneuploidies as a means of obtaining triazole or echinocandin resistance (G. Chen et al., 2012; Yang, L. Zhang, et al., 2017; Brimacombe et al., 2019; Yang, Teoh, et al., 2019; Bing et al., 2020; Sah, Hayes, and Rustchenko, 2021). However, until very recently, species of *Aspergilli* were previously supposed not to utilize this specific strategy to provide enhanced survival to triazole stress (Handelman and Osherov, 2022; Barda et al., 2023).

This belief was generally accepted until two studies published in 2023 revealed for the first time clear evidence of an aneuploidy-based mechanism associated with adaptation to triazoles in two separate *Aspergillus* species, one revealing aneuploidies which appear in the pathogen *Aspergillus flavus* following triazole exposures, and the other revealing clinically relevant triazole resistance based on specific potentially beneficial aneuploidies in *A. fumigatus* patient isolates (Barda et al., 2023; Khateb et al., 2023). *A. flavus* represents the second most common causative agent of human aspergillosis infections behind *A. fumigatus*. Revelation that this previously unknown mechanism might support survival and adaptation of *Aspergillus* species to medical triazoles, a notion which had been generally assumed impossible in the field of study up to this point, opened the door for a clearer understanding of how this important human and animal pathogen might acquire triazole resistance. However, neither publication demonstrated whether the susceptibility level of strains to triazole could be linked to gains and loss of aneuploidy within the population, and neither proposed a reasonable explanation for how such aneuploidies had arisen in the first place. These only reported that aneuploidy could be detected in a subset of isolates which had become resistant to triazole. In the **Appendix C** article, my work revealed that loss of components of the key pathway regulating the cell cycle mitotic Spindle Assembly Checkpoint (SAC), a quality control sensing and signalling pathway responsible for ensuring chromosomes are sorted correctly during anaphase, was sufficient to result in

aneuploidy among strain progeny, and simultaneously reduced the strains' susceptibility to voriconazole. Furthermore, I found that subjecting *wild type A. fumigatus* to repeated triazole exposures produced a similar level of aneuploidy as that of my SAC deficient mutants. Finally, I found that repeated exposure to voriconazole caused both SAC mutants and *wild type A. fumigatus* to select for the same specific aneuploidy at identical rates. These results show that in response to voriconazole, *wild type A. fumigatus* induces chromosomal instability that is comparable to deletion of SAC components, and then selects certain of the aneuploid progeny to be preferentially represented during the process of adapting to triazole. Removal of voriconazole from the medium showed that both MIC increases and the level of aneuploidy within the populations were generally unstable; at least partially lost once the stress of triazole is removed. I propose that transient SAC dysregulation, potentially through changes in expression or regulation of the key regulatory kinase SldA, represents a likely source and a directed mechanism whereby *A. fumigatus* induces, accumulates, and selects for aneuploidy during the process of triazole resistance acquisition, and that these may represent an unstable mechanism contributing to resistance which may often disappear (sometimes rapidly) when removed from the context of triazole. This transient resistance could potentially partly account for why the occurrence of aneuploidy-associated triazole resistance has not been well documented or studied until now in this species, especially within clinical isolates. While many factors outside of pathogen-specific variables can influence therapeutic outcomes, we propose that aneuploidies could potentially help to account for such temporary, context-specific triazole resistance in *A. fumigatus* clinical isolates. Moreover, this theory has been proposed previously for another fungal pathogens (R. T. Todd and A. Selmecki, 2020).

The discovery of an *sldA* mutant which exhibited reduced triazole susceptibility resulted from a screen of our protein kinase disruption mutant library described in the **Appendix B** article (**Figure B.1**). In the process of screening this library for mutants differently susceptible to the mold-active medical triazole antifungal voriconazole, we discovered two mutants which exhibited MIC shifts by at least two dilutions (representing a four-fold change in susceptibility). However, unlike what was seen in our previous study, rather than accentuating the activity of the antifungal, loss of gene function caused both mutants to be less susceptible to voriconazole. To confirm that this phenotype was truly a result of loss of gene function, we utilized the same method of gene editing described previously to construct whole-gene deletion mutants for the gene that was predicted to encode an ortholog of the *A. nidulans* SldA kinase. (As the other gene product, Ssn3, had been characterized previously and its influence on triazole susceptibility was already known, we did not further pursue study of this kinase). Moreover, to ascertain whether the *A. fumigatus* SldA kinase has a conserved roles supporting the function of the SAC, and whether defects in the SAC resulting from loss of the kinase were related to the loss of susceptibility to triazole antifungal, I also constructed a deletion of one of the other components of the mitotic checkpoint complex (MCC); a binding partner of SldA kinase orthologs in other species known as SldB (**Figure C.2** and **Figure C.3**) (Altschul et al., 1990;

Farkasovsky and Kuntzel, 1995; Funabiki, Kumada, and Yanagida, 1996; Starr et al., 1998; Yanagida, 2000; Tytell and Sorger, 2006; X. Wu, Xiang, and Hammer, 2006; Cheeseman and Desai, 2008; Gassmann et al., 2008; Gonczy, 2008; M. S. Skrzypek and Hirschman, 2011; Zheng et al., 2013; Kitagawa and S. H. Lee, 2015; Jin, Bokros, and Y. Wang, 2017; Basenko et al., 2018; Edwards et al., 2018; Gupta et al., 2018; Suzuki et al., 2018; Wimbish and DeLuca, 2020; Kiyomitsu and Boerner, 2021; P. Singh et al., 2021; T. Kim, 2022; Roy et al., 2022; White et al., 2022; Pitayu-Nugroho et al., 2023). I also constructed complemented strains for each wherein the coding sequence for the gene was re-integrated into the native locus of the deletion strain to restore the gene as close to its original state as was possible **Figure C.15**). These mutants were initially studied to determine whether loss of either component impacted the biological processes which regulate vegetative growth to a degree which results in a growth defect. This had been reported previously for an *A. nidulans* SldA mutant. However, upon analysis, neither our our Δ*sldA* nor our Δ*sldB* mutant exhibited biologically relevant differences in the capacity for growth in *Aspergillus* minimal medium or apparent defects in the timing or rate of conidial germination (**Figure C.4**). Previous studies had reported that loss of orthologs of either SldA or SldB, or in fact loss of various other SAC components, results in a heightened susceptibility to compounds which physically interfere with the dynamic stability of microtubules, including the benzimidazoles. To confirm that the functions of SldA and SldB are likely conserved for the SAC, we evaluated whether our Δ*sldA* and Δ*sldB* mutants exhibited this hypersusceptibility phenotype using the compound benomyl. Both mutants produced this expected phenotype, though the impact was more severe for loss of the SldA kinase than for loss of SldB, likely owing to the more vital role of the kinase for various SAC-related interactions (**Figure C.5**). We also evaluated whether loss of either gene might impact the production or distribution of nuclei following nuclear division and found that the mean nuclei per hyphal compartment was not significantly different between either mutant and the parental CEA10 strain (**Figure C.6**). We then wanted to confirm whether the reduced triazole susceptibility phenotype of the original disruption strain *sldA*-1 was, in fact, due to complete abrogation of SldA kinase function, and begin to reveal whether the phenotype was linked to an impact on the SAC, or on some other unrelated activity of the *A. fumigatus* SldA kinase. Analysis of susceptibility revealed that the phenotype present within the original *sldA* disruption strain was shared to approximately the same degree in both Δ*sldA* and Δ*sldB*, indicating that the phenotype likely related to defective SAC pathway function. We also confirmed that loss of either gene produced a pan-azole phenotype; reducing susceptibility to each mold-active medical triazole antifungal that we tested (**Figure C.7**). Furthermore, we found that the phenotype was also generalizable to other compounds which act as inhibitors of ergosterol biosynthesis, but which target other points of the pathway upstream of the triazole target enzyme Cyp51A/B. However, neither Δ*sldA* nor Δ*sldB* showed a difference in susceptibility to the antifungal compound Amphotericin B, which rather than inhibiting ergosterol biosynthesis, binds directly to the final product ergosterol and disrupts its impact for the fungal membranes (**Figure C.7H**). ((Or at least, if there was any difference, it was

not sufficient to produce a shift in the susceptibility level of either strain detectable by minimum inhibitory concentration (MIC) assays)). Similarly, Δ*sldA* and Δ*sldB* showed no alteration in the susceptibility level to a variety of other forms of stress, including those which produce DNA damage, oxidative or osmotic stress, cell wall stress, or host stress as modeled through in-host infection murine model of invasive aspergillosis (**Figure C.8**). Again, this deviated slightly from what we expected to find. Previous studies have reported that loss of the kinase ortholog of SldA can result in mutants with heightened sensitivity to DNA damage or the osmotic stressors sucrose and NaCl. However, whatever impact loss of *sldA* or *sldB* has within *A. fumigatus*, neither appeared to influence susceptibility, either positively or negatively, to forms of stress besides that of ergosterol biosynthesis inhibitors (EBIs), (again, with the qualifier that if changes can occur when either gene is lost that influence susceptibility to any of these forms of stress, the influence was insufficient to produce a clear shift in the MIC or MEC or other measure of outcome used in our analyses). Then, I noted something interesting when I observed the growth of these mutants within the wells of a voriconazole MIC assay. According to the Clinical and Laboratory Standards Institute (CLSI) protocols for triazole MIC assays, the results are recorded based on whether growth is visible within the well while simply observing the plate from beneath. The first well concentration in each row for which no fungal growth is visible is scored as the MIC for that row. However, I was curious to see how the strains were growing within the assay, whether any clear differences could be seen, such as differences in branching patterns, diameter of the filaments, etc. What I noticed was that for both Δ*sldA* and Δ*sldB*, not all conidia behaved the same way. Only a very few conidia were observed germinating within the wells, especially at higher concentrations of the drug. This implied that, rather than the entire population inheriting a reduced susceptibility to the action of triazoles, only some of the progeny exhibited resistance while the majority behaved like the *wild type* CEA10 strain (**Figure C.9**). Flow cytometric analyses then confirmed that both Δ*sldA* and Δ*sldB* exhibit some degree of spontaneous aneuploidy at baseline compared to the CEA10 genome, which produced net increases in average chromosomal content packed into the strain's conidia (**Figure C.10**).

Loss of the ortholog of the SldA kinase was reported to confer hyperadaptability to triazole stress in a *C. albicans* study. We predicted that if we repeatedly exposed Δ*sldA* to voriconazole, the SAC defect inherent due to loss of SldA function and the apparent predisposition to CIN at baseline might promote enhanced adaptation compared to the background CEA10 strain through increased capacity to select for fitness-conferring aneuploidies. We also expected that *in vitro* adaptation experiments might drive the acquisition of more common and previously known mutation-based resistance mechanisms in the *wild type* strain, and expected aneuploidy-associated adaptation to be less common for *A. fumigatus* that has an intact SAC pathway. What we instead discovered was somewhat unexpected.

First, the dynamics of MIC increases were very similar between *wild type* and Δ*sldA*

lineages after about the second "Generation" of transfer over voriconazole-embedded media (glucose minimal medium (GMM)) (**Figure C.11**). Second, *in vitro* adaptation to voriconazole caused aneuploid conidia to accumulate in both our *SldA* deletion strain and in CEA10, indicating that CEA10 had somehow begun to produce chromosomal instability in response to voriconazole stress similarly to our SAC deficient Δ*sldA* strain. (Although, aneuploidy production in our Δ*sldA* strain did appear to be somewhat additive with the combination of both loss of *sldA* and voriconazole exposure, as anecdotally, the DNA indices (DI) in our adapted Δ*sldA* lineages tended to be equal to that of the base, unadapted Δ*sldA* strain (averaged DI of 1.09, for an increase of about +.09), plus the average DI increase produced in an adapted *wild type* strain (averaged DI of 1.087, for an increase of about +.087) to end up at a DI averaging 1.173 for Δ*sldA* adapted lineages (**Figure C.12**) and (**Table C.4**). We interpreted this outcome as indicating that in both *wild type* and Δ*sldA*, voriconazole exposure increased the rate of chromosomal instability above the baseline rate of the strain. This finding was consistent with previous reports of triazoles increasing the rate of aneuploidy in other fungal pathogens (and more specifically the accumulation of aneuploid progeny exhibiting triazole heteroresistance) (Semighini et al., 2011; Kukurudz et al., 2022). This was also consistent with the observation that in *C. albicans*, deletion of an MCC component which associates with the SldA kinase ortholog Bub1, Mad2, caused a baseline level of aneuploidy that enabled enhanced survival in the context of fluconazole stress, but this baseline level of CIN and aneuploidy was shown to increase even further in this mutant during exposure to fluconazole (Vossen et al., 2019). In an effort to determine which kinds of changes had occurred within the voriconazole adapted lineages which could represent mechanisms contributing to their acquired resistance, for each parental strain and its adapted lineages, we collected genomic DNA from population conidia or from homogeneous hyphal biomass and sent these for whole genome sequencing (WGS) with variant analysis. We examined the variant analysis results for each strain to determine whether any mutations had occurred within genes previously known or suspected to influence triazole susceptibility, as well as searching various genes which putatively relate to the cell cycle or the SAC. Examination of the variant calling revealed a suprising result; although each of the three lineages of both parental strains had obtained MICs to voriconazole of between 16 and 32 µg/ml in *Aspergillus* minimal medium, only two lineages had acquired a mutation in a gene previously associated with resistance (**Table C.5**). Moreover, one of the lineages was from the Δ*sldA* background, which produced a mutation in the coding sequence of the *cyp51A* gene that results in a T50I substitution (**Figure C.13A**). The T50I mutation in the sequence for Cyp51A is very close to known mutations which are blamed for triazole resistance impacting the residue G54 (**Figure A.2**). Meanwhile, a single CEA10 lineage possessed a mutation in the coding sequence of the *hmg1* gene which results in a deletion of G483 (**Figure C.13B**). The only other mutations found in genes that we included in the search was the expected deletion impacting the coding sequence of the *sldA* gene in all Δ*sldA*-related lineages. We then subjected the WGS data for analysis to determine whether any copy number variations were detectable in any

of the strains or lineages. Two Δ*sldA* lineages and two CEA10 lineages each preferentially selected for aneuploidies in the population which produced detectable variances in copy number (CN). Surprisingly, all four strains shared a duplication of a region of chromosome two in common (**Figure C.14**). It remains unclear which genes or combinations of genes in this region might be influencing triazole susceptibility through increased gene dosage. Future work will be needed to dissect how, (and in fact if), duplication of this region was directly contributing to the triazole susceptibility loss in these four voriconazole adapted lineages. However, the fact that four out of six total lineages preferentially selected for progeny with this specific aneuploidy independent of each other strongly suggests that this aneuploidy provides beneficial gene dosage changes in the context of triazole stress adaptation.

Based on our findings, we propose that *wild type A. fumigatus* responds to triazole stress by increasing the incidence of chromosomal instability. We suggest that directed (but likely reversible) suppression of SAC checkpoint fidelity likely represents a primary strategy and mechanism whereby *A. fumigatus* allows for CIN in this context. We base this claim on findings from the present study, but which are supported by results from studies in other (generally better studied) species of pathogenic fungi. Evidence supporting our hypothesis includes several known facts:

1. Defects in SldA kinase orthologs or defects in components which regulate the SAC at points upstream or downstream of the SldA kinase ortholog are previously shown to cause some degree of SAC failure and chromosomal instability (**Table C.1**, **C.2**, and **C.3**).

2. In both *C. neoformans* and *C. albicans*, aneuploidy often enables the production of sub-populations with triazole heteroresistance within the total population of phenotypically drug susceptible fungi (Sionov, Y. C. Chang, et al., 2009; Sionov, H. Lee, et al., 2010; Y C Chang, Khanal Lamichhane, and Kwon-Chung, 2018; Bing et al., 2020; Kukurudz et al., 2022).

3. In *C. albicans*, SAC defect due to loss of the SldA kinase ortholog Bub1 can enable the acquisition of and selection for known resistance-conferring chromosomal duplication aneuploidies (Ch. 5L) at increased rates over *wild type* (Brimacombe et al., 2019).

4. Defects in SldA kinase ortholog or defects in components which regulate the SAC at points upstream or downstream of the SldA kinase ortholog are previously shown to reduce triazole susceptibility, and in some cases that reduction was shown to involve heteroresistant aneuploid subpopulations within the strains (Brimacombe et al., 2019; Varshney and Sanyal, 2019; Vossen et al., 2019).

While our study was not the first to reveal aneuploidy is associated with triazole tolerance and triazole adaptation in *A. fumigatus*, ours was the first to propose a mechanistic route whereby this species is able to generate aneuploidies through purposeful cell cycle checkpoint dysregulation. Future work should explore whether *A. fumigatus* can induce SAC

defects when desired simply by modifying the expression or activity of components like SldA and SldB, reveal which specific alterations in the content of chromosomes, portions of chromosomes and associated gene CNV underlie loss of triazole susceptibility, and attempt to appreciate the extent to which aneuploidy might impact triazole susceptibility in clinical isolates of this human and animal fungal pathogen.

## 4.3 Assessment for Differential Gene Expression by RNA sequencing

Samples of *wild type* CEA10 (in triplicate) were cultured overnight (24 hours) then cultured for an additional 6 hours in the presence or absence of 0.5x CEA10 MIC voriconazole and RNA extraction was performed as described in **Appendix C** following previously established protocols (Martin-Vicente et al., 2020). Samples were then submitted for Illumina-based RNA sequencing (Novogene, Sacramento, CA). Analysis for differential expression of genes was performed and genes which showed a log2 fold change greater than or equal to +1 (corresponding to a 2-fold increase or more) were considered significantly upregulated while genes exhibiting a log2 fold change of -1 or less were considered significantly downregulated. Genes which met this criteria were further analyzed by gene ontology (GO) analysis (FungiFun2) (Priebe et al., 2011) to determine which associated functional categories are enriched in the strains.

## 4.4 Results

### 4.4.1 Deletion of *sldA* does not alter transcription of most resistance-associated genes in comparison to *wild type*

In the prepared-for-publication article included in **Appendix C**, we assayed both *wild type* CEA10 and our Δ*sldA* strain to compare the relative levels of transcription of several genes by RT-qPCR. We included several genes whose overexpression has been previously associated with triazole resistance in *A. fumigatus*, including the *A. fumigatus* gene *abcC* (AKA *cdr1B*, AFUB_013880 in the strain A1163), which is predicted to encode an efflux pump in the ABC superfamily. (Enhanced baseline or induced expression of this gene following triazole exposure as measured by RT-qPCR had been reported previously in isolates with apparrently non-*cyp51A*-mediated triazole resistance). The protein sequence of *A. fumigatus* AbcC/Cdr1B is highly homologous to the Cdr1 of *C. albicans* (52% identity and 67% positivity (similarity) to the gene gene C3_05220W_A in *C. albicans* SC5314) (Fraczek et al., 2013; Basenko et al., 2018). To compare induction of the genes in the context of triazole stress, we examined cDNA produced from RNA extracted from both unstressed cultured hyphae and hyphae which had been subjected to subinhibitory voriconazole concentrations. In our RT-qPCR-based transcriptional analysis, there were no significant

differences between the baseline expression levels between Δ*sldA* and CEA10 or induction of the genes following triazole exposure that implied the triazole phenotype of Δ*sldA* was due to an altered capacity to express these genes compared to *wild type* (**Figure C.18**). While expression of several genes increased following voriconazole exposure, the levels of expression were increased to comparable levels between CEA10 in the voriconazole-exposure group and Δ*sldA* in the voriconazole exposure group at both timepoints for the genes *cyp51A*, *cyp51B*, and an *atrF* ortholog. In terms of changes which could explain why ΔsldA is less susceptible to voriconazole, our RT-qPCR results of expression did not point to a so called "smoking gun".

However, in addition to this RT-qPCR analysis, to thoroughly assess the Δ*sldA* strain for differential gene expression at baseline or in response to voriconazole stress, we also performed an RNA sequencing analysis. We cultured hyphae overnight, then for an additional six hours either with no added triazole or with subinhibitory voriconazole added (again, 0.5 x CEA10 MIC), and extracted RNA to send for RNA sequencing and analysis.

We analyzed the sets of differently expressed genes (DEG) in each strain following subinhibitory voriconazole exposure using the online tool FungiFun2 to determine which functions were significantly enriched among the upregulated and downregulated gene IDs. In response to 0.50µg/ml voriconazole stress, CEA10, Δ*sldA*, and Δ*sldB* upregulated 886 genes in common (**Figure** 4.1A).

GO analysis for enriched associated biological processes, cellular component, and molecular function terms show that of these 886 genes, the categories oxidation-reduction process, monooxygenase activity, oxidoreductase activity, oxidoreductase activity acting on paired donors with incorporation or reduction of molecular oxygen, metabolic process, phosphoric diester hydrolase activity, heme binding, iron ion binding, ATPase activity coupled to transmembrane movement of substances, oxidoreductase activity acting on CH-OH group of donors, response to stress, lipid catabolic process, and carbon-nitrogen ligase activity with glutamine as amido-N-donor were the 13 categories which were considered significantly enriched (**Figure** 4.2).

Meanwhile, 416 genes were downregulated in all three strains following voriconazole exposure (**Figure 4.1B**). GO analysis revealed that the categories of ribosome, structural constituent of ribosome, translation, intracellular, ribonucleoprotein complex, small ribosomal subunit, rRNA binding, large ribosomal subunit, ubiquinol-cytochrome-c reductase activity, RNA binding, structural constituent of cytoskeleton, protein complex, and protein polymerization were the 13 categories significantly enriched among these 416 genes (**Figure** 4.3).

In CEA10, 281 genes were upregulated in response to voriconazole exposure which were not considered upregulated in either Δ*sldA* or Δ*sldB*. Among these genes, only the GO categories of lyase activity and catalytic activity were considered significantly enriched (**Figure** 4.4).

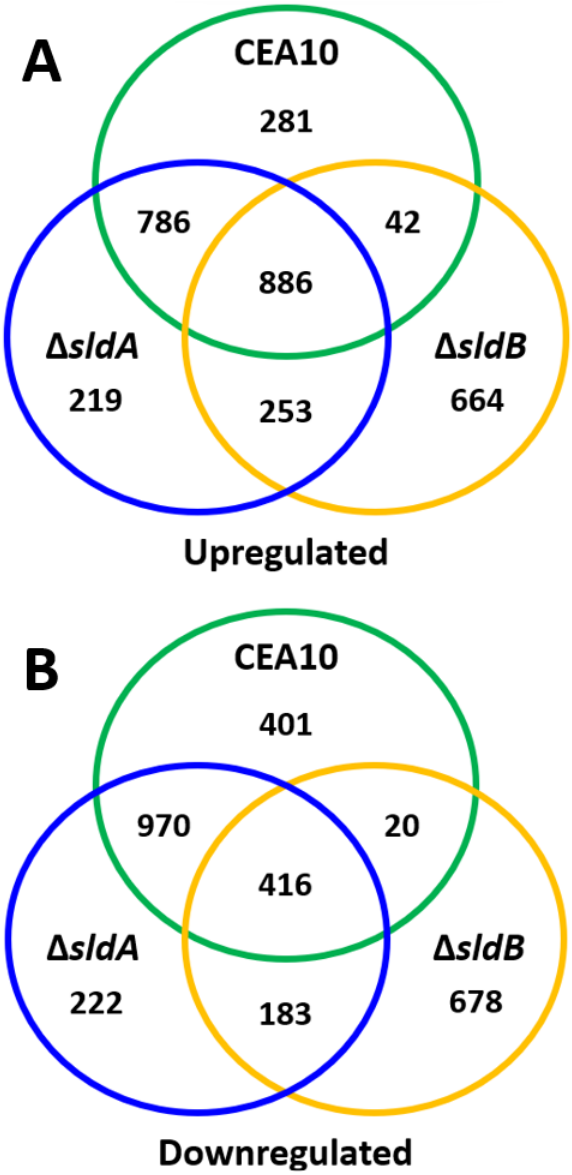


**Figure 4.1: Differently expressed genes (DEG) following voriconazole exposure.**

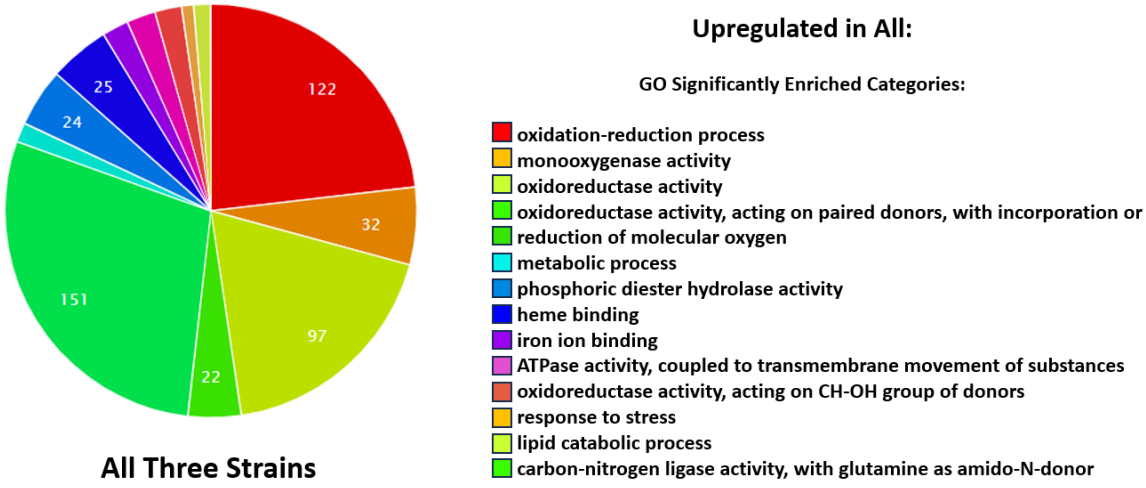


**Figure 4.2: Enriched GO categories shared between all three strains for upregulated genes.**

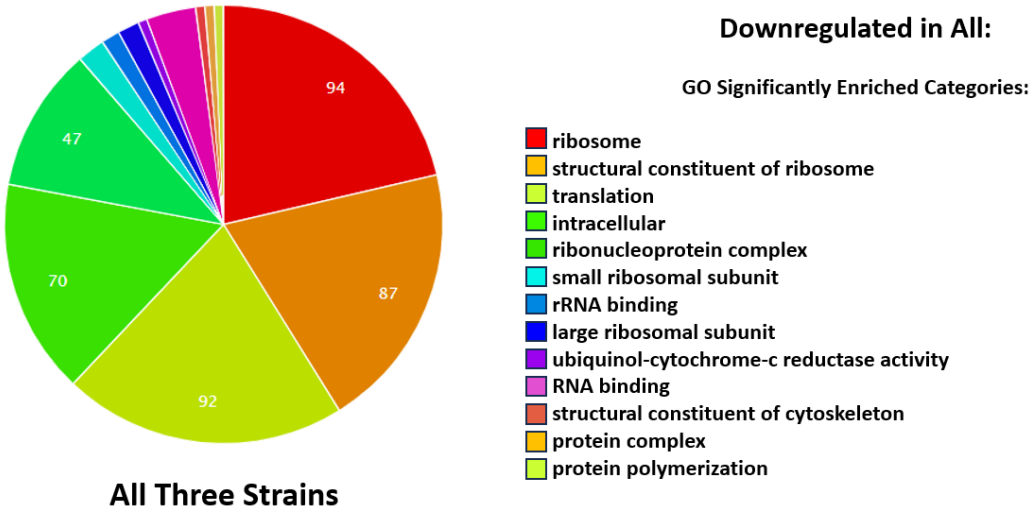


**Figure 4.3: Enriched GO categories shared between all three strains for downregulated genes.**

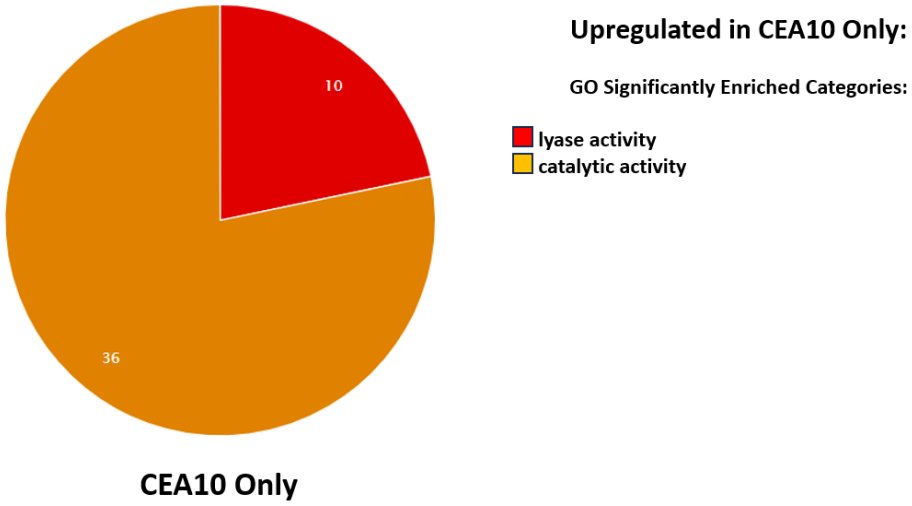


**Figure 4.4: Enriched GO categories for genes upregulated in CEA10 only.**

Meanwhile, 401 genes were only downregulated in CEA10 in response to the voriconazole exposure. Of these, only eight categories were considered significantly enriched in the analysis. These were the categories transmembrane transport, cell wall organization, polysaccharide catabolic process, transmembrane transporter activity, substrate-specific transmembrane transporter activity, hydrolase activity acting on glycosyl bonds, integral component of membrane, and transporter activity were significantly enriched (**Figure** 4.5).

According to GO analysis of DEGs, there were 253 genes that were considered upregulated in Δ*sldA* and Δ*sldB* by equal to or greater than log2 of +1, and 183 genes that were downregulated by equal to or less than log2 of -1, which were not considered upregulated or downregulated in CEA10. Among the genes upregulated in both mutants but not CEA10, GO analysis indicated that no categories could be considered to be significantly enriched within the group of 253 genes.

However, among the 183 genes which were downregulated in both mutants, eighteen GO categories were listed as significantly enriched. These included the terms translation, ribosome, structural constituent of ribosome, septin complex, formation of translation preinitiation complex, eukaryotic translation initiation factor 3 complex, eukaryotic 48S preinitiation complex, regulation of translational initiation, eukaryotic 43S preinitiation complex, NAD binding, tricarboxylic acid cycle, cytoplasm, pyruvate dehydrogenase (acetyl-transferring) activity, oxidoreductase activity, acting on NAD(P)H, dolichyl-diphosphooligosaccharide-protein glycotransferase activity, isoleucine biosynthetic process, isoprenoid biosynthetic process, and cell cycle (**Figure** 4.6).

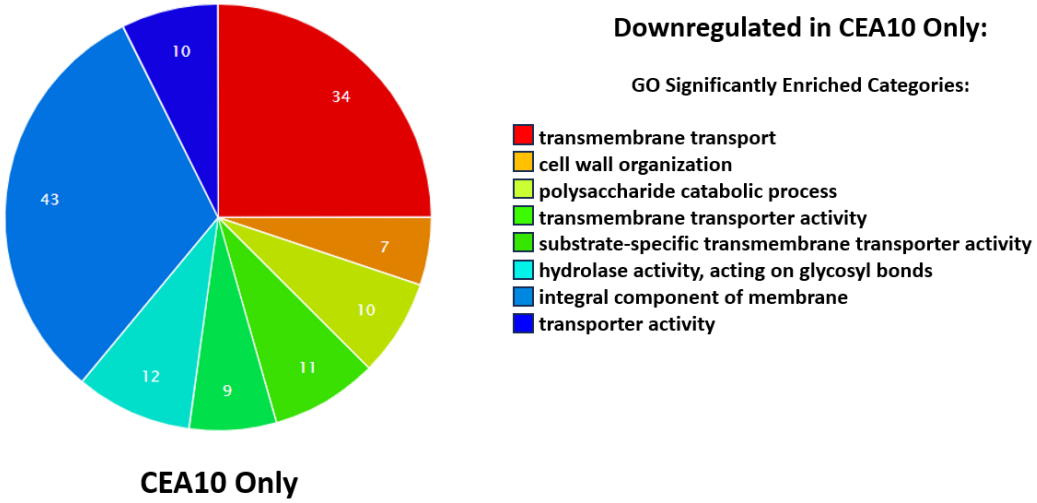


**Figure 4.5: Enriched GO categories for genes downregulated in CEA10 only.**

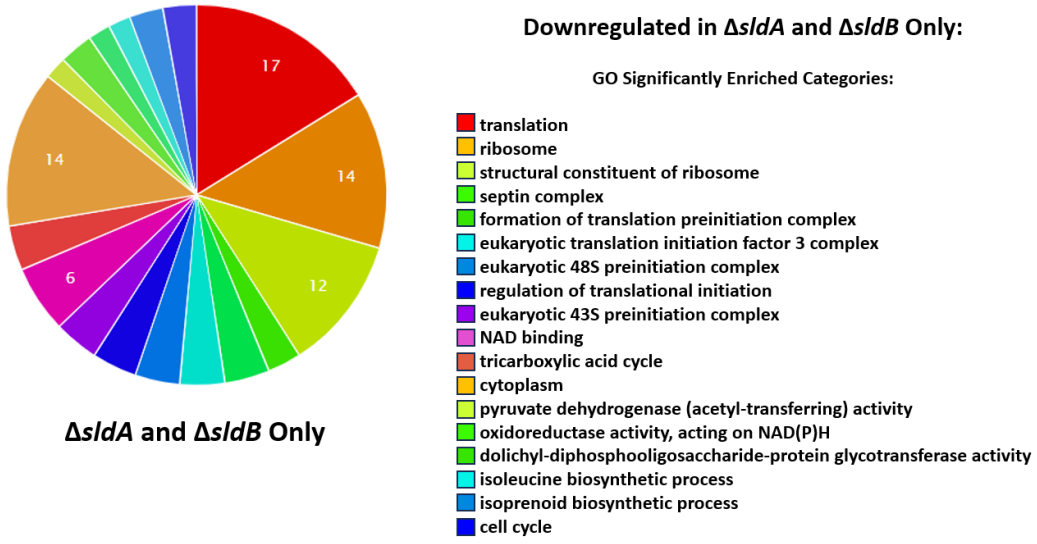


**Figure 4.6: Enriched GO categories for genes downregulated in Δ*sldA* and Δ*sldB* only.**

It was previously known that aneuploidy can provide tolerance or resistance to antifungals and genotoxic stress in yeast species (Stemmann et al., 2002; Davies and Kaplan, 2010; G. Chen et al., 2012; Pennisi, Ascenzi, and Masi, 2015; A. Singh and Xu, 2016; Yang, Teoh, et al., 2019). Previous studies of chromosome 5 aneuploidy-based triazole resistance in *Candida* species report that these aneuploidies conferred resistance by enhancing the transcription of certain genes, including overexpression of the 14$\alpha$-lanosterol demethylase enzyme Erg11, which was detectable both in RT-qPCR and RNA sequencing datasets (Bing et al., 2020; X. Fan et al., 2023). According to our RNA sequencing results, in the absence of drug the genes encoding *cyp51A* or *cyp51B* in *A. fumigatus*, (AFUB_063960 and AFUB_089270 respectively), were not considered upregulated or downregulated by two-fold or more in comparison to *wild type* in our Δ*sldA* strain at baseline. Neither did the Δ*sldA* mutant express *cyp51A* or *-B* differently from *wild type* in the context of subinhibitory voriconazole exposure; Both strains expressed these genes to approximately the same degree. This result harmonized with those from our previous RT-qPCR analysis, where neither *cyp51A* nor *cyp51B* transcript levels were significantly different between the CEA10 and Δ*sldA* at baseline, or between the CEA10 and Δ*sldA* in the context of voriconazole stress (**Figure C.18**).

We also reviewed the RNA sequencing results to examine for transcriptional differences of various genes predicted to encode orthologs of efflux pumps in *A. fumigatus*. Of the genes assessed, only one gene, AFUB_071280, (predicted to be an ortholog of *mdr1*), appeared differentially expressed in our Δ*sldA* strain in comparison to the *wild type*. The expression of this gene was just over two-fold increased in our Δ*sldA* strain over that of the *wild type* following voriconazole exposure (upregulated, log2 fold change of +1.009). There was no difference in the expression of this gene at baseline. None of the other resistance-associated genes we examined met the criteria to be considered differently expressed in Δ*sldA* in comparison to *wild type* either at baseline or in the context of subinhibitory voriconazole exposure. The genes we assessed included those predicted to encode orthologs of the AtrF drug transporter, predicted orthologs of the AbcC/Cdr1B transporter, and predicted orthologs of Mdr1. (Genes evaluated include predicted *atrF* and *abcC/cdr1B* orthologs AFUB_013880, AFUB_093930, AFUB_016810, AFUB_030790, AFUB_041770, and predicted *mdr1* orthologs AFUB_045530, AFUB_012160, AFUB_044470, AFUB_044820, AFUB_053630, AFUB_067110, AFUB_071280, AFUB_087060, AFUB_094820, and AFUB_095220, data not shown). Unsurprisingly, similar to our RT-qPCR results, our RNA sequencing data indicated that the gene encoding *sldA* was not expressed by our *sldA* deletion strain.

### 4.4.2 Exposing *wild type* to voriconazole may produce changes in expression of certain components supporting SAC including SldA kinase

Interestingly, several results stand out when analyzing the results of CEA10 transcription changes in response to voriconazole, both among individual DEG and among genes which

participate in the same GO category. Though genes relating to the category of "cell cycle" were not considered enriched among the upregulated or downregulated transcripts in CEA10 following incubation with voriconazole, the *wild type* CEA10 downregulated expression of *sldA*, (log2 fold change of -1.013). The gene encoding *sldB*, however, was not differently expressed in response to the drug. Furthermore, transcript of the gene predicted to encode a downstream phosphorylational target of SldA, Cdc20, (encoded by AFUB_014280), was also downregulated (Log2 FC of -1.483). The genes predicted to encode orthologs of upstream regulators of SldA in the SAC pathway, Aurora kinase B (encoded by AFUB_023600) and the kinetochore component Ndc80 (encoded by AFUB_038980), were also downregulated in response to voriconazole (Log2 FC of -1.353 and -1.002 respectively). Importantly, previous studies in other species have demonstrated that underexpression of SldA kinase orthologs, Aurora B orthologs, and Ndc80 orthologs each result in some level of aneuploidy in other eukaryotes. In fact, defects or altered expression of various components which support chromosome alignment and segregation result in some level of CIN (**Figure C.2** and **C.3** and **Table C.1**, **C.2**, and **C.3**). Expression of predicted orthologs of the kinetochore components Ndc80 and CENP-A/Cse4 also appeared reduced in *A. fumigatus* in response to voriconazole according to one dataset available, further implying downregulation of certain key components controlling the fidelity of chromosome segregation as a natural response to triazole stress (Kline-Smith, Sandall, and Desai, 2005; Basenko et al., 2018; Furukawa et al., 2020). Furthermore, defects in the function of Aurora B, Sgo1, or Mad2 orthologs are each previously associated with reduced susceptibility to the triazole fluconazole in *C. albicans* (Brimacombe et al., 2019; Varshney and Sanyal, 2019; Vossen et al., 2019). In fact, alterations to the SldA kinase orthologs Bub1 or BubR1, Bub3, Sgo1, Aurora B/Ipl1, Mad2, Hsp90, histone H2A, or CENP-A, all of which support chromosome alignment and segregation, have each been previously shown to produce a reduction in susceptibility to medical triazole antifungals (**Figure C.3** and **Table C.1**, **C.2**, and **C.3**).

## 4.5 Conclusion

It was previously known that aneuploidy can provide tolerance or resistance to antifungals and genotoxic stress in yeast species (Stemmann et al., 2002; Davies and Kaplan, 2010; G. Chen et al., 2012; Pennisi, Ascenzi, and Masi, 2015; A. Singh and Xu, 2016; Yang, Teoh, et al., 2019). Previous studies of chromosome 5 aneuploidy-based triazole resistance in *Candida* species report that these aneuploidies conferred resistance by enhancing the transcription of certain genes, including overexpression of the 14$\alpha$-lanosterol demethylase enzyme Erg11, which was detectable both in RT-qPCR and RNA sequencing datasets (Bing et al., 2020; X. Fan et al., 2023).

We conclude from these results that, at least in the triazole concentration and the duration of exposure used in this analysis, our Δ*sldA* strain likely does not produce its loss of susceptibility phenotype simply by possessing an enhanced capacity for expression of

genes commonly associated with triazole resistance. However, the concentration used were subinhibitory to the CEA10. Δ*sldA* possesses an MIC to voriconazole of approximately 4µg/ml in glucose minimal medium (GMM). Future work would be needed that examine the transcriptional profile of these mutants in response to voriconazole exposure closer to the MIC of Δ*sldA*, and possibly at timepoints more reminiscent of the culture conditions used for conventional MIC analyses (48 hours) to better understand the transcriptional differences which may underlie the loss of susceptibility phenotype of our SAC mutant strains.

We originally assumed that fungal pathogens may simply maintain a constant low-level production of aneuploid progeny as insurance policy, so that in the event stress that overwhelms the *wild type* capacity to survive suddenly appears, some of the strain population may survive. Indeed, *Candida* species do appear to maintain some low level of spontaneous aneuploidy. However, previous work in yeast fungal pathogens shows that the rate of aneuploidy generation appears to increase in a given strain in response to triazole stress. Researchers have posited that these organisms likely have a means for regulatable and inducible production of aneuploidy in response to certain forms of stress, but few publications have attempted to suggest a mechanism for how this occurs (Brimacombe et al., 2019). Studies have suggested *Candida* species may duplicate the entire genome to become tetraploid, then progeny undergo a process of random chromosome loss resulting in a variety of aneuploid progeny (Harrison et al., 2014; Brimacombe et al., 2019). However, one study of *C. albicans* showed that changes which cause the SAC pathway to fail may be a good candidate for purposefully increasing the rate of imbalanced chromosome sorting in mitosis (Brimacombe et al., 2019). In this study, researchers show that loss of Bub1 phosphoregulatory activity, either through deletion of the kinase itself, or through changes to either of two targets of Bub1 phosphorylation, (deletion of shugoshin Sgo1 or through a naturally-occurring non-phosphorylatable allelic variant of the target histone H2A), each increases survival in the presence of fluconazole (Fernius and K. G. Hardwick, 2007) The researchers suggest that *C. albicans* carrying one normal H2A allele and one allele encoding the non-phosphorylatable H2A can control the level of SAC failure by preferential allele expression. However, the study also revealed that changes to an SAC-regulating component upstream in the pathway also controls the fidelity of chromosome distribution. This mechanism which causes SAC dysfunction was based simply on the level of expression of the protein CENP-A, which introduces the concept that fungi may be able to control the rate of aneuploidy simply by transcriptional regulation of certain SAC-related elements. Therefore, while fungi may naturally maintain some low level of aneuploid offspring, they might also naturally increase that rate when needed by altering the function or expression level of certain SAC regulating genes.

Analysis for ploidy changes by conventional flow cytometric analyses of mature *Aspergillus* hyphae is not possible, due to inconsistency in size and shape of hyphal filaments and the tendency of such to clog microfluidic systems (R T Todd, Braverman, and Selmecki,

2018). Moreover, *A. fumigatus* cells are multinucleate and nuclear division is not directly synchronized with septation. This species does not possess a yeast phase and cytokinesis does not occur to separate cells. Instead, the hyphae share a common cytoplasm in which materials and even nuclei can be shared throughout a filament. These factors combine to make interrogation of *A. fumigatus* for ploidy changes under various circumstances difficult, though not impossible. In our study, we leveraged analyses of fungal conidia as a means to assay the outcomes of thousands of nuclear divisions.

Similar to the Δ*sldA* strain, each of our *wild type* CEA10 lineages experimentally adapted to triazoles produced aneuploid signatures according to flow cytometry. This implied that, as suggested in yeast fungal pathogens, *wild type A. fumigatus* likely possesses some mechanism for inducible CIN. We wondered if this mechanism might leverage SAC dysregulation, but in a way which, rather than producing an irreversible loss of a key component's function (as is likely the situation within our *sldA* and *sldB* deletion strains), instead depends on a temporary and reversible response such as transcriptional changes. Previous studies show that alterations in the expression of the BUB1/BUBR1/MAD3 kinase ortholog (or of other SAC components) is sufficient to induce CIN and aneuploidy (Cahill et al., 1998; Hernando et al., 2001; Kops, Weaver, and Cleveland, 2005; Bolanos-Garcia and Blundell, 2011; Basenko et al., 2018). For example, in mice, dysregulation of BUB1 kinase expression, either increased or decreased, is sufficient to cause chromosome missegregation errors and aneuploidy, showing that tight regulation of this kinase is essential for the mitotic checkpoint to operate correctly (R M Ricke et al., 2012). We considered whether reducing expression of *sldA* could be a strategy whereby *A. fumigatus* acquires aneuploidy in the context of triazole stress and found that a limited number of publicly available transcriptomics datasets appear to support this hypothesis (**Supplemental Figure 7 C.21**, **C.22**, **C.23**, **C.24**, and **C.25**).

In the study which produced the RNA sequencing dataset referenced above, *C. auris* lineages which had obtained known resistance-conferring chromosome 5 aneuploidy exhibited reduced expression of the Bub1 ortholog. These adapted strains were shown to lose their triazole resistance and also subsequently lost the aneuploidy and returned to a *wild type* level of triazole susceptibility once the stress of fluconazole was no longer present in the growth medium (Bing et al., 2020). In fact, studies of pathogenic yeast strains which have acquired such aneuploidies in the context of chemical antifungal stress often find the aneuploidy, and the adapted-level MIC, are both simultaneously lost once the stress is taken away (Selmecki, Forche, and Berman, 2006; Selmecki, Gerami-Nejad, et al., 2008; A. M. Selmecki et al., 2009; Yang, Teoh, et al., 2019). It is previously known that aneuploidy provides triazole resistance (and heteroresistance phenotype) which is transient in *C. neoformans* (Semighini et al., 2011; Zafar et al., 2019; Handelman and Osherov, 2022). Often, resistance-conferring aneuploidies in this species, such as affecting chromosomes 1, 4, 6, and 10 in *C. neoformans* are only retained by the cells as long as the triazole stress remains (Semighini et al., 2011; Zafar et al., 2019). This transient resistance associated with transient

aneuploidies is also observed in *C. albicans* isolates which have been experimentally adapted to triazoles then passaged in drug-free medium in much the same experimental protocol as we utilized in this work (R. T. Todd and A. Selmecki, 2020). Moreover, when *C. glabrata* isolates which obtained an additional minichromosome/isochromosome in the context of patient infection are passaged in the absence of stress, they too often lose both the aneuploidy and the resistance (Polakova et al., 2009). In one case, the strain possessing an aneuploidy exhibited an MIC to fluconazole of 129.6mg/L. Following the loss of its acquired minichromosome after passaging in the absence of stress, the MIC was reduced to only 14.4mg/l. We observed that a similar pattern of context-dependent resistance-associated aneuploidy was seen within several lineages in our experimentally adapted *A. fumigatus* strains.

This may support our assumption that the changes which may occur when SAC component expression is altered in the context of triazole are likely transient and reversible. Such an expression-based strategy would be more advantageous than deletion of a key SAC component. Once the stress is removed, the strain can likely restore SAC function, rather than remain with permanently affected genome stability. However, previous studies have proposed that aneuploidy can provide an initial tolerance to antifungals which allows for the eventual development of true genetically-fixed resistance (Bing et al., 2020). In fact, aneuploidy may allow for genetically fixed mutation-based resistance to arise, as the genomic instabilities that result in aneuploidies can either potentiate further genomic instability as a result of CNV imbalances in gene dosage, or because the pathways that must be altered to allow for aneuploidies to occur are also connected to pathways which participate in DNA damage responses and repair (Chan and Botstein, 1993; Stemmann et al., 2002; Davies and Kaplan, 2010; G. Chen et al., 2012; Pennisi, Ascenzi, and Masi, 2015; R. T. Todd, A. Forche, and A. Selmecki, 2017). These types of resistance mechanisms could be retained by the strain even once triazole is removed. (Unless they carry fitness defects, in which case that may cause progeny that have the mutation or aneuploidy to be selected against and gradually reduced or eliminated from the population).

Two of our voriconazole adapted strains indeed might have developed a mutation which contributed to their resistant-level MIC. The approximate position of the T50I substitution in the predicted structure of the Cyp51A enzyme (which is available through a link in the FungiDB page for the A1163 SldA protein sequence was modeled using the online AlphaFold structure prediction tool (available at https://alphafold.ebi.ac.uk/entry/B0Y5N0) based on comparison to resolved structure of the Cyp51B enzyme) (Basenko et al., 2018; Jumper et al., 2021). This substitution occurred very close to a hotspot for resistance conferring Cyp51A mutations (**Figure C.13A**). Mutations in the residues G54 and S52 have been previously reported to contribute to clinical and environmental triazole resistance in *A. fumigatus* (Snelders, Karawajczyk, et al., 2010; Ballard et al., 2019; Sharma et al., 2019; Y. Fan et al., 2021). The mutation did not appear in the WGS analysis of hyphae from the lineage. The other mutation identified appears to result in a deletion of the amino acid Glycine at

position 483 in Hmg1 (gene ID AFUB_020770 in A1163). This mutation is also visualized in the predicted structure of the Hmg1 (3-hydroxy-3-methylglutaryl coenzyme A reductase) modeled using AlphaFold (available at https://alphafold.ebi.ac.uk/entry/B0XUP2) (**Figure C.13B**). Hmg1 mutations are found in strains often in conjunction with *cyp51A* modifications, but are also reported to confer resistance to triazoles in strains lacking mutations in Cyp51A (Hagiwara, Arai, et al., 2018; J M Rybak, Ge, et al., 2019; Sharma et al., 2019; C. J. Wu et al., 2020). This G483 deletion mutation was called in both analysis of DNA from both conidia and hyphae in the strain. Unsurprisingly, the original Δ*sldA* strain and all three of its voriconazole-adapted lineages show a deletion in the sequence encoding *sldA*.

Besides these mutations, no other mutational variations were found in the voriconazole-adapted lineages in any of the select genes we examined that were previously confirmed or suspected to impact triazole susceptibility that did not also exist in the *wild type* parental strain (**Table C.5**). These included genes related to ergosterol biosynthesis and putative genes encoding efflux mechanisms. Among the select genes examined relating to the cell cycle/the SAC, the only mutations identified were the expected deletion within the sequence of *sldA* in each Δ*sldA*-related strain.

Whole genome sequencing followed by analysis of copy number is currently the gold standard by which to identify specific aneuploidies within cells of an organism, and this can be accomplished with genomic DNA samples from hyphal biomass or even collected from conidia (R. T. Todd and A. Selmecki, 2023). Our rationale for completing WGS analysis of genomic DNA collected from both mature hyphal growth and dormant spores for each strain was based on several assumptions made based on information from the literature. The WGS data from dormant conidia would provide a snapshot of the result of many mitotic divisions. Each *A. fumigatus* spore is normally packaged with the representative content of one nucleus (Adams, Wieser, and J. H. Yu, 1998; Deak et al., 2011). However, if our *sldA* and *sldB* component mutants display CIN and aneuploidy similar to that reported for loss of these two SAC components in other species, the spores produced may possess a wide range of aberrant genome sizes. WGS of genomic DNA extracted from the conidial population directly can assess the degree of aneuploidy within progeny of a voriconazole-selected strain, detecting even the contribution of subpopulations of aneuploid conidia which represent inviable or fitness-defect-associated chromosomal abnormalities. In contrast, analysis of genomic DNA collected from mature hyphal cultures will only select for growth of spores with viable genome content. Moreover, the genomes which provide the best fitness in the specific culture medium will likely become the primary represented genomes within the hyphal biomass. Lastly, mitotic divisions during hyphal growth in the absence of voriconazole have the potential to resolve any aneuploidies which previously were providing a fitness benefit to the adapted strain. Thus, the aneuploidies which may have been present in the voriconazole-adapted lineages during voriconazole stress may become underrepresented in WGS analysis of mature hyphal cultures grown without triazole stress.

With the observation that the majority of triazole adapted lineages showed at least

some reduction of voriconazole MIC (and often apparent reduction in the magnitude of aneuploid conidia production) in the absence of triazole, we would expect that future analysis would show that some of our lineages which were allowed to adapt to voriconazole and then grow for several cycles without voriconazole likely lost the aneuploidies associated with the resistant-level voriconazole MICs, and we would predict that expression of the *sldA* gene would likely show a return to pre-exposure levels. Future work would be needed to address this possibility.

Combined with the indication within our own RNA sequencing dataset that *wild type* CEA10 downregulates *sldA* expression in response to voriconazole exposure, we propose that regulation of SAC component expression may be a natural response to triazole exposures, just as, coincidentally, increased CIN appears to be a natural response to triazole exposure in *A. fumigatus* and other fungal pathogens. In fact, we propose this is not a coincidence, but points to a likely route whereby directed dysregulation of the SAC by gene expression changes allows fungal pathogens to produce such observed chromosomal instability. This assumption may be supported by the fact that according to our RNA sequencing dataset, the downregulation of *sldA* was not occurring as part of a general suppression of cell cycle genes, but occurred without similar changes to expression of many other SAC components. Previous studies in various eukaryotes from fungi to humans has illustrated that unbalancing the levels of active, available SAC components, including orthologs of the SldA kinase, is sufficient to cause chromosome segregation errors (with the levels of CIN varying slightly depending on the component in question) (**Table C.1**, **C.2**, and **C.3**).

Future work should be done to examine the transcriptional profiles of *wild type A. fumigatus* and Δ*sldA* in response to higher level triazole stress. Studies should also examine gene transcription within lineages experimentally adapted to acquire triazole resistance-level MICs to determine whether transcriptional repression of SAC components including the SldA kinase is a common characteristic in *A. fumigatus* triazole adaptation. Further analyses will also be required to confirm whether whether genes present within any of the chromosomal regions affected by aneuploidy within our triazole adapted strains exhibit altered expression due to the CNV, and to begin to dissect how each aneuploidy might have provided for better fitness in the process of adaptation to medical triazole antifungal.

# Chapter 5

# Discussion

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Ctrl/Alt+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command+Right Arrow, respectively. See the Preface for further details.

The collective theme of my student research has revolved around studying the problem of *A. fumigatus* adaptation to medical antifungals. My studies began with a survey of the literature to gain an understanding of what was known about the acquisition of resistance to mold-active medical triazole antifungals in *A. fumigatus*, along with the mechanisms which were known or suspected to confer resistance to these frontline therapeutics. This work produced the collaborative literature review which is presented in whole in **Appendix A** of my ETD. This work summarized what was known at that point concerning the drivers of resistance development, the routes through which patients may obtain a resistant *A. fumigatus* infection, and some of the best known mechanisms which had been identified up to that point which were suspected or confirmed to reduce the susceptibility level of *A. fumigatus* to one or more mold-active triazole antifungals. I was simultaneously participating in the project which produced the results outlined within the **Appendix B** article. This study recounted the project in which our entire lab coordinated to systematically create a library of mutants made in a wild type background strain of *A. fumigatus*, in which we identified all putative genes predicted to encode protein kinases in the genome, and utilize our modified CRISPR-Cas9 gene editing system to cause individual disruptions of a single gene per mutant. This study evaluated the susceptibility of library mutants to the echinocandins, and characterized several mutants for which the gene was required for echinocandins to cause fungistatic antifungal activity. The information obtained during these two prior works contributed the groundwork on which my own student research project was later built, as both studies played significant roles to guide the aims, hypotheses, and experimental design of my own research project, the results of which are included in their current prepared-for-publication draft form in **Appendix C**.

In fungi, the main sterol within the cell membrane is ergosterol rather than the cholesterol present in mammalian cell membranes. Ergosterol is a vital component in the membrane, contributing to appropriate membrane fluidity and enabling membrane-associated proteins to be oriented correctly (Alcazar-Fuoli and Mellado, 2012; J M Rybak, J R Fortwendel, and Rogers, 2019). It is assumed that the chief cause of triazole-mediated cell death in fungi derives from the depletion of this key membrane component, which leads to membrane instability and ultimately cell lysis, though additional stress may be placed on the cells by other factors such as the accumulation of toxic sterol intermediates (Alcazar-Fuoli and Mellado, 2012; Herrick and Hashmi, 2023). Because of the necessity of ergosterol for fungal cell viability, the majority of antifungal compounds currently available for clinical applications target ergosterol biosynthesis at some point in the pathway (Alcazar-Fuoli and Mellado, 2012; Rodrigues, 2018). Therefore, an adaptation which reduces the effect of EBIs in general would be greatly advantageous to a fungal organism, but potentially disastrous to the patient in the context of treating clinical fungal infection.

**Appendix A** revealed that in this species, most clinically detected triazole resistance is likely to originate as a result of strains which become resistant outside of a host system and before the patient exposure. Due to similarities in the mode of action of azole antifungals used widely in agriculture and industry, strains in the environment obtain cross-resistance to medical triazole drugs after exposure to fungicides drives adaptation and selection. At the time the article Mechanisms of Triazole Resistance in *Aspergillus fumigatus* was published, the mechanisms which had been reported to provide resistance to triazoles revolved almost exclusively around changes in the expression or mutations to the sequences of enzymes that participate in the process of ergosterol biosynthesis in *A. fumigatus*. Overexpression or enhanced removal of drug from the fungal cells was also known to be involved, and enhanced biofilm formation that prevents drug from reaching areas of fungal growth was further suspected to contribute to a loss of susceptibility.

The article provided in **Appendix B** revealed the impact that phosphoregulatory activities of protein kinases are required to maintain viability in the presence of echinocandin stress. In this study, we created and screened a collection of mutants genetically modified for disruption of individual genes predicted to encode protein kinases, most of which had not been studied previously in this species. We discovered through phenotypic screening for altered echinocandin susceptibility that several kinases involved in the uncharacterized tripartite cascade regulating the initiation of septation (the SIN pathway), and two regulatory components which guide their activity all support the fungistatic activity of echinocandins. In their absence, septa are unable to form, which leads to a loss of the protective barriers which normally prevent hyphal damage from continuing from affected compartments through the entire mycelium (Thorn et al., 2024).

As an interesting note, some components of the SIN cascade in budding yeast share homology with components in the mitotic exit network (MEN), a signal transduction cascade which regulates the events necessary to guide a cell through the end stages of

the cell cycle (Tamborrini et al., 2018). The MEN is further connected downstream of the SAC pathway, as the APC/C associated with Cdh1 promotes the silencing of the MEN by marking the polo-like kinase Cdc5 for degradation (Tamborrini et al., 2018). (I mention this simply to highlight the interconnected nature of many signalling pathways *A. fumigatus* and their dependence on the correct function and regulation of various protein kinases).

At the time the review of the literature described above was published (several years ago), the general consensus in the literature at this time was that *A. fumigatus*, and in fact filamentous fungal pathogens collectively, were unable to, or simply did not, utilize aneuploidy to provide positive genomic combinations that promote survival during antifungal stress. While I touched on the known contributions aneuploidy has for common yeast pathogens such as *C. albicans*, no filamentous fungus had ever been reported to possess an aneuploidy associated with triazole resistance.

My project, which began around the time this article was published (mid to late 2020), conflicted with and questioned this generally assumed theory. My results increasingly suggested that chromosomal instability could be involved and likely explained the lessened susceptibility of my mutants to triazoles, later identified as a a pan-azole heteroresistance phenotype also impacting susceptibility to other forms of ergosterol biosynthesis inhibitors. Both fortunately and unfortunately, in 2023, two independent publications vindicated my own assertions by revealing aneuploidies existed within subsets of triazole resistant *A. fumigatus* and *A. flavus*. While my project is now not the first to report this occurrence, it is the first to illustrate that a specific aneuploidy is selected for in the majority of lineages during voriconazole adaptation *in vitro*, and is the first to propose a reasonable mechanism that explains how *A. fumigatus* (and other fungal pathogens) can induce CIN and obtain aneuploidies in the first place, (which can then be selected for and the best combinations become the most represented in the context of antifungal stress).

At the onset of the research described in **Appendix C**, we predicted that various protein kinases play vital roles for stress responses to triazole antifungals in *A. fumigatus*. We originally expected to discover increases in triazole susceptibility resulting from loss of certain kinases. Instead, our screen of the kinase disruption library previously constructed in our lab (**Appendix B**) revealed only two mutants for which the MIC was shifted by more than a single dilution, and in both cases was indicative of a reduction in triazole susceptibility (**Figure C.1**). The identification of a triazole phenotype for loss of Ssn3 was not surprising, as this kinase has been previously characterized in *A. fumigatus* and the phenotype is known (Long et al., 2018). Loss of azole efficacy in *ssn3* mutants was previously linked to a multi-faceted resistance strategy: Deletion resulted in overexpression of resistance-associated genes such as *cyp51A* and drug efflux transporters which can actively remove triazole from the fungal cell, combined with an enhanced capacity for biofilm formation which could preclude drug entry into the cells (Long et al., 2018). Identifying *ssn3* in our screen validated the usefulness of the disruption library for identifying antifungal stress phenotypes associated with loss of protein kinase function in *A. fumigatus*.

Moreover, the discovery of a triazole phenotype when the *A. fumigatus* kinase ortholog SldA is non-functional was novel. This discovery warranted further study to expound on the impacts of SAC pathway dysfunction on *A. fumigatus* susceptibility to antifungals, and begin to explore whether it might contribute to loss of susceptibility observed in *wild type A. fumigatus*.

The spindle assembly checkpoint, also referred to simply as the mitotic checkpoint, is a highly conserved eukaryotic quality control pathway which is vital to protect genomic integrity (Musacchio and Salmon, 2007). This checkpoint becomes active during metaphase of the cell cycle and prevents the incorrect distribution of chromosomes to daughter nuclei during anaphase by monitoring the status of associations between microtubules and the kinetochores, proteinaceous structures that associate with the centromere of condensed chromosomes (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). Occurrence of (or predisposition to) chromosomal missegregation is referred to as chromosomal instability (CIN) and can result in aneuploidy (Thompson, Bakhoum, and Compton, 2010). Aneuploidy is the loss or gain of sections of the DNA within chromosomes or of entire chromosomes within a nucleus (Selmecki, Forche, and Berman, 2010; Bolanos-Garcia and Blundell, 2011; Garribba and Santaguida, 2022). The SAC prevents this by facilitating the resolution of errors in the associations of microtubules emanating from each spindle pole body (SPB) to the kinetochores of duplicated chromosomes, and arresting mitosis until correct attachments are accomplished (**Figure C.2** and **C.3**). A complex series of sensing and signaling interactions involving various components supports this pathway. While the exact responsibilities and associations of components may differ between species, their essential functions are generally highly conserved across eukaryotes (Musacchio and Salmon, 2007; T. Kim and Gartner, 2021).

Components of the kinetochore facilitate signalling pathway events that guide the progression of mitosis. The kinetochore is a large complex of various proteins which assembles onto the centromere of a condensed chromosome during mitosis (**Figure C.3**). This structure contains the point to which microtubules emanating from a spindle pole will attach. The KMN network, composed of Knl1/Mis12 and Ndc80 (Hec1 in human cells), resides at the kinetochores to mediate microtubule attachment (Kline-Smith, Sandall, and Desai, 2005; T. Kim and Gartner, 2021). The Ndc80 complex recruits the kinase Mps1 to the kinetochore. In budding yeast, Mps1 is required for formation of the mitotic checkpoint complex at the kinetochore (Brady and K G Hardwick, 2000). The mitotic checkpoint complex (MCC) is a diffusible SAC effector complex composed of the components Mad2, Cdc20, Bub3 (SldB in *A. nidulans*), and a key SAC kinase which is required for checkpoint integrity known by names such as BUB1, BUBR1, MAD3, or SldA depending on the species involved (Edgerton, Paolillo, and Oakley, 2015; T. Kim and Gartner, 2021). In order for the MCC to assemble at unattached kinetochores, several events first occur. In various species, Mps1 (Mph1 in some species) phosphorylates the KMN component Knl1, which recruits other SAC components to the kinetochore including Aurora B (Ipl1 in budding

yeast), which is the enzymatic component core of the Chromosomal Passenger Complex (CPC), and the MCC components Bub1 and Bub3 in complex (Efimov and Morris, 1998; Fisk, Mattison, and Winey, 2004; Vader, Medema, and Lens, 2006; De Souza et al., 2013; Leontiou et al., 2019; T. Kim and Gartner, 2021). Mps1 also directly phosphorylates the central SAC kinase Bub1, (orthologous to SldA), at the kinetochore, which enables Aurora B to also phosphorylate Bub1. The combined activities of the kinases Mps1, Bub1, and Aurora B subsequently promote the recruitment of Mad1 and the MCC component Mad2 to unattached kinetochores (Chan and Botstein, 1993; Abrieu et al., 2001; Fisk, Mattison, and Winey, 2004; T. Kim and Gartner, 2021; Roy et al., 2022). Bub1 and/or BubR1 kinase in mammalian cells also recruits the MCC component Cdc20 and other SAC effectors including the kinase Plk1 (PlkA in *A. nidulans*) and the Rod/Zw10/Zwilch (RZZ) complex to unattached kinetochores to promote SAC activation (Basenko et al., 2018; T. Kim and Gartner, 2021; Roy et al., 2022). Moreover, activated Bub1 in some species is known to in turn phosphoregulate Aurora B, which regulates the turnover of kinetochore-microtubule attachments to facilitate error correction (Cimini et al., 2006; R. M. Ricke, Jeganathan, and Deursen, 2011). (In human cells, Bub1 directly regulates Cdc20 and also facilitates phosphorylation of Cdc20 by Plk1 to promote inhibition of the E3 ubiquitin ligase anaphase promoting complex (APC/C) (L. Jia, B. Li, and H. Yu, 2016)).

Defects which impact the function or even level of gene expression of many kinetochore components in other eukaryotic species has been previously shown to result in some level of detectable failure to sort chromosomes correctly during mitosis. (A list of components of the kinetochore and SAC for which alteration results in CIN in at least one species studied was provided in **Table C.1**, **C.2**, and **C.3**). As a note, the *A. fumigatus* ortholog of Mps1, likely encoded by AFUB_041010 in CEA10/A1163 or Afu3g08100 in Af293, appears to remain uncharacterized but is reported as essential in *A. nidulans* (De Souza et al., 2013; Basenko et al., 2018). The ortholog of Knl1 is Spc105 in *S. cerevisiae* or Spc7 in *Schizosaccharomyces pombe* and *Fusarium oxysporum*. The Knl1 ortholog appears uncharacterized in both *A. fumigatus* and *A. nidulans* but likely encoded by AFUB_021210 in A1163 and AN5221 in *A. nidulans* strain FGSC A4 (Basenko et al., 2018; Roy et al., 2022). The *A. fumigatus* ortholog of Aurora B is likely encoded by the gene AFUB_023600 in A1163, but, again, appears to remain yet uncharacterized. The *A. fumigatus* ortholog of Plk1, likely encoded by the gene AFUB_081870 in Cea10/A1163 appears also to remain uncharacterized but deletion results in lethality in *A. nidulans* (Bachewich, Masker, and Osmani, 2005; Basenko et al., 2018)). We were unable to generate a viable disruption mutant for the predicted orthologs of Mps1, Aurora B, or Plk1 after three separate transformation attempts, indicating that these are likely also essential in *A. fumigatus* (**Appendix B**). Therefore, the *A. fumigatus* SAC and various components supporting the correct attachment and segregation of the chromosomes remains an area that is in need of further study.

Even disruption of the formation of the kinetochore, the anchoring of the spindle to the cell's periphery, the positioning of the spindle, or the connections between microtubules

and the SPB can overwhelm the SAC's ability to prevent chromosomal instability (Chan and Botstein, 1993; Maier et al., 2013; Tame et al., 2014; R. T. Todd, A. Forche, and A. Selmecki, 2017) (**Table C.1**, **C.2**, and **C.3**). Centrosomes in humans (and SPB in fungi) are anchored to the actin filaments of the cell cortex via dynein, with the help of several other proteins including Num1/NuMA/LIN-5 orthologs (Maier et al., 2013; McNally, 2013; Tame et al., 2014; Jez, 2021) (**Figure C.2**). In human and *C. elegans* cells, dynein maintains the tension required by acting as a minus-end directed motor protein positioning and pulling the astral microtubules of the centrosomes/SPB toward the cortex (Morin and Bellaiche, 2011; Kotak, Busso, and Gonczy, 2012; Maier et al., 2013). Defects impacting the stability of the cortex to which the SPB anchors via astral microtubules, or the linkages between the astral microtubules and the polar cortical actin (via the motor proteins dynein and Myo10) can result in misoriented spindle and mitotic errors (Maier et al., 2013; Tame et al., 2014; Balkunde and Dixit, 2021; Jez, 2021). At higher concentrations, benomyl is proposed to entirely block the formation of the mitotic spindle (Edgerton, Paolillo, and Oakley, 2015). Some evidence exists which suggests that exposure to benzimidazoles, which compromise the structural integrity of the spindle and can result in CIN, could produce spontaneous triazole resistance in the species *Aspergillus flavus* (Akbari Dana et al., 2019). In the study referenced, ten isolates were evolved *in vitro* to a benzimidazole fungicide compound. Following this process, MIC assays revealed that three lineages spontaneously gained resistance to triazoles (voriconazole and itraconazole) and reduced susceptibility to amphotericin B (Akbari Dana et al., 2019). This further supports the assumption that spontaneous aneuploidy formation, whether as a result of SAC component deficiency or chemical spindle stress, can provide spontaneous triazole adaptation. In *wild type* cells of various species, dosage with nocodazole or benomyl activates the SAC by causing defects in spindle tension, prompting the BUB1/BubR1/MAD3 kinase ortholog to signal for mitosis to arrest (Bolanos-Garcia and Blundell, 2011; Goto et al., 2011). In organisms lacking SAC components such as an BUB1/BUBR1/MAD3 ortholog, cells become hypersensitive to microtubule destabilizing agents such as benzimidazoles or paclitaxel. Moreover, in fungal species, loss of SAC components becomes synthetically lethal with loss of components which maintain the structure and/or the positioning of the spindle, including motor proteins which maintain spindle tension such as dynein or kinesin-like motor proteins such as BimC (Efimov and Morris, 1998; Glotzer, 2009; Redemann et al., 2010; Morin and Bellaiche, 2011; Maier et al., 2013; McNally, 2013; Tame et al., 2014; Balkunde and Dixit, 2021). The basis for these phenotypes is likely shared: A combined impact of KT-MT errors with further spindle disruption likely creates a level of mitotic instability which becomes inviable. With both deficiency of a vital SAC component and spindle stability, the process of chromosome sorting likely crashes. The chaperone Hsp90 functions for assembly of the kinetochore and of the components of the kinetochore to which KT-MT attach. Depletion or chemical inhibition of Hsp90 both result in CIN and spontaneous aneuploidies which confer reduced susceptibility to fluconazole, as well as to benzimidazoles and forms of genotoxic stress (Stemmann et al., 2002; Davies and Kaplan, 2010; G. Chen et al., 2012; Pennisi, Ascenzi,

and Masi, 2015). Defects in the Ndc80 complex cause chromosome missegregation due to instability of KT-MT attachments and prolonged SAC activation. In *C. albicans*, deletion of the kinase Bub1 or the downstream target Sgo1 were already known to reduce fluconazole susceptibility (Brimacombe et al., 2019). Deletion of BUB1 or Sgo1 both reduce fluconazole susceptibility in *C. albicans* (Brimacombe et al., 2019). In eukaryotes from fungi to humans, depletion or mutation of the conserved members of the $Ndc80^{Hec1}$ complex or the Histone H3-like centromeric protein A (CENP-A/Cse4) both result in unstable KT-MT attachments, misaligned chromosomes, and incorrect chromosome segregation (Kline-Smith, Sandall, and Desai, 2005; Brimacombe et al., 2019). (CENP-A is a variant of histone H3 which replaces H3 in the nucleosomes organizing centromeric chromatin and is the site of assembly of the "inner kinetochore" subcomplex) (Orthaus et al., 2008) (**Figure C.3**). Imbalances due to overexpression of CENP-A in human cells also result in CIN, lagging chromosomes and formation of micronuclei (Shrestha et al., 2021).

The genome of eukaryotic organisms encode a key kinase involved in the SAC, the functions of which are highly conserved across eukaryotes (Suijkerbuijk et al., 2012; T. Kim and Gartner, 2021). Depending on the species, an organism may possess two paralogous genes encoding proteins which combined have the active domains required for the checkpoint functions, or a single kinase which alone must possess all of the required domains (Bolanos-Garcia and Blundell, 2011; Suijkerbuijk et al., 2012). For example, both humans and *Drosophila melanogaster* possess the two kinases BUB1 and BUBR1 (Musacchio and Salmon, 2007; Suijkerbuijk et al., 2012). Meanwhile, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* possess the kinase proteins BUB-1, Bub1p, and Bub1 respectively, and the paralogous non-kinase proteins SAN-1, Mad3p, and Mad3 respectively, in which the kinase active domain was lost (Suijkerbuijk et al., 2012). However, some eukaryotic organisms instead retain only a single kinase ortholog. This is the case for *Apis mellifera* (honeybees, uncharacterized gene LOC726325), and *Cryptococcus neoformans* (BUB1), *Neurospora crassa* (div-1/Mad3), *Fusarium oxysporum* (bub1), and *Aspergillus* species including *A. flavus* (uncharacterized gene F9C07_2225604), *A. niger* (uncharacterized gene ATCC64974_88510), and *A. nidulans* (SldA) (Efimov and Morris, 1998; Suijkerbuijk et al., 2012; De Souza et al., 2013; Edgerton, Paolillo, and Oakley, 2015; Basenko et al., 2018). In the study outlined in **Appendix C**, we confirmed that *Aspergillus fumigatus* encodes a single ortholog of the kinase which we have named SldA (Basenko et al., 2018).

In other species, orthologs of the BUB1/BUBR1/MAD3 kinase are known to play vital roles for the SAC including protection of cohesion between sister chromosomes prior to anaphase, sensing for the correct tension attained when chromosomes are correctly oriented and attached to spindle microtubules, and signalling to recruit the appropriate error resolution machinery to the kinetochore (Straight et al., 1996; Kawashima et al., 2010; Brimacombe et al., 2019; Roy et al., 2022). In response to detection of errors, the orthologs of the kinase in other species participate in several crucial phosphoregulatory signaling events. When the SAC kinase senses unsatisfactory tension of the spindle (which indicates improper

chromatid bi-orientation or kinetochore attachments), the kinase plays a role as part of the diffusible SAC effector MCC, which has the responsibility to inhibit the APC/C until the checkpoint is satisfied (Musacchio and Salmon, 2007; Suijkerbuijk et al., 2012) (**Figure C.3**). Orthologs of this central SAC kinase also uphold the SAC by phosphoregulation of targets such as the centromere-associated histone H2A, the shugoshin Sgo1, and Aurora B in other eukaryotic species (Fernius and K. G. Hardwick, 2007; Kawashima et al., 2010; Jin, Bokros, and Y. Wang, 2017; Brimacombe et al., 2019). This phosphorylation of H2A signals for recruitment of shugoshin, which preserves the cohesion between the centromeres of the sister chromatids by promoting the retention of cohesin (Y. Watanabe and Kitajima, 2005; Kawashima et al., 2010; Jin, Bokros, and Y. Wang, 2017). The shugoshins also in turn recruit to the centromere protein phosphatase 2A (PP2A) and the Chromosomal Passenger Complex (CPC), of which the kinase Aurora B is a component (Siemeister et al., 2017; Brimacombe et al., 2019). Aurora B promotes the resolution of chromosomes incorrectly attached to microtubules (Chan and Botstein, 1993). Once the kinetochores are correctly attached to KT-MT and adequate tension is sensed in the spindle due to the alignment and bi-orientation of all chromosome pairs, the BUB1/BUBR1/MAD3 kinase activity is silenced and the APC/C is released from inhibition. At this point, the APC/C ubiquitinates various substrates including Cyclin B and Securin to allow chromosome cohesion to be removed and anaphase to proceed (Vanoosthuyse and K. G. Hardwick, 2009; T. Kim and Gartner, 2021).

The spindle assembly checkpoint requires the support and participation of many individual enzymes and complexes. Defects within various components of the checkpoint or of the function of the mitotic spindle can disrupt the entire system, leading to imbalanced chromosome sorting during anaphase (Chan and Botstein, 1993; Basu et al., 1999; Fisk, Mattison, and Winey, 2004; Kamthan et al., 2014; Sherwin and Y. Wang, 2019). These defects can come in the form of entire gene loss, mutation, or even altered levels of transcription, and can impact chromosome segregation at multiple points of the process, including disrupting the movements of the chromosomes along the microtubules, sabotaging the ability to recruit components to the kinetochore, and disabling the interactions between or activity level of components. As mentioned in **Appendix C**, abnormal Bub1 or BubR1 expression is linked to aneuploidy and cancer in human cells, illustrating again how careful regulation of SAC component expression is required for chromosomal stability (Dai et al., 2004; Pinto et al., 2008; Ando et al., 2010). (Changes in expression of Mad1 and Mad2 are also implicated in human cancers) (Pinto et al., 2008). In the context of cancer, BUB1 alteration appears to combine both the enhanced generation of aneuploidies which confer fitness benefits to the cancerous cells including enhanced proliferation and the loss of the surveillance system which normally would put the brakes on erroneous mitotic progressions (Bolanos-Garcia and Blundell, 2011; Garribba and Santaguida, 2022). Unfortunately, studies also link such BUB1 defects to acquisition of resistance to anti-cancer chemotherapeutic drugs (Kops, Weaver, and Cleveland, 2005). As an interesting note, screening for mutations in the genes encoding the kinase orthologs BUB1 and BUBR1 in humans may be used to inform cancer

prognosis (unfortunately as a marker of lower chance of survival) and the human BUB1 kinase is now being explored as a potential target for anti-cancer drugs (Siemeister et al., 2017; Bolanos-Garcia and Blundell, 2011).

Aneuploidy has long been associated with adaptation to antifungals in yeast species. Many clinical isolates of Candida species are found to be aneuploid (Polakova et al., 2009). First discovered as the mechanism conferring fluconazole resistance within a *C. albicans* patient isolate, chromosome 5 (Ch. 5) aneuploidy is now one of the most common triazole-resistance associated aneuploidies reported in *Candida* species (Selmecki, Forche, and Berman, 2006; Selmecki, Gerami-Nejad, et al., 2008; McTaggart et al., 2020; X. Fan et al., 2023). A study of 90 triazole resistant clinical isolates of *C. albicans* found that 50% possessed some form of aneuploidy, half of which possessed the isochromosome 5L aneuploidy (i(5L)) (Selmecki, Forche, and Berman, 2010). Chromosome 5 contains the gene encoding 14$\alpha$-sterol demethylase enzyme of *C. albicans*, ERG11 (ortholog of *A. fumigatus* Cyp51A/B) (Basenko et al., 2018). The chromosome also contains the TAC1 transcription factor, which upregulates the expression of drug genes encoding the ABC superfamily multidrug transporters CDR1 and CDR2 (M S Skrzypek et al., 2017; McTaggart et al., 2020). Studies of *Candida* species possessing additional Ch. 5 reveal triazole susceptibility loss is directly due to increased expression of these genes. This triazole resistance-conferring aneuploidy can appear in several forms: As a supernumerary chromosome (SNC), a an isochromosome, or as an additional portion of the left arm of chromosome 5 incorporated into existing chromosome 5 after the telomere (Selmecki, Forche, and Berman, 2006; Selmecki, Gerami-Nejad, et al., 2008; A. M. Selmecki et al., 2009; McTaggart et al., 2020). While this aneuploidy occurs in *C. albicans*, *C. auris*, and *C. neoformans* isolates both spontaneously and through *in vitro* and *in vivo* triazole evolution, the mechanism was initially discovered isolates from patients with *C. albicans* infection who were failing triazole therapy (Selmecki, Forche, and Berman, 2010; J. Kim et al., 2012; Brimacombe et al., 2019; R. T. Todd and A. Selmecki, 2020; Kukurudz et al., 2022; X. Fan et al., 2023; R. T. Todd, Soisangwan, et al., 2023). Its important to note that certain chromosome 5 aneuploidies in yeast species can also confer resistance or tolerance to echinocandins, and studies have suggested the potential for aneuploidy to confer increased virulence (Polakova et al., 2009; Sah, Hayes, and Rustchenko, 2021; Sun et al., 2023).

*C. albicans* is often found to accumulate aneuploidies when exposed to fluconazole, and defects in the cell cycle have been previously suggested as the route for these changes to occur (Hill et al., 2013; Harrison et al., 2014). In one study, every lineage of *C. albicans* acquired a chromosome 5L aneuploidy during *in vitro* adaptation to fluconazole, while none were detected in the triazole susceptible parent strain. These strains were confirmed to overexpress ERG11 and TAC1 (Selmecki, Forche, and Berman, 2006; Selmecki, Gerami-Nejad, et al., 2008; A. M. Selmecki et al., 2009). (Additionally, some of the populations accumulated other aneuploidies, specifically impacting chromosomes 3-7, and cells which had acquired several of these additional chromosomes or chromosomal portions in addition to the over-representation of Chromosome 5L typically possessed the highest levels of

triazole resistance). Moreover, once these adapted strains were transferred to grow in drug-free medium, the aneuploidies and the adaptation to triazole were commonly lost. However, this was not always the case. The aneuploidy became a fixed characteristic in several of the lineages, remaining in the cells and their progeny even in the absence of triazole stress.

Prior studies of aneuploidy-mediated triazole resistance in yeast have proposed that triazole exposure might produce heterogeneous populations of progeny; some possessing aneuploidies and others retaining a *wild type* genome size (Bing et al., 2020). In one study, eight fluconazole-adapted *C. auris* lineages produced WGS results that were consistent with the entire lineage population exhibiting a whole chromosome 5 duplication (a CNV of exactly or very close to 2, indicative of a duplication of the chromosome in the vast majority of progeny cells). In contrast, eleven strains exhibited an increase in copy numbers across the entirety of chromosome 5, but which amounted to CNV levels that was somewhere in between the haploid and the diploid number. This indicated that these strains possessed a mixture of *C. auris* progeny with and without Ch. 5 duplication (Bing et al., 2020). Moreover, the researchers point to the possibility that the aneuploidy development may provide heteroresistance that allows for eventual development of genetically-fixed mutation-based resistance. While lineages removed from triazole stress generally lost both aneuploidy and adaptation, lineages which were maintained in triazole-containing media retained the aneuploidies and eventually acquired resistance-associated mutations. This article supported our assumption that a small sub-population of cells possessing a variety of beneficial CNV are responsible for the shift in MIC within our *A. fumigatus* Δ*sldA* and Δ*sldB* strains.

These previously known findings harmonize with the results of my own student research project. Just as deletion of the SldA kinase ortholog Bub1 in *C. albicans* allowed the mutants to show increased aneuploidy levels, loss of SldA or its binding partner SldB resulted in increased baseline production of aneuploid progeny detectable by flow cytometry (Brimacombe et al., 2019) (**Figure C.10B**). Moreover, in the case of *C. albicans* Bub1 deletion, mutants were less susceptible to the triazole compound fluconazole. Fluconazole does not have good activity against *A. fumigatus*, but our assays using mold-active triazole antifungals showed that Δ*sldA* and Δ*sldB* showed reduced susceptibility to every triazole tested (**Figure C.7D** through **G**). Moreover, we found that the SAC mutants exhibited a heteroresistance phenotype in which only a few progeny were fit to survive at higher concentrations of triazole past the wild type MIC, while the majority lacked whatever change these few offspring possessed. This phenotype is commonly reported in other yeast fungal pathogens in cases where sub-populations show reduced triazole susceptibility due to the possession of certain aneuploidies conferring fitness benefits (Sionov, Y. C. Chang, et al., 2009; Sionov, H. Lee, et al., 2010; Y C Chang, Khanal Lamichhane, and Kwon-Chung, 2018; Bing et al., 2020; Kukurudz et al., 2022; Sun et al., 2023). (Moreover, the hyphae observed to grow within the MIC assay wells appeared to possess a range of phenotypic triazole

susceptibility, with some hyphae growing seemingly indifferent to the presence of the drug, while others grow past the MIC of the *wild type*, but poorly. These observed behaviors likely are due to the inherent unpredictability of chromosome missegregation events. Only subsets of aneuploid conidia will inherit the "right" chromosomal abnormalities to confer a fitness benefit, and of these, some combinations would produce more profound impacts than others). So, an increased predisposition to chromosomal instability likely explained the phenotype of our SAC mutants. Moreover, exposure of *C. albicans* to triazoles increases the rate of aneuploidy, and in my project, I found the level of aneuploidy to increase not only in my SAC deficient mutants, but also in *wild type A. fumigatus* during triazole exposure, indicating that triazoles also increase the incidence of CIN in *Aspergilli* as in *Candida* species. Also, just as *C. albicans* with SAC defects due to insufficient Bub1-mediated phosphoregulation show a capacity to select for chromosome 5 duplications in the context of triazole stress, both my Δ*sldA* and *wild type* lineages selected for a specific chromosomal duplication impacting nearly the exact same region of chromosome two in four out of six independent lineages. If the *wild type* leveraged suppression of SldA kinase expression in order to achieve a similar level of CIN as our SldA deletion strain, this would fall perfectly in line with previous literature. Downregulation of the kinase orthologs of SldA has been demonstrably shown to cause SAC defects by leaving the cells with insufficient amounts of active Bub1 kinase to perform required duties. Specifically, this lack of Bub1 causes failure to recruit and/or regulate components at the kinetochore. And furthermore, this tends to result in a failure to regulate chromosome cohesion and KT-MT attachment error resolution, leading to shearing and fragmentation of chromosomes and formation of structurally abnormal chromosomes, just like the abnormal chromosome two detected in my triazole adapted lineages. And, as covered in **Chapter 4**, I found in my preliminary analysis of RNA sequencing data, that expression of *sldA* was downregulated in wild type CEA10, while many other genes involved in the SAC remained unchanged in regard to expression level. I am not assuming or proposing that altered transcription of the SldA kinase is the only route, or possibly even the chief route for SAC defects that allow for aneuploidy-based adaptation. Indeed, altered expression of CENP-A was already reported to enable *C. albicans* to show loss of fluconazole susceptibility in a very similar degree to deletion of Bub1 or Sgo1, and so changes to the expression of other SAC-regulating components is likely to be involved (Brimacombe et al., 2019). In fact, based on my search of the available transcriptomics datasets reporting changes in gene expression in triazole adaptation contexts, it appears that changes such as downregulation of multiple genes at once, without changes to the remaining SAC-supporting components, may be involved in destabilizing the SAC. So, then, just as several different mechanisms may develop in a strain that each contribute to a portion of the observed resistant phenotype, it is likely that slight changes to several SAC effectors occur to ultimately cause a larger overall failure of the pathway that prevents chromosome missegregation in mitosis. However, based on the collected facts, I propose that altered transcription of the SldA kinase is a natural response to triazole exposures, is likely sufficient by itself to cause some low-level of CIN (though is likely often combined

with simultaneous changes in several other components), and represents one route whereby *wild type A. fumigatus* promotes the acquisition of aneuploidies which are beneficial during adaptation to medical triazole antifungals. Future work should explore the possibility that aneuploidy may underlie the occurrences of patient infection which is not cleared by triazole therapy, but which, after being removed from the patient and cultured (in the absence of triazole stress), produces a susceptible-level triazole MIC. It may be that in *A. fumigatus*, aneuploidies contribute to context-dependent triazole phenotypes which reduce or disappear entirely once removed from the context of triazole stress.

# Appendix A

# Chapter 2 Article

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Ctrl/Alt+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command+Right Arrow, respectively. See the Preface for further details.

## A.1 Introduction

Final submission reproduced with open access permission. A. V. Nywening et al. (Dec. 2020). "Mechanisms of triazole resistance in Aspergillus fumigatus". In: *Environ Microbiol* 22.12, pp. 4934–4952. ISSN: 1462-2912 (Print) 1462-2912. DOI: 10.1111/1462-2920.15274.
**Author's Pre-Publication Manuscript:**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7828461/pdf/nihms-1662156.pdf

This appendix provides the pre-print final author manuscript submission of the published article, for which the author of this ETD was the primary author. This manuscript is included as an appendix as it provides a clear and detailed overview of the issue of triazole antifungal resistance in the fungal pathogen *Aspergillus fumigatus* as it was understood at the time of publication. Therefore, the content of this article are directly relevant to the premise, rationale, interpretation of the data and results, and conclusions presented within the body chapters of this ETD.

## A.2 Article

### Mechanisms of Triazole Resistance in *Aspergillus fumigatus*

Ashley V. Nywening [1,2], Jeffrey M. Rybak [1], P. David Rogers [1], and Jarrod R. Fortwendel [1]

[1]Department of Clinical Pharmacy and Translational Sciences, The University of Tennessee Health Science Center, 881 Madison Avenue, Memphis, TN, USA. [2]College of Graduate

Health Sciences, Integrated Biomedical Sciences Program, The University of Tennessee Health Science Center, Memphis, TN 38163, USA.

## Contact Address:

Jarrod R. Fortwendel, PhD University of Tennessee Health Science Center College of Pharmacy 881 Madison Avenue, Rm 343 Memphis, TN 38163

## Key Words:

Aspergillus, antifungal, triazole, resistance, ergosterol

### Originality-Significance Statement:

This work addresses the understudied but significant issue of triazole antifungal resistance in the human and animal pathogen *Aspergillus fumigatus*. Though resistance within this species represents a threat to ecology and public health, much information concerning its origination and causes is limited, frustrating comprehension and progress in this area. This work concisely summarizes the process of triazole antifungal resistance progression within both patients and environmental settings as it is currently understood, as well as discussing multiple reported biological mechanisms associated with clinical and environmental triazole resistance in *Aspergillus fumigatus*.

## Abstract

The ubiquitous fungal pathogen *Aspergillus fumigatus* is the primary cause of opportunistic mold infections in humans. Aspergilli disseminate via asexual conidia passively traveling through air currents to germinate within a broad range of environs, wherever suitable nutrients are found. Though the average human inhales hundreds of conidia daily, *A. fumigatus* invasive infections primarily affect the immunocompromised. At-risk individuals can develop often fatal invasive disease for which therapeutic options are limited. Regrettably, the global insurgence of isolates resistant to the triazoles, the frontline antifungal class used in medicine and agriculture to control *A. fumigatus*, is complicating the treatment of patients. Triazole antifungal resistance in *A. fumigatus* has become recognized as a global, yet poorly comprehended, problem. Due to a multitude of factors, the magnitude of resistant infections and their contribution to treatment outcomes are likely underestimated. Current studies suggest that human drug-resistant infections can be either environmentally acquired or *de novo* host selected during patient therapy. While much concerning development of resistance is yet unknown, recent investigations have revealed assorted underlying mechanisms enabling triazole resistance within individual clinical and environmental isolates. This review will provide an overview of triazole resistance as it is currently understood, as well as highlight some of the prominent biological mechanisms associated with clinical and environmental resistance to triazoles in *A. fumigatus*.

## Introduction

Among species of human filamentous fungal pathogens, one of the most pestilent is *Aspergillus fumigatus*, a saprophytic fungus extant in much of the world (Anastasi, Varese, and Marchisio 2005; Nierman et al. 2005; Denning 1998). This hyaline septate mold is ubiquitous, found in a plethora of natural climates and ecosystems as well as urban settings. While every human is estimated to inhale hundreds of conidia daily of this opportunistic pathogen, invasive aspergilloses, (IA,) primarily affect humans with a compromised immune system. *A. fumigatus* represents a major cause of filamentous fungal infections among susceptible patient populations as well as affecting many domesticated animals including dogs, wild animals such as birds, and even insects (Seyedmousavi et al. 2015; Beernaert et al. 2009; Verweij, Chowdhary, et al. 2016; Bowyer, Bromley, and Denning 2020; Valdes et al. 2018). Infection culminates in a spectra of disease severity ranging from allergic conditions to invasive and disseminated aspergillosis (Groll and Walsh 2001; van der Linden et al. 2015). Overall mortality varies depending on the severity and type of underlying infection and on patient-specific factors such as severity of immunosuppression. Regardless of treatment, invasive disease poses a universally high mortality rate ranging from 30% to 90% for most patient types even with current antifungal therapies (Rybak, Fortwendel, and Rogers 2019; Denning et al. 2011; Verweij, Chowdhary, et al. 2016; Tekaia and Latge 2005; Falcone et al. 2011). Aspergillosis can be chronic, requiring continual and prolonged antifungal treatment lasting years in duration. In the recent past, invasive aspergilloses have seen a dramatic rise in incidence in multiple areas of the world. This epidemiological pattern of increasing incidence is largely due to prolonged survival of immunocompromised and immunosuppressed individuals, but is also linked to other shifting host-specific variables, changing environmental conditions, and to pathogenic adaptation to selective pressures (Brissaud et al. 2012; Groll et al. 1996; Groll and Walsh 2001; Lass-Florl and Cuenca-Estrella 2017; Verweij, Chowdhary, et al. 2016).

*Aspergillus fumigatus* is a saprophytic mold which primarily acts as a decomposer of organic matter (Latge 1999; Dagenais and Keller 2009). Aspergilli primarily reproduce asexually by producing many tiny structures known as conidia which are designed for dispersal through air (Levetin 2004). When some force such as weather, construction, landscaping, agricultural activity, animal activity or wind disturbs the mold's environment, conidia are dislodged. These structures can travel in contaminated material, air, and/or water and will germinate to grow wherever conditions and nutrients permit. Consequently, *A. fumigatus* is commonly found growing in both outdoor spaces and human structures. The fungus can be found subsisting on a wide range of ecological spaces such as within farmlands, greenhouses and in aquatic habitats including on surfaces including leaves, leaf litter, rotting wood, in soils and compost (Paulussen et al. 2017; Basenko et al. 2018; Ren et al. 2017; Zhang et al. 2017). Moreover, the species exists within indoor locales circulating in air as buoyant spores or in bioaerosols, on various surfaces, and within virtually any material or substance with adequate organic residues (Paulussen et al. 2017). *A. fumigatus*

is even found growing within concrete and flooring material, especially following water damage (Andersen et al. 2011). For example, one source suggests that between 10% and 50% of buildings within North America and Europe exhibit levels of moisture suitable for fungal growth and between 15% and 40% of homes harbor mold within the structure (Andersen et al. 2011).

Conidia of *A. fumigatus* can be two to three micrometers in diameter, small enough to become lodged in lung alveoli. Both animals and humans commonly inhale conidia from *A. fumigatus*. The fungal spores are usually cleared by innate immune mechanisms. However, conidia that withstand host defenses can germinate, grow, and subsist by destroying surrounding tissue for nutrients (Dagenais and Keller 2009). The pathogen can spread and disseminate to further sites via hyphal growth or angioinvasion and even conidiate inside a host within an aspergilloma (Eggimann et al. 2006; Dagenais and Keller 2009). While allergic bronchopulmonary aspergillosis (ABPA) and aspergillomas can occur, infections in healthy individuals are rare (Latge 1999).

One of the most common causes of invasive fungal infections in immunocompromised humans, *A. fumigatus* poses a threat to populations with predisposing conditions including solid-organ and stem cell transplant recipients, individuals undergoing certain chemotherapies, steroid use, those with acquired or inherited immunodeficiencies, severe liver disease, and with conditions affecting the lungs such as asthma, COPD, cystic fibrosis, bronchiectasis, sarcoidosis, respiratory infections such as influenza and very likely the COVID-19 coronavirus (Koehler et al. 2017; Lopez-Medrano et al. 2016; Steinbach et al. 2012; Wiederhold and Verweij 2020; Kosmidis and Denning 2015; Falcone et al. 2011; Resendiz Sharpe et al. 2018). In such individuals, infections typically begin in the lung following inhalation of fungal conidia but may then disseminate through tissue and bone to other locales. While the general inoculum required to cause disease and the incubation period for development of aspergillosis are not defined, infection likely depends on various aspects including the size of the fungal exposure, virulence of the strain, and host-specific factors (CDC 2019; Dagenais and Keller 2009; Paulussen et al. 2017). Excessive contact with conidia, for example, can lead to invasive disease for even immunocompetent humans (Kosmidis and Denning 2015). Moreover, while the link between fungal exposures and development of allergy such as ABPA, allergic *Aspergillus* sinusitis (AAS,) or *Aspergillus*-induced asthma (AIA) are clear, host specific factors such as genetics appear to determine susceptibility to allergic disease more than any external factor (Andersen et al. 2011; Shah and Panjabi 2016).

With developments and advances in modern medical strategies came the extended survival of immunocompromised populations and concurrent increases in certain diseases such as IA (Latge 1999). Multiple studies have attempted to approximate the global impact of aspergilloses and to discern any appreciable changes in incidence over time. Surveys have recently estimated the current burden of aspergilloses worldwide, approximating 4.8 million cases of ABPA, 3 million cases of chronic pulmonary aspergillosis (CPA,) and between 250,000 and 400,000 cases of IA annually (Brown et al. 2012; Bongomin et al. 2017;

Bongomin et al. 2020; CDC 2019). IA occurs in up to 14% of heart transplant recipients, up to 7% of bone marrow transplant recipients, and up to 10% of hematological malignancy and lung transplant patients (Eggimann et al. 2006).

Unfortunately, within the United States, aspergillosis is not considered a reportable infection. Certain areas such as Seattle, Washington, where a children's hospital has had fourteen cases and lost seven patients since 2001 to nosocomial aspergillosis linked to contaminated air circulation systems, are moving to require reporting of aspergillosis to promote better responses to the disease (Staff 2019). However, the CDC does not require medical personnel to recount cases of confirmed or suspected disease in human patients, (though the CDC did prompt passive surveillance specifically for triazole resistant clinical isolates of *A. fumigatus* beginning in 2011) (Pham et al. 2014). Consequently, accurate numbers of active or past cases in the U.S. are difficult to procure and often depend on data from retrospective studies. However, reasonable approximations for the weight of these infections on public health have been proposed based on available literature.

One of the first active evaluations in the U.S. to detect the burden of invasive fungal infections assessed data within the San Francisco Bay area from 1992 to 1993 and predicted the incidence of IA as 12.4 cases per million people annually. This study found aspergillosis to be the fourth most common invasive fungal infection (IFI) at that time. However, this study was limited to 45 hospital laboratories in a specific region of California (Rees et al. 1998). According to one study which evaluated information on a national hospital database, hospitalizations associated with IA rose by about 3% annually in the U.S. between the years 2000 and 2013, (approximately 169,110 IA-related hospitalizations total.) Using current census data, the same study estimated the rate of IA related hospitalizations to have increased in that time frame from about 32.8 cases per million people to 46 cases per million (Vallabhaneni et al. 2017). In a study utilizing available data from 2001 to 2006 to estimate the burden of IFI in the U.S., IA was the most common IFI detected (Kontoyiannis et al. 2010). Of the 16,200 hematopoietic stem cell transplant (HSCT) patients within the 23 participating centers of the study, 425 proven or probable cases of IA were found, with 187 confirmed as *A. fumigatus* specifically. The median time from transplant to onset of invasive aspergillosis infection was 99 days. For the 15,820 HSCT patients for which follow-up data was available, the total 12-month cumulative incidence (CI) of IA was 1.6%, though the incidence tended to show an increase over time. The 1-year mortality in this study among patients with IA was reported as 25.4% (Kontoyiannis et al. 2010). Unfortunately, details concerning patient treatment were unavailable for many of the cases and this study only evaluated HSCT patients. Another study of data from participating healthcare centers within Idaho and Utah from 2006 to 2016 found IA to be the third most common IFI, detecting 301 cases total, (40 confirmed specifically as *A. fumigatus*,) and estimating a mean annual incidence of 2.4 cases per 100,000 patients (Webb et al. 2018). This study reported a crude 1-year mortality for IA patients of 48.8% (Webb et al. 2018). The occurrence of IA among the healthcare centers remained stable throughout the period of study. However,

this study only includes information within southern Idaho and Utah (Webb et al. 2018). The overall consensus in the literature is that incidence of IA both within the U.S. and across the globe has increased over the past thirty years, although approximations of the burden of aspergilloses on public health may vary depending on regional and local variables (Pfaller et al. 2018; CDC 2019; Hokken et al. 2019).

The list of approved therapies to combat aspergilloses include three specific classes of antifungals comprised of the polyenes, the echinocandins, and the triazoles. Primary therapy for IA begins with the triazoles voriconazole and isavuconazole, followed by the polyene amphotericin B (lipid formulations) and the echinocandin caspofungin. For patients who show no clinical response to primary treatment, salvage therapies also include other triazoles as well as amphotericin B, caspofungin, or combination therapy with multiple antifungals (Patterson et al. 2016). Antifungal prophylaxis, such as with the triazole posaconazole, is commonly used for at-risk individuals to preclude fungal infection. Moreover, surgery to remove infected tissue is sometimes required, such as for pulmonary aspergilloma (Walsh et al. 2008; Bellete et al. 2010; Patterson et al. 2016). Treatment approach depends largely on the type of infection and patient-specific variables. For example, patients with ABPA may require treatment with anti-inflammatory corticosteroids to moderate lung function in addition to triazole antifungal doses sufficient to clear infection (Walsh et al. 2008; Denning, Pleuvry, and Cole 2013).

The triazole class of antifungals were introduced for medical use in the late 1990's and are currently utilized for therapy in human infections as well as certain animal species including birds (Beernaert et al. 2009; Hokken et al. 2019). This class of antimicrobial can be given orally with relatively low patient toxicity and effective activity against many fungal pathogens (Denning et al. 1989; Oakley, Moore, and Denning 1998; Heeres, Backx, and Van Cutsem 1984; George, Miniter, and Andriole 1996; Denning, Radford, et al. 1997; Verweij, Chowdhary, et al. 2016). Triazoles chiefly act to inhibit a key enzyme of ergosterol biosynthesis in fungi known as lanosterol 14$\alpha$-demethylase, (Erg11 in many fungal species or the Cyp51 enzymes in *Aspergillus*) (Basenko et al. 2018; Arnaud et al. 2010). These compounds possess affinity for the substrate binding site of the fungal enzymes, thus blocking access for the natural substrate and inhibiting the synthesis of ergosterol, the key membrane sterol of fungi.

Unfortunately, the continued utility of the triazoles against *Aspergillus* spp. infections is now threatened by the global insurgence of triazole resistance. Studies indicate that triazole resistant invasive infection is positively associated with treatment failure and with patient mortality ranging from 30% to approaching 90% for some patient types (van der Linden et al. 2015; Lestrade et al. 2019; Falcone et al. 2011). Clinical cases of documented and confirmed *A. fumigatus* infections resistant to triazoles have originated from outdoor locales and within medical centers in various countries across the globe (Denning 1998; Groll and Walsh 2001; Chowdhary et al. 2015; Snelders et al. 2008; Snelders et al. 2009). While susceptibility of a strain to an azole antifungal is difficult to determine in-vivo, the

common method by which antifungal activity is measured in-vitro involves the broth microdilution method, though methods such as disk-diffusion are also useful. Much of the literature employs minimum inhibitory concentration (MIC) values obtained through this method to approximate the susceptibility of an isolate to a given compound. Two standardized protocols exist whereby MIC values may be obtained; the European Committee on Antibiotic Susceptibility Testing (EUCAST) method, and a protocol provided by the Clinical and Laboratory Standards Institute (CLSI) (Alastruey-Izquierdo et al. 2015). While the CLSI has not yet set clinical breakpoints (CBP) of MIC values to mold-active medical triazoles for filamentous fungi, epidemiological cutoff values (ECV) based on the CLSI and EUCAST methods suggest that strains of *A. fumigatus* possessing an MIC for voriconazole or itraconazole above 1.0 µg/mL, isavuconazole above 2 µg/mL, or posaconazole above 0.25 µg/mL may be non-wild-type and should be considered for resistance (Alastruey-Izquierdo et al. 2015).

Investigations have attempted to uncover the prevalence of azole resistance among clinical and environmental isolates and estimate local or global incidence of resistant IA cases. A global surveillance study of 497 *A. fumigatus* isolates collected from 2008 to 2009 detected a total of 29 isolates with heightened triazole MIC values (Lockhart et al. 2011). A study of 1,972 isolates of *Aspergillus* from across the globe identified eight isolates of *A. fumigatus* with an MIC to itraconazole exceeding 8ug/mL (Pfaller et al. 2018). Another group evaluated isolates submitted to the Mycology Reference Centre Manchester from 1992 to 2007 and then again between 2008 and 2009. Of the 519 isolates submitted to the center between 1992 and 2007, a total of 34 isolates were deemed resistant to at least one triazole, (possessing an MIC greater than 2µg/mL to itraconazole or voriconazole or >0.5µg/mL to posaconazole.) Interestingly, most of the patient isolates possessed *cyp51A* gene mutations, including TR34/L98H, G54(E,V, or R,), G138, M220(K or T,) and G448S, which are associated with triazole resistance in *A. fumigatus* and which will be discussed further in this review (Howard et al. 2009). At least 5 of the patient's deaths in this earlier study were blamed on progression of aspergillosis despite treatment with triazoles and, for some, efforts with alternative therapy. The frequency of resistance was only 1% from 1999 to 2004, but then rose to 8% from 2004-2007. The latter study found that resistance among isolates was 14% in 2008 and jumped to 20% in 2009 (Howard et al. 2009; Bueid et al. 2010). Another work published in 2008 reported similarly prominent *cyp51A* mutation occurrences among resistant isolates from the Netherlands, however in this investigation, the majority of isolates possessed only the TR34/L98H mutation rather than a variety of mechanisms (Snelders et al. 2008). Again in the Netherlands, a retrospective investigation into the prevalence of triazole resistance within patients in a university hospital ICU suffering from IA from 2011 to 2013 determined that of the 38 patients whose patient cultures grew *A. fumigatus*, 10 patients' isolates were deemed azole resistant. The 90-day mortality among the 28 patients with azole susceptible isolates was 82% (23 out of 28,) while mortality for the 10 patients with resistant disease was 100% (van Paassen et al. 2016). Isolates from the 10 patients with resistant IA harbored the *cyp51A* TR34/L98H and TR46/Y121F/T289A

mutations. Microsatellite typing indicated the isolates were not likely related (van Paassen et al. 2016). In another survey for resistant *A. fumigatus* isolates within 16 participating hospitals and medical centers in the Netherlands published in 2016, the rate of resistance among tested isolates ranged from 5-10% overall, but was especially elevated within certain patient subsets where rates reached 30% (Lestrade et al. 2016). Resistant strains of *A. fumigatus* have now been identified within six of the seven continents (Dos Reis et al. 2019; Resendiz Sharpe et al. 2018).

Though the first *A. fumigatus* isolates found with resistance to triazoles originated in California in the late 1980's, some studies indicate that triazole resistance is less prevalent within the U.S. than in other regions of the world such as in Europe and the Netherlands (Walker et al. 2018). While the U.S. has fewer investigations than in European countries where active surveillance for triazole resistance has occurred regularly, some data is available for prevalence of resistant *A. fumigatus*. In a study of transplant recipients within the United States, occurrence of resistance within patient-isolated *Aspergillus* reached 7% (Baddley et al. 2009). Another study screened 1,026 clinical isolates of *Aspergillus* collected from 22 states in the U.S. from October of 2011 to October of 2013 for resistance to itraconazole (Pham et al. 2014). Of the 51 isolates which produced an MIC above 1ug/mL to the drug, one isolate with an MIC above 32ug/mL possessed an M220 mutation, (which, again, will be discussed later in this review.)

The true scope of triazole resistance in this pathogen and its effect on public health are likely underestimated due to the profound lack of regular screening for resistance by laboratories and centers both within the United States and around the world (Krishnan Natesan et al. 2012). Moreover, the frequency of negative patient cultures for this pathogen, despite confident diagnosis of aspergillosis supported by serological diagnostics, renders detection of clinical resistance problematic (Denning et al. 2011). For example, systematic analysis of patient health records and matching autopsy records of adult patients admitted to ICU from 1966 to 2011 within the U.S. revealed that aspergillosis was one of the four most common potentially lethal patient misdiagnoses which likely resulted in patient death (Winters et al. 2012; Webb et al. 2018). To conclusively diagnose aspergillosis typically demands a positive culture from a normally sterile sample location as well as histological evidence for a patient *Aspergillus* infection, (though radiological scans, PCR-based assays, and blood galactomannan or beta-D-glucan assays can also aid diagnosis.) Consequently, infections with either resistant or wild-type *Aspergillus* strains are often misdiagnosed or otherwise undetected, making accurate assumptions of the true national and global impact of this pathogen difficult.

The origins, drivers, and effects of clinically and environmentally resistant isolates have been a subject of intense research since the first report of clinically resistant isolates correlated with patient treatment failure in 1997 (Denning, Venkateswarlu, et al. 1997). Unfortunately, due to the similarity in essential chemical structure and antifungal biomolecular activity, even agricultural fungicides can select for resistance to medical triazole drugs

(Snelders et al. 2009; Schoustra et al. 2019; Faria-Ramos et al. 2014; Zhang et al. 2017). Indeed, this environmentally selected resistance is blamed for more clinically resistant patient infections than *de novo* resistance acquisition during extended medical triazole therapy (van der Linden et al. 2013). Regardless of the route of acquisition, mechanisms underpinning triazole resistance in *A. fumigatus* often come at no pathogenic fitness cost (Buil et al. 2019). Regrettably, much concerning the specific biomechanical causes, (whether genetically based or epigenetic,) clinical versus environmental derivation, and overall burden of triazole resistant *A. fumigatus* to public health within the United States and globally remains undetermined. This brief review aims to provide a succinct description of the selection of resistance in this species as well as to highlight some of the known environmental and clinical resistance mechanisms, especially those contributing to patient treatment failures and associated with increased mortality among infected immunocompromised patients.

**Section One:** The Rise of Resistance The first instances of well-documented triazole-resistant *A. fumigatus* associated with treatment failure occurred in California during 1989-1990 and the results were published in 1997 (Denning, Venkateswarlu, et al. 1997). This study identified itraconazole resistant isolates from two separate patients with invasive aspergilloses undergoing triazole therapy. The isolates each exhibited an *in vitro* broth microdilution MIC value to itraconazole exceeding 16 µg/mL. *In vitro* resistance was confirmed to translate *in vivo* through experiments utilizing murine neutropenic models of infection (Denning, Venkateswarlu, et al. 1997). Differences in the phenotypes and sterol profiles of the resistant patient isolates pointed to the existence of at least two entirely separate stratagems of triazole drug resistance. One likely involved the increased expression, altered drug binding affinity, and / or enhanced activity of the fungal 14$\alpha$-demethylase to counteract the drug's action whereas the other likely involved reduced intracellular accumulation of drug potentially as a result of increased membrane transporter activity. Observations of this initial report have held true to the current view that multiple mechanistic strategies putatively underlie triazole resistance in *A. fumigatus* (Denning, Venkateswarlu, et al. 1997).

It is now understood that there are likely two general routes for selection of resistance in *A. fumigatus*: *de novo* selection within the host during therapy with triazoles or through selection in the environment (Verweij, Chowdhary, et al. 2016; Snelders et al. 2009; Buil et al. 2019; Verweij et al. 2013; Camps, van der Linden, et al. 2012; Hagiwara et al. 2014). Environmental saprobic fungi such as *A. fumigatus* are typically both phenotypically and genotypically plastic, a trait that allows for continual acclimation to ever-changing and generally adverse conditions (Groll and Walsh 2001). In turn, stressful stimuli within the environment often cause enduring adaptive responses and can select for beneficial and often heritable genetic or epigenetic changes within such organisms (Buil et al. 2019; Sniegowski and Gerrish 2010; Yona, Frumkin, and Pilpel 2015). Fungi can survive stress and outcompete other microbes within their environment through intracellular changes enabled by genetic alterations or via responses such as activation of efflux mechanisms or stress response pathways. Unfortunately, when demethylase-inhibiting fungicides are

introduced into natural settings, as during agricultural work, fungi within these locales become exposed to the compounds and can readily adapt to the selective antifungal pressures (Groll and Walsh 2001; Chowdhary et al. 2015; Snelders et al. 2009). Though *A. fumigatus* is not a phytopathogen, the species acts as an important decomposer of organic matter, thriving wherever suitable nutrients and conditions are found including within outdoor environments and human-inhabited settings (Verweij, Chowdhary, et al. 2016). Multiple agriculturally employed azole fungicides are chemically similar and each possess the same primary mechanism of action against the fungal ergosterol biosynthetic pathway (Verweij, Snelders, et al. 2009; Stensvold, Jørgensen, and Arendrup 2012; Snelders et al. 2012; Zhang et al. 2017). Once introduced into water or soil, triazole fungicides can persist for at least several months (Ribas et al. 2016). Consequently, *A. fumigatus* present in such settings may become strained by these agricultural fungicides and counter with adaptive stress responses (Buil et al. 2019; Alvarez-Moreno et al. 2019). For example, three separate laboratory-based studies have reported the induction of target gene mutations similar to those found in clinical isolates via exposure to agricultural triazole compounds including tebuconazole, metconazole, bromuconazole, difenoconazole, propiconazole, hexaconazole and epoxiconazole. These compounds have been in use for agricultural applications and have been shown to select for strains possessing heightened MIC to the medical drugs voriconazole, itraconazole, posaconazole, or multi- or pan-azole resistance (Zhang et al. 2017; Snelders et al. 2012; Ren et al. 2017).

Exposures to resistant strains derived from the environment are blamed for a preponderance of the causal mechanisms identified within patient isolates (van der Linden et al. 2015; Chowdhary et al. 2015; Chowdhary et al. 2013; Verweij, Snelders, et al. 2009; van der Linden et al. 2009; Verweij, Chowdhary, et al. 2016; Verweij et al. 2013; van der Linden et al. 2013). However, the remainder of resistant clinical infections seem to result primarily due to prolonged or successive triazole treatments selecting for resistant fungi within the host (Howard et al. 2009; Chryssanthou 1997; Camps, Dutilh, et al. 2012; Verweij et al. 2002; Howard et al. 2013). Extended phases of antifungal treatments even spanning several months can provide sufficient time for the development of resistant strains within the human host (Camps, Dutilh, et al. 2012; Denning et al. 2003). This is especially pertinent given that patients have been known to suffer from chronic aspergilloses requiring therapy exceeding 12 years in duration (Denning et al. 2003). A *de novo* resistant infection will likely no longer respond to treatment with the original triazole used and may additionally exhibit pan-azole resistance (van der Linden et al. 2015; Howard et al. 2009; Camps, Dutilh, et al. 2012; Verweij et al. 2002). Consequently, development of resistant infection during therapy can contribute to treatment failures and increased mortality (Dannaoui et al. 2001; Camps, Dutilh, et al. 2012).

It is, however, often difficult to prove the origins of a resistant strain and the occurrence of later infection with a resistant strain from the patient's environment cannot always be dismissed (Dannaoui et al. 1999). Analyses of the genomes of cultured strains are often

utilized to propose the origins of a resistant infection (Dannaoui et al. 1999; Camps, Dutilh, et al. 2012). The presence of genetically diverse isolates possessing the same resistance mechanism in azole-naïve patients can imply isolates were selected for resistance within the environment (Wiederhold et al. 2016; Snelders et al. 2009; Walsh et al. 2008; Verweij et al. 2002). However, whole-genome sequencing experiments indicate *A. fumigatus* can undergo rather drastic genomic changes during invasive growth within the host niche, bringing such assumptions into question (Hagiwara et al. 2014). To provide clarity on isolate lineage, microsatellite typing has been employed to indicate whether serially collected isolates from a patient infection are "isogenic," having identical or very similar genotypes. As in the work by Hagiwara et. al., though related strains accumulated many separate mutations detectable through sequencing, they retained identical microsatellite results (Hagiwara et al. 2014). When genetic relationships are confirmed utilizing this method, a series of isogenic patient isolates which become resistant during extended patient treatment may suggest *de novo* resistance (Camps, Dutilh, et al. 2012). Regardless of the mode of resistance acquisition, infection with an azole-resistant strain of *A. fumigatus* is associated with an escalated cost of hospitalization and care, treatment failures, and increased mortality (Zilberberg et al. 2018; van der Linden et al. 2009; Lestrade et al. 2019; van der Linden et al. 2013). Resistant invasive *Aspergillus* infection elevates the observed mortality rate from about 50% for an azole-susceptible strain to approximately 90% for a resistant infection (van der Linden et al. 2011; Howard et al. 2009; Lestrade et al. 2019). Some of the most common resistance-associated mechanisms within both environmental and de-novo originated strains include triazole target or non-target alterations and are summarized in **Table A.1**.

Note to Editor: Preferred position of **Table A.1** here.

**Section Two: Target gene-dependent resistance**

Mutations affecting the target enzymes of triazoles, the fungal sterol 14$\alpha$ demethylases, represent the most commonly identified mechanisms associated with triazole resistance in *A. fumigatus* (Snelders et al. 2010; Denning et al. 2011; van der Linden et al. 2013). *A. fumigatus* possesses two Cyp51 sterol 14$\alpha$ demethylase enzymes; Cyp51A and Cyp51B. Studies predict that Cyp51B has better binding affinity with triazole compounds. Aberrations have been identified within both genes encoding 14$\alpha$ demethylases of *A. fumigatus* isolates. However, only alterations affecting the *cyp51A* sterol-demethylase gene and its protein product have thus far been convincingly associated with clinically relevant triazole resistance in this pathogen (Rybak, Fortwendel, and Rogers 2019; Ferreira et al. 2005). Target enzyme lesions have been found within both environmental and clinical isolates and have been recovered both from patients who have had extended contact with triazoles, as well as from infections of azole-naïve individuals (Wiederhold et al. 2016; Howard et al. 2006; Verweij, Chowdhary, et al. 2016). Sterol 14$\alpha$ demethylase mutations have been identified in patient isolates and implicated in treatment failures among clinical patients in multiple countries (Wiederhold et al. 2016; Bueid et al. 2010; Howard et al. 2006; Chowdhary et al. 2015; van der Linden et al. 2013).

Depending on the type of mutation and its location in the genetic sequence, the resulting strain can exhibit a range of resistant phenotypes including a spectrum of triazole susceptibilities as well as single or pan-azole resistant qualities (Wiederhold et al. 2016; Howard et al. 2009; Chowdhary et al. 2015; van der Linden et al. 2013). Several principal stratagems exist whereby alterations in the coding sequence or upstream regulatory sequence of *cyp51A* enable resistance. The most commonly found Cyp51A mutations associated with triazole resistance occur at the gene sequence codons G54, L98, Y121 and T289, G138, and M220, with nonsynonymous mutations at position 98, 121 and 289 typically accompanied by a tandem repeat within the upstream genetic regulatory promoter region (Denning et al. 2011; Wiederhold et al. 2016). This category includes the mutation combinations TR34/L98H and TR46/Y121F, and TR46/T289A, which exist within patient derived and environmental isolates of *A. fumigatus* from multiple continents (Chowdhary et al. 2015; Verweij, Mellado, and Melchers 2007; Mellado et al. 2007; Snelders et al. 2008; Camps, Rijs, et al. 2012; Pham et al. 2014; Verweij, Howard, et al. 2009; Bader et al. 2013; Snelders et al. 2010; Wiederhold et al. 2016; Chowdhary et al. 2014; Snelders et al. 2009; Snelders et al. 2015; Stensvold, Jørgensen, and Arendrup 2012). Several of these will be discussed in more detail.

TR34/L98H is named such because of a tandem repeat of a length spanning 34 base pairs within the upstream promoter region of *cyp51A* and is also characterized by a substitution of a leucine at the 98th amino acid position to a histidine in the resultant protein (Gsaller et al. 2016b, 2016a; Bader et al. 2013; Snelders et al. 2010; Verweij, Mellado, and Melchers 2007; Abdolrasouli et al. 2015; Lockhart et al. 2011; Mellado et al. 2007; Rybak et al. 2019; Fuhren et al. 2015). This mechanism enhances transcription of the *cyp51A* gene, as well as altering the resultant protein enzyme's structure (**Figure A.1**) (Mellado et al. 2007). In *A. fumigatus*, the residue at position 98 lies near the first recognition site for the enzyme's substrate within the Cyp51A enzyme (Mellado et al. 2007; Snelders et al. 2011). Isolates possessing this mechanism tend to show reduced susceptibility to multiple medical triazoles including voriconazole, posaconazole, and itraconazole as well as the experimental azole compound ravuconazole and to multiple agricultural and industrial azole fungicides (Mellado et al. 2007; Mann et al. 2003; Snelders et al. 2008; Snelders et al. 2011). Cyp51A TR34/L98H has been well established as a functional cause of increased triazole resistance within human infections and is often associated with patient treatment failures and increased mortality (Pham et al. 2014). Strains with a TR34/L98H mechanism also retain virulence in a mouse model of invasive aspergillosis and possess no distinct defects in growth in comparison to wild-type isolates (Mavridou et al. 2013; Wang et al. 2018). The mechanism has been suggested to be the dominant causal contributor to azole resistance among both environmental and clinical isolates of *A. fumigatus* and exists in samples from across the globe including the U.K., China, the Netherlands, Belgium, Austria, Norway, Denmark, Spain, France, Germany, Iran, India, and the U.S. (Gsaller et al. 2016b, 2016a; Bader et al. 2013; Snelders et al. 2010; Verweij, Mellado, and Melchers 2007; Abdolrasouli et al. 2015; Lockhart et al. 2011; Mellado et al. 2007; Chowdhary et

al. 2012; Snelders et al. 2009; Chowdhary et al. 2015; Wiederhold et al. 2016; Fuhren et al. 2015; Pham et al. 2014). Among clinically resistant infections, TR34/L98H appears to be the result of both *de novo* resistance in azole-naïve patients and from environmental resistance involving strains selected in nature prior to human infection, the latter situation being more likely (Gsaller et al. 2016b, 2016a; Verweij, Mellado, and Melchers 2007; Verweij et al. 2002; Mellado et al. 2007; Snelders et al. 2008). Studies have illustrated the potential for this mechanism to arise in response to antifungal pressures. For example, duplication of the 34bp tandem repeat can be induced in an isolate possessing a TR34/L98H mutation following exposure to demethylase-inhibiting agricultural fungicides in a laboratory setting (Snelders et al. 2012).

While introduction of only the tandem repeat into a previously susceptible strain of *A. fumigatus* increases mRNA transcription in laboratory mutant strains, the introduction of both lesions simultaneously is required to recreate the phenotype of the clinically resistant strains (Mellado et al. 2007). Studies reveal that the 34bp repeat in the promoter region is bound by several important transcription factors which are known to affect the transcription of genes related to sterol biosynthesis as well as other pathways (Gsaller et al. 2016a, 2016b). One such transcriptional regulator, SrbA, binds to the repeated sequences in the *cyp51A* promoter in this mutant (Gsaller et al. 2016b). SrbA acts as a sterol regulatory element binding protein or "SREBP." Increased SrbA binding to the *cyp51A* promoter results in increased expression, thus likely exacerbating triazole resistance observed within these mutant strains (Gsaller et al. 2016b). Moreover, SrbA competes with the transcriptional repressor CCAAT-DNA binding complex (CBC) for DNA binding on the *cyp51A* promoter. Increased binding of SrbA blocks binding of the repressor resulting in de-repression of *cyp51A* transcription (Gsaller et al. 2016b).

The TR46/Y121F/T289A resistance mechanism also involves a tandem repeat of bases within the regulatory region as well as nonsynonymous mutations within the *cyp51A* gene (Wiederhold et al. 2016; Chowdhary et al. 2014; Snelders et al. 2015; van der Linden et al. 2013; Fuhren et al. 2015). The TR46/Y121F/T289A mechanism was initially found in the Netherlands within multiple voriconazole-resistant isolates of fifteen individual patients from six individual geographically distant hospitals, the earliest of which dated to December of 2009 (van der Linden et al. 2013). These patients suffered a high incidence of treatment failures and mortality through the study, especially among those given voriconazole. The same study sampled ten separate locations and recovered isolates possessing the same mechanism at six of the ten environmental locales (van der Linden et al. 2013). This resistance mechanism can spontaneously arise in response to antifungal pressure, as a study has reported generation of TR46/Y121F/T289A and G448S mutation combinations upon exposure of *A. fumigatus* strains to agricultural triazoles (Ren et al. 2017). The G448S mutation will be discussed in more detail later in this review. Another study found that introduction of the 46-basepair repeat and the Y121F mutation together without the T289A

substitution into a previously susceptible strain appears sufficient to recapitulate the multi-azole resistant nature of clinically obtained resistant isolates with the TR46/Y121F/T289A mutation pattern (Snelders et al. 2015). Isolates bearing this mutation typically have elevated MIC values for voriconazole and also attenuation of itraconazole and posaconazole susceptibility (van der Linden et al. 2013). The residue Y121 supposedly hydrogen bonds with the heme group within Cyp51A according to homology models, supporting the assumption of this residue as the more important site altering antifungal susceptibility when mutated (**Figure A.2A**) (Snelders et al. 2015). In contrast, genetic introduction of either TR46 and the T289A mutation or the T289A mutation alone had little effect on susceptibility, though it may alter the enzyme's association with voriconazole (Snelders et al. 2015).

Mutations in the sequence for *A. fumigatus* Cyp51A at position G54 are also associated with cross-resistance to multiple triazole compounds including posaconazole and itraconazole (Chowdhary et al. 2015; Diaz-Guerra et al. 2003; Verweij, Howard, et al. 2009; Camps, van der Linden, et al. 2012; Mann et al. 2003; Nascimento et al. 2003; Bader et al. 2013; Mellado et al. 2007; Snelders et al. 2010; Pfaller et al. 2008; Rodriguez-Tudela et al. 2008; Krishnan Natesan et al. 2012; Denning et al. 2011). In the predicted tertiary structure of Cyp51A, the glycine residue at position 54 appears to interact directly with triazole drugs and is situated within the substrate access channel (**Figure A.2B**) (Xiao et al. 2004; Snelders et al. 2010; Rybak, Fortwendel, and Rogers 2019; Diaz-Guerra et al. 2003). Multiple amino acid substitutions at glycine 54 are known including G54E, G54W and G54R (Mellado et al. 2007; Diaz-Guerra et al. 2003; Xiao et al. 2004; Tashiro and Izumikawa 2016). Replacement of the glycine residue at position 54 with an arginine (R) or a glutamate (E) in a previously azole-susceptible strain has been shown to increase MIC values to posaconazole by 30-fold while replacement with a tryptophan residue (W) produces a 250-fold MIC increase in laboratory mutants. These mutations also greatly increase MIC values to itraconazole but typically have no effect on *in vitro* voriconazole susceptibility (Xiao et al. 2004; Mann et al. 2003; Nascimento et al. 2003; Manavathu et al. 2000; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Tashiro and Izumikawa 2016).

Another known mutation occurs at position G138 of the enzyme (Snelders et al. 2010; Manavathu et al. 2000; Xiao et al. 2004; Howard et al. 2006; Denning et al. 2011). The *cyp51A* G138C mutation was identified in a patient with chronic cavitary aspergillosis undergoing extended triazole therapy whose isolates simultaneously developed resistance to voriconazole and itraconazole (Howard et al. 2006). Isolates containing this amino acid substitution tend to be resistant to voriconazole but not to posaconazole according to both *in vivo* and *in vitro* measures of susceptibility or response to treatment (Xiao et al. 2004; Manavathu et al. 2000; Rybak, Fortwendel, and Rogers 2019). The residue at 138 is also situated within the ligand access channel near the heme cofactor of Cyp51A (Rybak, Fortwendel, and Rogers 2019). A G138R substitution likely disrupts the heme cofactor environment in the active site, disrupting the binding of voriconazole to the enzyme. The side-chain interactions of posaconazole may accommodate better association with Cyp51A

and overcome the altered active site conformation (Xiao et al. 2004; Rybak, Fortwendel, and Rogers 2019). The G138 mutation has been spontaneously generated through exposure of *A. fumigatus* to triazoles in a laboratory setting, hinting at the potential for triazole antifungal stress to select for this mechanism. One such study induced the formation of a G138L mutation by exposing strains to itraconazole (Snelders et al. 2012).

Furthermore, one common lesion associated with azole resistance occurs at position M220 of the Cyp51A protein sequence (Mellado et al. 2004; Chen et al. 2005; Snelders et al. 2010; Mellado et al. 2007; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Rodriguez-Tudela et al. 2008; Denning et al. 2011). Amino acid substitutions replacing the Methionine at residue 220 of the gene for Cyp51A have also been associated with an itraconazole resistant phenotype *in vivo* and elevated MIC values to itraconazole, voriconazole, and posaconazole *in vitro* (Mellado et al. 2004; Chen et al. 2005; Snelders et al. 2010; Mellado et al. 2007; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Rodriguez-Tudela et al. 2008). This amino acid substitution has been detected within cultures recovered from diseased patients as well as through ultra-sensitive, real-time PCR assays for *A. fumigatus cyp51A* within patient samples which were not culturable (Denning et al. 2011; Howard et al. 2013; Howard et al. 2009). One such group of isolates were collected from a patient with chronic pulmonary aspergillosis (CPA) undergoing extended itraconazole treatment who developed a resistant infection. Upon the patient's death, researchers found that the individual's left lung had nearly entirely been destroyed; reduced to fibrotic tissue and interconnected aspergillomas (Howard et al. 2013). Individual strains cultured from separate aspergillomas displayed *cyp51A* mutations M220K or M220T and exhibited elevated MIC to itraconazole as well as to either voriconazole or posaconazole (Howard et al. 2013).

Finally, isolates possessing a G448S mutation have been associated with resistance to voriconazole and itraconazole (Krishnan Natesan et al. 2012; Bellete et al. 2010; Fuhren et al. 2015; Howard et al. 2009) and these mutants tend to remain susceptible to posaconazole (Xiao et al. 2004; Manavathu et al. 2000; Pfaller et al. 2008; Mellado et al. 2004; Howard et al. 2009; Krishnan Natesan et al. 2012). The residue at position G448 is also located near the heme cofactor in Cyp51A (Xiao et al. 2004). The mutation G448S has been established as a causal contributor to triazole resistance within the context of patient infections as well as an animal model of invasive pulmonary aspergillosis (Howard et al. 2009; Verweij, Howard, et al. 2009; Krishnan Natesan et al. 2012; Xiao et al. 2004; Manavathu et al. 2000). One individual developed a voriconazole resistant infection bearing the *cyp51A* G448S mutation after a year of treatment with voriconazole (Bellete et al. 2010). Though the patient was switched to lipid formulation amphotericin B and caspofungin therapy following the susceptibility testing results, in this case the patient ultimately died shortly after this modification of antifungal therapy. Sequencing of isolates recovered during the patient's extended care revealed that though the strains were identical according to microsatellite genotyping, the earlier collected strains which were recovered during treatment were susceptible to itraconazole and voriconazole while the later isolates possessed resistance to

itraconazole, voriconazole, or both (Bellete et al. 2010). Among the four patient isolates and a reference strain, no other mutations existed in the *cyp51A* sequence in either the sense or antisense directions (Bellete et al. 2010). Laboratory mutant strains ectopically expressing *cyp51A* containing a G448S mutation introduced into a previously susceptible strain also recapitulate voriconazole resistance *in vitro* (Hagiwara et al. 2018).

**Section Three: Target gene mutation-independent resistance**

Often, sequence analysis of isolates with heightened MIC values unveils multiple strains which possess no Cyp51A-related lesions (Arendrup et al. 2010; Wiederhold et al. 2016; Fraczek et al. 2013; Verweij, Chowdhary, et al. 2016; Buil et al. 2019). Indeed, a poorly understood shift from target enzyme-based mechanisms to alternative resistance mechanisms appears to be occurring in certain areas of the world (Fraczek et al. 2013). Common non-target gene mechanisms currently recognized include genetic and epigenetic occurrences such as alterations to transcription factor components, alteration of the sterol biosynthetic pathway, overexpression of specific drug efflux pumps or other means by which intracellular accumulation of triazole is reduced in the fungal cells, and enhanced adaptability due to biochemical or genomic plasticity (Gsaller et al. 2016a, 2016b; Camps, Dutilh, et al. 2012; Bellete et al. 2010). Selected genetic lesions which are not within the sequences or promoter regions for 14$\alpha$ demethylases have been well studied and convincingly associated with drug resistance and treatment failures in patients (Nascimento et al. 2003; Camps, Dutilh, et al. 2012; Fraczek et al. 2013; Ferreira et al. 2005). While a comprehensive list of all known or suggested azole resistance mechanisms is beyond the scope of this review, a selection of such methods will be discussed.

One such resistance mechanism involves a single point mutation in the protein coding sequence for a subunit of the fungal CCAAT-binding transcription factor complex, CBC, known as HapE in *A. fumigatus*. The CBC acts as a transcriptional repressor affecting the expression of genes such as *cyp51A* (Gsaller et al. 2016b; Camps, Dutilh, et al. 2012). The *hapE* P88L mutation was initially discovered within the sequences of multiple isolates obtained from a single patient with invasive pulmonary aspergillosis given extended triazole therapy (Camps, Dutilh, et al. 2012). In the case of this study, the patient ultimately failed therapy and succumbed to the invasive pulmonary infection. This outcome was very likely due to the resistant nature of the infection (Camps, Dutilh, et al. 2012). Microsatellite typing and sequencing analyses confirmed the isogenic nature of the early susceptible patient isolates of *A. fumigatus* and the later resistant samples. Analyses also revealed no changes within the gene sequences for Cyp51A and Cyp51B nor their promoter regions and only the *hapE* P88L mutation was able to recapitulate the invulnerable phenotype of the isolates. The P88L mechanism of resistance was reasonably verified as the causal mutation conferring a pan-azole resistant phenotype in the patient isolates through homologous gene replacement and sexual crossing studies with subsequent analyses of susceptibility and growth (Camps, Dutilh, et al. 2012). The repercussions of the P88L mutation involve loss of CBC activity in the mutant strain which results in de-repression thus allowing a higher

level of *cyp51A* transcription within the pathogen, enabling resistance to triazole treatment (**Figure A.1C**) (Gsaller et al. 2016b; Camps, Dutilh, et al. 2012).

A recent finding showing that altered sterol biosynthesis pathway activity underpins resistance in *A. fumigatus* clinical isolates came from work with the pathway rate-limiting HMG-CoA reductase, *hmg1* (Buil et al. 2019; Rybak et al. 2019; Hagiwara et al. 2018). One study which screened clinical isolates collected from multiple patients and from multiple medical centers in Japan for novel resistance mechanisms identified *hmg1* mutations among azole-resistant isolates (Hagiwara et al. 2018). The mutation S269F was present in each isolate which exhibited elevated triazole MIC collected from one patient with chronic pulmonary aspergillosis. The residue at S269 of *hmg1* resides within the beginning of the enzyme's predicted sterol-sensing domain (Hagiwara et al. 2018). However, several of the patient's isolates from this study also bore a G448S mutation in *cyp51A* and all resistant isolates from the individual also possessed an A350T mutation in *erg6*, a major sterol methyltransferase (Hagiwara et al. 2018). This study further showed that ectopic expression of mutant *hmg1* and *erg6* alleles caused no shift in triazole MIC, suggesting that these mutations are not drivers of resistance (Hagiwara et al. 2018). However, another study positively linked mutations residing in the predicted sterol-sensing domain of *hmg1* within clinically resistant isolates to triazole resistance (Rybak et al. 2019). In this work, one of three mutations identified as unique to triazole resistant isolates or a wild-type *hmg1* allele were directly swapped with the native *hmg1* genomic locus of a previously susceptible laboratory strain (Rybak et al. 2019; Al Abdallah, Ge, and Fortwendel 2017). The resulting mutants with either an S305P or I412S mutation or an in-frame deletion of F262 showed increased MIC at least 4-fold to voriconazole, itraconazole, posaconazole, and isavuconazole, whereas the control wild-type allele matched the susceptibility of the parental strain. Furthermore, genetic knock-in of a wild-type *hmg1* sequence into the backgrounds of the *hmg1*-mutant resistant clinical isolates abrogated resistance (Rybak et al. 2019). These findings suggested that the sterol-sensing function of *hmg1* is an essential element for mediation of pathway activity and triazole susceptibilities in *A. fumigatus*. Further supporting this, sterol profiling confirmed an altered accumulation of precursors to ergosterol in strains carrying *hmg1* mutations, though the expression of *A. fumigatus* sterol demethylases was not abnormal (Rybak et al. 2019).

Apart from transcriptional or enzyme activity-based de-regulation of sterol biosynthesis, increased triazole export is another commonly identified mechanism of resistance. Multiple putative drug efflux transporters are transcriptionally upregulated in response to itraconazole treatment in *A. fumigatus*, implying a role in stress responses in wild-type strains (Fraczek et al. 2013). Drug efflux pump overexpression has been well-established in yeast species as a triazole resistance mechanism, but their contribution to resistance in molds has been somewhat less studied. However, multiple reports have suggested that constitutive overexpression of specific efflux pumps may be a mechanism of triazole resistance in clinical isolates of *Aspergillus* (Denning, Venkateswarlu, et al. 1997; Slaven et al.

2002; Nascimento et al. 2003; Rajendran et al. 2011; Fraczek et al. 2013; Nierman et al. 2005; Ferreira et al. 2005). The *A. fumigatus* genome contains genes encoding efflux transporters of the multidrug resistance (MDR) variety including major facilitator superfamily (MFS) and ATP-binding cassette (ABC) superfamily, few of which are currently associated with clinical or environmental resistance to azole compounds (Fraczek et al. 2013; Paul, Diekema, and Moye-Rowley 2013; Ferreira et al. 2005).

One study of resistant clinical isolates obtained both from patients who had prior triazole exposure and from azole-naïve individuals revealed clinically resistant strains overexpressing the gene *cdr1B* and which possessed increased MIC values to itraconazole, posaconazole, and/ or voriconazole (Fraczek et al. 2013). The *A. fumigatus* efflux pump-encoding gene *cdr1B*, also known as *abcB*, *abcC*, or *abcG1*, encodes an ATP-binding cassette (ABC) transporter (Fraczek et al. 2013; Rybak et al. 2019). Deletion of *cdr1B* in susceptible and itraconazole resistant isolates results in reduced itraconazole MIC values relative to controls, showing that loss of *cdr1B* increases susceptibility to the triazole (Fraczek et al. 2013; Hagiwara et al. 2016). Another study confirmed a genetic deletion of *cdr1B* within a triazole resistant background containing the TR34/L98H mutation resulted in a significant decrease of resistance (Paul, Diekema, and Moye-Rowley 2013). Likewise, constitutive overexpression of the ABC transporter atrF also has been implicated in resistance (Slaven et al. 2002; Fraczek et al. 2013; Nascimento et al. 2003; Wang et al. 2018).

Several putative multidrug resistance (MDR) transporters in *A. fumigatus* have also been suggested to contribute to triazole resistance (Nascimento et al. 2003; Rajendran et al. 2011). The putative *A. fumigatus* efflux pumps Mdr1 and Mdr2, coded by the genes *mdr1* and *mdr2*, may underlie resistance (Tobin, Peery, and Skatrud 1997; Nascimento et al. 2003). In a study of 20 putative efflux transporters in *A. fumigatus*, levels of mRNA transcript for *mdr1* in the wild-type strain Af293 were found to be increased 14-fold over basal expression following four hours exposure to 1 mg/L itraconazole (Fraczek et al. 2013). Furthermore, gene deletion of *mdr1* in a wild-type strain of *A. fumigatus* results in increased susceptibility to itraconazole according to MIC results, indicating that this transporter is at least important in response to triazole-induced stress (Fraczek et al. 2013). Aberrations in the transcriptional levels of additional putative MDR efflux pumps in *A. fumigatus* have also been reported and have been suggested to play a role in resistance (Nascimento et al. 2003; Wang et al. 2018). A transcriptional analysis of a collection of isolates with high itraconazole MIC values identified strains possessing increased expression of a gene with homology to a major facilitator (MFS) transporter mdr3 as well as to a gene with homology to an ABC transporter, mdr4 (Nascimento et al. 2003). These strains displayed either constitutive expression of these transporters or heightened induction upon triazole exposure. However, genetic evidence confirming their roles in increased triazole MICs is currently lacking.

The same biochemical or genetic plasticity which enables fungi to adapt to stressors ranging from climate changes and nutrient availability to internalized toxins from competing microbiota can also allow for adaptation to antifungals (Verweij, Chowdhary, et al. 2016;

Covo 2020; Hokken et al. 2019; Buil et al. 2019). Loss of genetic stability in fungal species such as *Candida albicans* and *A. fumigatus* has been associated with lessened susceptibility to triazole antifungals (Buscaino 2019; Selmecki et al. 2009). While frequent genomic changes can often result in inviable or weakened isolates under normal conditions, a moderate level of genetic instability can allow for selection of isolates inherently resistant to an adverse condition (Verweij, Zhang, et al. 2016; Dos Reis et al. 2018; Buscaino 2019). Genetic instability can result in ploidy changes, chromosomal rearrangement, and mutations in the genetic code. Mutations or disruption of genes encoding elements of DNA damage and cell cycle checkpoint pathways have been found to affect genomic stability and susceptibility to triazoles. For example, deletion of the genes encoding the AtmA and AtrA kinases in *Aspergillus fumigatus*, which are involved in the response to DNA damage and activation of DNA repair and cell cycle checkpoint pathways (Dos Reis et al. 2018). Moreover, loss of either gene *atmA* or *atrA* enhances adaptability to triazole stress to higher levels than wild-type, indicating that alterations in DNA damage or cell checkpoint players may affect evolvability and acquisition of resistant phenotypes (Dos Reis et al. 2018). The same group also reported that loss of the gene encoding DNA mismatch repair (MMR) protein MshA in *A. fumigatus* results in genomic instability that promoted enhanced evolvability upon exposure to posaconazole to resistant level MIC. Moreover, researchers detected the presence of *mshA* genetic variants among clinical and environmental isolates (Dos Reis et al. 2019). This study mirrors studies of *C. albicans* strains lacking the related MMR protein MSH2 which similarly resulted in genomic instability and frequent occurrence of triazole resistant mutant strains, as well as a report that half of sequenced *C. glabrata* strains possess mutations in MSH2 which may contribute to resistance acquisition (Legrand et al. 2007; Healey et al. 2016). Moreover, study of *Cryptococcus neoformans* isolates which mutated at a high rate revealed strains bearing MSH2 mutations (Boyce et al. 2017). Although mutations that alter genetic stability have not been yet described as drivers of resistance in clinical isolates, these examples validate the possibility that genetic instability from aberrations in pathways such as DNA repair may enable acquisition of triazole resistance within *A. fumigatus*.

In addition to the genetic means outlined in this review, other mechanisms have been put forth as strategies by which *A. fumigatus* may undermine and survive the effects of triazole exposures. These include mitochondrial dysfunction, the formation of biofilms, epigenetic changes, RNA interference, alterations in intracellular networks, and impermeability to drug due to cell wall changes (Matthaiou et al. 2018; Bowyer, Bromley, and Denning 2020; Li et al. 2020; Hokken et al. 2019; Bromley et al. 2016; Valdes et al. 2018; Abdolrasouli et al. 2015). Many other stratagems likely exist which have yet to be detected or linked to resistance in environmental or clinical strains of *A. fumigatus* (Buil et al. 2019). While a discussion of every potential contributor to triazole antifungal resistance is beyond the scope of this review, such additional pathogen attributes may well impact the health of aspergillosis patients or alter the makeup of ecological niches and fragile microbiomes.

## Conclusions

Fungal pathogens have become a considerable threat to human health as well as to animals and ecosystems globally. The rise of antifungal resistance only exacerbates this situation. The impact that *A. fumigatus* triazole resistance has on environmental microbiomes and patient treatment outcomes is only recently being appreciated. It is clear from the data collected thus far that drug resistance in *A. fumigatus* is associated with multiple distinct adaptive strategies. Often, the aberrant induction of gene transcription enables a strain to withstand drug activity by overproducing the drug's target enzyme or drug efflux mechanisms (Nascimento et al. 2003). Overexpression can also occur concomitantly with ORF mutations in the *A. fumigatus* gene encoding the triazole target, Cyp51A, to alter the binding affinity of triazoles to the enzyme. Such mutations can occur without detected changes in enzyme expression to confer triazole resistance. Moreover, resistance has also been associated with the overexpression of drug efflux transporters and various additional stratagems, including alteration of sterol biosynthesis in the mevalonate pathway, have been suggested to enable resistance to triazole compounds in this opportunistic pathogen. It is likely that we are only scratching the surface of the myriad mechanisms that filamentous fungi can utilize to subvert the activity of triazole compounds.

Although triazole resistance in *A. fumigatus* appears to be selected both during prolonged exposure to azoles within a host and in the environment by agricultural triazoles, the incidence of each route and the potential for common mechanisms acquired through each is still unclear. Increased surveillance for resistant *A. fumigatus* isolates in agricultural settings, compost, indoor ventilation, and patient samples will be of high value in order to appreciate and respond to this threat to public health (Verweij, Mellado, and Melchers 2007; Zilberberg et al. 2018; Chowdhary et al. 2012). Adequate methods of surveillance and detection of pathogenic *Aspergillus* species, especially drug-resistant variants, are needed to predict the onset of allergic symptoms, nosocomial infections, and to guide treatment decisions in patient centers densely concentrated with at-risk individuals (Streifel et al. 1983; Rose 1972; Patterson et al. 2016). Unfortunately, studies on the fiscal and mortal burdens of *Aspergillus* diseases within the United States are few in number and often outdated (Zilberberg et al. 2018; Dasbach, Davies, and Teutsch 2000; Fraser et al. 1979; Steinbach et al. 2012). Improved surveillance to determine the prevalence of resistant strains, epidemiology, patient outcomes with resistant infections, contemporary effective treatment stratagems to combat invasive aspergillosis, and the occurrence of triazole resistance within both environmental samples and clinical patient isolates of *A. fumigatus* is essential (Steinbach et al. 2012; Neofytos et al. 2010; Patterson et al. 2016). This will enable effective study of the distribution and impact of resistant pathogens on ecosystems and human health (Fisher et al. 2012). Better understanding of the process of resistance development within *A. fumigatus* is also needed (Verweij, Chowdhary, et al. 2016). Probes into the process whereby strains become resistant may reveal stratagems to halt the rise of resistance in this opportunistic pathogen. Moreover, illuminating triazole-specific intracellular stress

responses could identify novel targets and inform strategies to address resistant infections in novel stand alone or combination therapies (Snelders et al. 2008).

## Acknowledgements

This work was supported by (R21AI142509 and R21AI139388) to JRF and by (R01AI143197) to PDR and JRF. All authors declare no conflict of interest.

## References:

Abdolrasouli, A., J. Rhodes, M. A. Beale, F. Hagen, T. R. Rogers, A. Chowdhary, J. F. Meis, D. Armstrong-James, and M. C. Fisher. 2015. 'Genomic Context of Azole Resistance Mutations in Aspergillus fumigatus Determined Using Whole-Genome Sequencing', mBio, 6: e00536.

Al Abdallah, Q., W. Ge, and J. R. Fortwendel. 2017. 'A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus: In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates', mSphere, 2.

Alvarez-Moreno, C., R. A. Lavergne, F. Hagen, F. Morio, J. F. Meis, and P. Le Pape. 2019. 'Fungicide-driven alterations in azole-resistant Aspergillus fumigatus are related to vegetable crops in Colombia, South America', Mycologia, 111: 217-24.

Anastasi, A., G. C. Varese, and V. F. Marchisio. 2005. 'Isolation and identification of fungal communities in compost and vermicompost', Mycologia, 97: 33-44.

Andersen, B., J. C. Frisvad, I. Sondergaard, I. S. Rasmussen, and L. S. Larsen. 2011. 'Associations between fungal species and water-damaged building materials', Appl Environ Microbiol, 77: 4180-8.

Arendrup, M. C., E. Mavridou, K. L. Mortensen, E. Snelders, N. Frimodt-Moller, H. Khan, W. J. Melchers, and P. E. Verweij. 2010. 'Development of azole resistance in Aspergillus fumigatus during azole therapy associated with change in virulence', PLoS One, 5: e10080.

Arnaud, M. B., M. C. Chibucos, M. C. Costanzo, J. Crabtree, D. O. Inglis, A. Lotia, J. Orvis, P. Shah, M. S. Skrzypek, G. Binkley, S. R. Miyasato, J. R. Wortman, and G. Sherlock. 2010. 'The Aspergillus Genome Database, a curated comparative genomics resource for gene, protein and sequence information for the Aspergillus research community', Nucleic Acids Res, 38: D420-7.

Baddley, J. W., K. A. Marr, D. R. Andes, T. J. Walsh, C. A. Kauffman, D. P. Kontoyiannis, J. I. Ito, S. A. Balajee, P. G. Pappas, and S. A. Moser. 2009. 'Patterns of susceptibility of Aspergillus isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network', J Clin Microbiol, 47: 3271-5.

Bader, O., M. Weig, U. Reichard, R. Lugert, M. Kuhns, M. Christner, J. Held, S. Peter, U. Schumacher, D. Buchheidt, K. Tintelnot, U. Gross, and D. Partners MykoLabNet. 2013. 'cyp51A-Based mechanisms of Aspergillus fumigatus azole drug resistance present in clinical samples from Germany', Antimicrob Agents Chemother, 57: 3513-7.

Basenko, E. Y., J. A. Pulman, A. Shanmugasundram, O. S. Harb, K. Crouch, D. Starns, S. Warrenfeltz, C. Aurrecoechea, C. J. Stoeckert, Jr., J. C. Kissinger, D. S. Roos, and C. Hertz-Fowler. 2018. 'FungiDB: An Integrated Bioinformatic Resource for Fungi and Oomycetes', J Fungi (Basel), 4.

Beernaert, L. A., F. Pasmans, L. Van Waeyenberghe, G. M. Dorrestein, F. Verstappen, F. Vercammen, F. Haesebrouck, and A. Martel. 2009. 'Avian Aspergillus fumigatus strains resistant to both itraconazole and voriconazole', Antimicrob Agents Chemother, 53: 2199-201.

Bellete, B., H. Raberin, J. Morel, P. Flori, J. Hafid, and R. T. Manhsung. 2010. 'Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma', Med Mycol, 48: 197-200.

Bongomin, F., L. G. Asio, J. B. Baluku, R. Kwizera, and D. W. Denning. 2020. 'Chronic Pulmonary Aspergillosis: Notes for a Clinician in a Resource-Limited Setting Where There Is No Mycologist', J Fungi (Basel), 6.

Bongomin, F., S. Gago, R. O. Oladele, and D. W. Denning. 2017. 'Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision', J Fungi (Basel), 3.

Bowyer, P., M. J. Bromley, and D. W. Denning. 2020. 'Linking calcium signaling and mitochondrial function in fungal drug resistance', Proc Natl Acad Sci U S A, 117: 1254-56.

Boyce, K. J., Y. Wang, S. Verma, V. P. S. Shakya, C. Xue, and A. Idnurm. 2017. 'Mismatch Repair of DNA Replication Errors Contributes to Microevolution in the Pathogenic Fungus Cryptococcus neoformans', mBio, 8.

Brissaud, O., J. Guichoux, J. Harambat, O. Tandonnet, and T. Zaoutis. 2012. 'Invasive fungal disease in PICU: epidemiology and risk factors', Ann Intensive Care, 2: 6.

Bromley, M., A. Johns, E. Davies, M. Fraczek, J. Mabey Gilsenan, N. Kurbatova, M. Keays, M. Kapushesky, M. Gut, I. Gut, D. W. Denning, and P. Bowyer. 2016. 'Mitochondrial Complex I Is a Global Regulator of Secondary Metabolism, Virulence and Azole Sensitivity in Fungi', PLoS One, 11: e0158724.

Brown, G. D., D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea, and T. C. White. 2012. 'Hidden killers: human fungal infections', Sci Transl Med, 4: 165rv13.

Bueid, A., S. J. Howard, C. B. Moore, M. D. Richardson, E. Harrison, P. Bowyer, and D. W. Denning. 2010. 'Azole antifungal resistance in Aspergillus fumigatus: 2008 and 2009', J Antimicrob Chemother, 65: 2116-8.

Buil, J. B., R. K. Hare, B. J. Zwaan, M. C. Arendrup, W. J. G. Melchers, and P. E. Verweij. 2019. 'The fading boundaries between patient and environmental routes of triazole resistance selection in Aspergillus fumigatus', PLoS Pathog, 15: e1007858.

Buscaino, A. 2019. 'Chromatin-Mediated Regulation of Genome Plasticity in Human Fungal Pathogens', Genes (Basel), 10.

Camps, S. M., B. E. Dutilh, M. C. Arendrup, A. J. Rijs, E. Snelders, M. A. Huynen, P. E. Verweij, and W. J. Melchers. 2012. 'Discovery of a HapE mutation that causes azole resistance in Aspergillus fumigatus through whole genome sequencing and sexual crossing', PLoS One, 7: e50034.

Camps, S. M., A. J. Rijs, C. H. Klaassen, J. F. Meis, C. M. O'Gorman, P. S. Dyer, W. J. Melchers, and P. E. Verweij. 2012. 'Molecular epidemiology of Aspergillus fumigatus isolates harboring the TR34/L98H azole resistance mechanism', J Clin Microbiol, 50: 2674-80.

Camps, S. M., J. W. van der Linden, Y. Li, E. J. Kuijper, J. T. van Dissel, P. E. Verweij, and W. J. Melchers. 2012. 'Rapid induction of multiple resistance mechanisms in Aspergillus fumigatus during azole therapy: a case study and review of the literature', Antimicrob Agents Chemother, 56: 10-6.

CDC. 2019. 'Aspergillosis Statistics', Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED), Accessed 8/24/2020. https://www.cdc.gov/fungal/diseases/aspergillosis/statistics.html.

Chen, J., H. Li, R. Li, D. Bu, and Z. Wan. 2005. 'Mutations in the cyp51A gene and susceptibility to itraconazole in Aspergillus fumigatus serially isolated from a patient with lung aspergilloma', J Antimicrob Chemother, 55: 31-7.

Chowdhary, A., S. Kathuria, H. S. Randhawa, S. N. Gaur, C. H. Klaassen, and J. F. Meis. 2012. 'Isolation of multiple-triazole-resistant Aspergillus fumigatus strains carrying the TR/L98H mutations in the cyp51A gene in India', J Antimicrob Chemother, 67: 362-6.

Chowdhary, A., S. Kathuria, J. Xu, and J. F. Meis. 2013. 'Emergence of azole-resistant aspergillus fumigatus strains due to agricultural azole use creates an increasing threat to human health', PLoS Pathog, 9: e1003633.

Chowdhary, A., C. Sharma, S. Kathuria, F. Hagen, and J. F. Meis. 2014. 'Azole-resistant Aspergillus fumigatus with the environmental TR46/Y121F/T289A mutation in India', J Antimicrob Chemother, 69: 555-7.

Chowdhary, Anuradha, Cheshta Sharma, Shallu Kathuria, Ferry Hagen, and Jacques F. Meis. 2015. 'Prevalence and mechanism of triazole resistance in Aspergillus fumigatus in

a referral chest hospital in Delhi, India and an update of the situation in Asia', Frontiers in Microbiology, 6.

Chryssanthou, E. 1997. 'In vitro susceptibility of respiratory isolates of Aspergillus species to itraconazole and amphotericin B. acquired resistance to itraconazole', Scand J Infect Dis, 29: 509-12. Covo, S. 2020. 'Genomic Instability in Fungal Plant Pathogens', Genes (Basel), 11.

Dagenais, T. R., and N. P. Keller. 2009. 'Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis', Clin Microbiol Rev, 22: 447-65. Dannaoui, E., E. Borel, M. F. Monier, M. A. Piens, S. Picot, and F. Persat. 2001. 'Acquired itraconazole resistance in Aspergillus fumigatus', J Antimicrob Chemother, 47: 333-40.

Dannaoui, E., F. Persat, M. F. Monier, E. Borel, M. A. Piens, and S. Picot. 1999. 'In-vitro susceptibility of Aspergillus spp. isolates to amphotericin B and itraconazole', Journal of Antimicrobial Chemotherapy, 44: 553-55.

Dasbach, E. J., G. M. Davies, and S. M. Teutsch. 2000. 'Burden of aspergillosis-related hospitalizations in the United States', Clin Infect Dis, 31: 1524-8.

Denning, D. W. 1998. 'Invasive aspergillosis', Clin Infect Dis, 26: 781-803; quiz 04-5.

Denning, D. W., S. Park, C. Lass-Florl, M. G. Fraczek, M. Kirwan, R. Gore, J. Smith, A. Bueid, C. B. Moore, P. Bowyer, and D. S. Perlin. 2011. 'High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease', Clin Infect Dis, 52: 1123-9.

Denning, D. W., A. Pleuvry, and D. C. Cole. 2013. 'Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults', Med Mycol, 51: 361-70.

Denning, D. W., S. A. Radford, K. L. Oakley, L. Hall, E. M. Johnson, and D. W. Warnock. 1997. 'Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of Aspergillus fumigatus infection', J Antimicrob Chemother, 40: 401-14.

Denning, D. W., K. Riniotis, R. Dobrashian, and H. Sambatakou. 2003. 'Chronic cavitary and fibrosing pulmonary and pleural aspergillosis: case series, proposed nomenclature change, and review', Clin Infect Dis, 37 Suppl 3: S265-80.

Denning, D. W., R. M. Tucker, L. H. Hanson, and D. A. Stevens. 1989. 'Treatment of invasive aspergillosis with itraconazole', Am J Med, 86: 791-800.

Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly. 1997. 'Itraconazole resistance in Aspergillus fumigatus', Antimicrob Agents Chemother, 41: 1364-8.

Diaz-Guerra, T. M., E. Mellado, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2003. 'A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in Aspergillus fumigatus', Antimicrob Agents Chemother, 47: 1120-4.

Dos Reis, T. F., L. P. Silva, P. A. de Castro, P. B. Almeida de Lima, R. A. do Carmo, M. M. Marini, J. F. da Silveira, B. H. Ferreira, F. Rodrigues, I. Malavazi, and G. H. Goldman. 2018. 'The Influence of Genetic Stability on Aspergillus fumigatus Virulence and Azole Resistance', G3 (Bethesda), 8: 265-78.

Dos Reis, T. F., L. P. Silva, P. A. de Castro, R. A. do Carmo, M. M. Marini, J. F. da Silveira, B. H. Ferreira, F. Rodrigues, A. L. Lind, A. Rokas, and G. H. Goldman. 2019. 'The Aspergillus fumigatus Mismatch Repair MSH2 Homolog Is Important for Virulence and Azole Resistance', mSphere, 4.

Eggimann, P., J. C. Chevrolet, M. Starobinski, P. Majno, M. Totsch, B. Chapuis, and D. Pittet. 2006. 'Primary invasive aspergillosis of the digestive tract: report of two cases and review of the literature', Infection, 34: 333-8.

Falcone, M., A. P. Massetti, A. Russo, V. Vullo, and M. Venditti. 2011. 'Invasive aspergillosis in patients with liver disease', Med Mycol, 49: 406-13.

Faria-Ramos, I., S. Farinha, J. Neves-Maia, P. R. Tavares, I. M. Miranda, L. M. Estevinho, C. Pina-Vaz, and A. G. Rodrigues. 2014. 'Development of cross-resistance by Aspergillus fumigatus to clinical azoles following exposure to prochloraz, an agricultural azole', BMC Microbiol, 14: 155.

Ferreira, M. E., A. L. Colombo, I. Paulsen, Q. Ren, J. Wortman, J. Huang, M. H. Goldman, and G. H. Goldman. 2005. 'The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus', Med Mycol, 43 Suppl 1: S313-9.

Fisher, M. C., D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw, and S. J. Gurr. 2012. 'Emerging fungal threats to animal, plant and ecosystem health', Nature, 484: 186-94.

Fraczek, M. G., M. Bromley, A. Buied, C. B. Moore, R. Rajendran, R. Rautemaa, G. Ramage, D. W. Denning, and P. Bowyer. 2013. 'The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in Aspergillus fumigatus', J Antimicrob Chemother, 68: 1486-96.

Fraser, D. W., J. I. Ward, L. Ajello, and B. D. Plikaytis. 1979. 'Aspergillosis and other systemic mycoses. The growing problem', JAMA, 242: 1631-5.

Fuhren, J., W. S. Voskuil, C. H. Boel, P. J. Haas, F. Hagen, J. F. Meis, and J. G. Kusters. 2015. 'High prevalence of azole resistance in Aspergillus fumigatus isolates from high-risk patients', J Antimicrob Chemother, 70: 2894-8.

George, D., P. Miniter, and V. T. Andriole. 1996. 'Efficacy of UK-109496, a new azole antifungal agent, in an experimental model of invasive aspergillosis', Antimicrob Agents Chemother, 40: 86-91.

Groll, A. H., P. M. Shah, C. Mentzel, M. Schneider, G. Just-Nuebling, and K. Huebner. 1996. 'Trends in the postmortem epidemiology of invasive fungal infections at a university hospital', J Infect, 33: 23-32.

Groll, A. H., and T. J. Walsh. 2001. 'Uncommon opportunistic fungi: new nosocomial threats', Clin Microbiol Infect, 7 Suppl 2: 8-24.

Gsaller, F., P. Hortschansky, T. Furukawa, P. D. Carr, B. Rash, J. Capilla, C. Muller, F. Bracher, P. Bowyer, H. Haas, A. A. Brakhage, and M. J. Bromley. 2016a. 'Correction: Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex', PLoS Pathog, 12: e1006106.

———. 2016b. 'Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex', PLoS Pathog, 12: e1005775.

Hagiwara, D., T. Arai, H. Takahashi, Y. Kusuya, A. Watanabe, and K. Kamei. 2018. 'Non-cyp51A Azole-Resistant Aspergillus fumigatus Isolates with Mutation in HMG-CoA Reductase', Emerg Infect Dis, 24: 1889-97.

Hagiwara, D., H. Takahashi, A. Watanabe, A. Takahashi-Nakaguchi, S. Kawamoto, K. Kamei, and T. Gonoi. 2014. 'Whole-genome comparison of Aspergillus fumigatus strains serially isolated from patients with aspergillosis', J Clin Microbiol, 52: 4202-9.

Hagiwara, D., A. Watanabe, K. Kamei, and G. H. Goldman. 2016. 'Epidemiological and Genomic Landscape of Azole Resistance Mechanisms in Aspergillus Fungi', Front Microbiol, 7: 1382.

Healey, K. R., C. Jimenez Ortigosa, E. Shor, and D. S. Perlin. 2016. 'Genetic Drivers of Multidrug Resistance in Candida glabrata', Front Microbiol, 7: 1995.

Heeres, J., L. J. Backx, and J. Van Cutsem. 1984. 'Antimycotic azoles. 7. Synthesis and antifungal properties of a series of novel triazol-3-ones', J Med Chem, 27: 894-900.

Hokken, M. W. J., B. J. Zwaan, W. J. G. Melchers, and P. E. Verweij. 2019. 'Facilitators of adaptation and antifungal resistance mechanisms in clinically relevant fungi', Fungal Genet Biol, 132: 103254.

Howard, S. J., D. Cerar, M. J. Anderson, A. Albarrag, M. C. Fisher, A. C. Pasqualotto, M. Laverdiere, M. C. Arendrup, D. S. Perlin, and D. W. Denning. 2009. 'Frequency and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure', Emerg Infect Dis, 15: 1068-76.

Howard, S. J., A. C. Pasqualotto, M. J. Anderson, H. Leatherbarrow, A. M. Albarrag, E. Harrison, L. Gregson, P. Bowyer, and D. W. Denning. 2013. 'Major variations in Aspergillus fumigatus arising within aspergillomas in chronic pulmonary aspergillosis', Mycoses, 56: 434-41.

Howard, S. J., I. Webster, C. B. Moore, R. E. Gardiner, S. Park, D. S. Perlin, and D. W. Denning. 2006. 'Multi-azole resistance in Aspergillus fumigatus', Int J Antimicrob Agents, 28: 450-3.

Koehler, P., A. Hamprecht, O. Bader, I. Bekeredjian-Ding, D. Buchheidt, G. Doelken, J. Elias, G. Haase, C. Hahn-Ast, M. Karthaus, A. Kekule, P. Keller, M. Kiehl, S. W. Krause, C. Kramer, S. Neumann, H. Rohde, P. La Rosee, M. Ruhnke, P. Schafhausen, E. Schalk, K. Schulz, S. Schwartz, G. Silling, P. Staib, A. Ullmann, M. Vergoulidou, T. Weber, O. A. Cornely, and M. J. Vehreschild. 2017. 'Epidemiology of invasive aspergillosis and azole resistance in patients with acute leukaemia: the SEPIA Study', Int J Antimicrob Agents, 49: 218-23.

Kontoyiannis, D. P., K. A. Marr, B. J. Park, B. D. Alexander, E. J. Anaissie, T. J. Walsh, J. Ito, D. R. Andes, J. W. Baddley, J. M. Brown, L. M. Brumble, A. G. Freifeld, S. Hadley, L. A. Herwaldt, C. A. Kauffman, K. Knapp, G. M. Lyon, V. A. Morrison, G. Papanicolaou, T. F. Patterson, T. M. Perl, M. G. Schuster, R. Walker, K. A. Wannemuehler, J. R. Wingard, T. M. Chiller, and P. G. Pappas. 2010. 'Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database', Clin Infect Dis, 50: 1091-100.

Kosmidis, C., and D. W. Denning. 2015. 'The clinical spectrum of pulmonary aspergillosis', Thorax, 70: 270-7.

Krishnan Natesan, S., W. Wu, J. L. Cutright, and P. H. Chandrasekar. 2012. 'In vitro-in vivo correlation of voriconazole resistance due to G448S mutation (cyp51A gene) in Aspergillus fumigatus', Diagn Microbiol Infect Dis, 74: 272-7.

Lass-Florl, C., and M. Cuenca-Estrella. 2017. 'Changes in the epidemiological landscape of invasive mould infections and disease', J Antimicrob Chemother, 72: i5-i11.

Latge, J. P. 1999. 'Aspergillus fumigatus and aspergillosis', Clin Microbiol Rev, 12: 310-50.

Legrand, M., C. L. Chan, P. A. Jauert, and D. T. Kirkpatrick. 2007. 'Role of DNA mismatch repair and double-strand break repair in genome stability and antifungal drug resistance in Candida albicans', Eukaryot Cell, 6: 2194-205.

Lestrade, P. P., R. G. Bentvelsen, Afad Schauwvlieghe, S. Schalekamp, Wjfm van der Velden, E. J. Kuiper, J. van Paassen, B. van der Hoven, H. A. van der Lee, W. J. G. Melchers, A. F. de Haan, H. L. van der Hoeven, B. J. A. Rijnders, M. T. van der Beek, and

P. E. Verweij. 2019. 'Voriconazole Resistance and Mortality in Invasive Aspergillosis: A Multicenter Retrospective Cohort Study', Clin Infect Dis, 68: 1463-71.

Lestrade, P. P., J. F. Meis, J. P. Arends, M. T. van der Beek, E. de Brauwer, K. van Dijk, S. C. de Greeff, P. J. Haas, C. J. Hodiamont, E. J. Kuijper, T. Leenstra, A. E. Muller, A. M. Oude Lashof, B. J. Rijnders, E. Roelofsen, W. Rozemeijer, M. Tersmette, E. M. Terveer, C. M. Verduin, M. J. Wolfhagen, W. J. Melchers, and P. E. Verweij. 2016. 'Diagnosis and management of aspergillosis in the Netherlands: a national survey', Mycoses, 59: 101-7.

Levetin, E. 2004. 'An atlas of fungal spores', J Allergy Clin Immunol, 113: 366-8.

Li, Y., Y. Zhang, C. Zhang, H. Wang, X. Wei, P. Chen, and L. Lu. 2020. 'Mitochondrial dysfunctions trigger the calcium signaling-dependent fungal multidrug resistance', Proc Natl Acad Sci U S A, 117: 1711-21.

Lockhart, S. R., J. P. Frade, K. A. Etienne, M. A. Pfaller, D. J. Diekema, and S. A. Balajee. 2011. 'Azole resistance in Aspergillus fumigatus isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene', Antimicrob Agents Chemother, 55: 4465-8.

Lopez-Medrano, F., M. Fernandez-Ruiz, J. T. Silva, P. L. Carver, C. van Delden, E. Merino, M. J. Perez-Saez, M. Montero, J. Coussement, M. de Abreu Mazzolin, C. Cervera, L. Santos, N. Sabe, A. Scemla, E. Cordero, L. Cruzado-Vega, P. L. Martin-Moreno, O. Len, E. Rudas, A. P. de Leon, M. Arriola, R. Lauzurica, M. David, C. Gonzalez-Rico, F. Henriquez-Palop, J. Fortun, M. Nucci, O. Manuel, J. R. Pano-Pardo, M. Montejo, P. Munoz, B. Sanchez-Sobrino, A. Mazuecos, J. Pascual, J. P. Horcajada, T. Lecompte, A. Moreno, J. Carratala, M. Blanes, D. Hernandez, M. C. Farinas, A. Andres, J. M. Aguado, the Group for the Study of Infection in Transplant Recipients of the Spanish Society of Clinical Microbiology Spanish Network for Research in Infectious Diseases, the Study Group for Infections in Compromised Hosts of the European Society of Clinical Microbiology Infectious Diseases, Diseases Infectious, and Study the Swiss Transplant Cohort. 2016. 'Clinical Presentation and Determinants of Mortality of Invasive Pulmonary Aspergillosis in Kidney Transplant Recipients: A Multinational Cohort Study', Am J Transplant, 16: 3220-34.

Manavathu, E. K., J. L. Cutright, D. Loebenberg, and P. H. Chandrasekar. 2000. 'A comparative study of the in vitro susceptibilities of clinical and laboratory-selected resistant isolates of Aspergillus spp. to amphotericin B, itraconazole, voriconazole and posaconazole (SCH 56592)', J Antimicrob Chemother, 46: 229-34.

Mann, P. A., R. M. Parmegiani, S. Q. Wei, C. A. Mendrick, X. Li, D. Loebenberg, B. DiDomenico, R. S. Hare, S. S. Walker, and P. M. McNicholas. 2003. 'Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase', Antimicrob Agents Chemother, 47: 577-81.

Matthaiou, E. I., G. Sass, D. A. Stevens, and J. L. Hsu. 2018. 'Iron: an essential nutrient for Aspergillus fumigatus and a fulcrum for pathogenesis', Curr Opin Infect Dis, 31: 506-11.

Mavridou, E., J. Meletiadis, P. Jancura, S. Abbas, M. C. Arendrup, W. J. Melchers, T. Heskes, J. W. Mouton, and P. E. Verweij. 2013. 'Composite survival index to compare virulence changes in azole-resistant Aspergillus fumigatus clinical isolates', PLoS One, 8: e72280.

Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, M. Cuenca-Estrella, and J. L. Rodriguez -Tudela. 2004. 'Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of Aspergillus fumigatus are responsible for resistance in vitro to azole antifungal drugs', Antimicrob Agents Chemother, 48: 2747-50.

Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, W. J. Melchers, P. E. Verweij, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2007. 'A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations', Antimicrob Agents Chemother, 51: 1897-904.

Nascimento, A. M., G. H. Goldman, S. Park, S. A. Marras, G. Delmas, U. Oza, K. Lolans, M. N. Dudley, P. A. Mann, and D. S. Perlin. 2003. 'Multiple resistance mechanisms among Aspergillus fumigatus mutants with high-level resistance to itraconazole', Antimicrob Agents Chemother, 47: 1719-26.

Neofytos, D., J. A. Fishman, D. Horn, E. Anaissie, C. H. Chang, A. Olyaei, M. Pfaller, W. J. Steinbach, K. M. Webster, and K. A. Marr. 2010. 'Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients', Transplant Infectious Disease, 12: 220-29. Nierman, W. C., G. May, H. S. Kim, M. J. Anderson, D. Chen, and D. W. Denning. 2005. 'What the Aspergillus genomes have told us', Med Mycol, 43 Suppl 1: S3-5.

Oakley, K. L., C. B. Moore, and D. W. Denning. 1998. 'In-vitro activity of voriconazole against Aspergillus spp. and comparison with itraconazole and amphotericin B', J Antimicrob Chemother, 42: 91-4.

Patterson, T. F., G. R. Thompson, 3rd, D. W. Denning, J. A. Fishman, S. Hadley, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, M. H. Nguyen, B. H. Segal, W. J. Steinbach, D. A. Stevens, T. J. Walsh, J. R. Wingard, J. A. Young, and J. E. Bennett. 2016. 'Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America', Clin Infect Dis, 63: e1-e60.

Paul, S., D. Diekema, and W. S. Moye-Rowley. 2013. 'Contributions of Aspergillus fumigatus ATP-binding cassette transporter proteins to drug resistance and virulence', Eukaryot Cell, 12: 1619-28.

Paulussen, C., J. E. Hallsworth, S. Alvarez-Perez, W. C. Nierman, P. G. Hamill, D. Blain, H. Rediers, and B. Lievens. 2017. 'Ecology of aspergillosis: insights into the

pathogenic potency of Aspergillus fumigatus and some other Aspergillus species', Microb Biotechnol, 10: 296-322.

Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema. 2008. 'In vitro survey of triazole cross-resistance among more than 700 clinical isolates of Aspergillus species', J Clin Microbiol, 46: 2568-72.

Pfaller, M. A., P. R. Rhomberg, N. P. Wiederhold, C. Gibas, C. Sanders, H. Fan, J. Mele, L. L. Kovanda, and M. Castanheira. 2018. 'In Vitro Activity of Isavuconazole against Opportunistic Fungal Pathogens from Two Mycology Reference Laboratories', Antimicrob Agents Chemother, 62.

Pham, C. D., E. Reiss, F. Hagen, J. F. Meis, and S. R. Lockhart. 2014. 'Passive surveillance for azole-resistant Aspergillus fumigatus, United States, 2011-2013', Emerg Infect Dis, 20: 1498-503.

Rajendran, R., E. Mowat, E. McCulloch, D. F. Lappin, B. Jones, S. Lang, J. B. Majithiya, P. Warn, C. Williams, and G. Ramage. 2011. 'Azole resistance of Aspergillus fumigatus biofilms is partly associated with efflux pump activity', Antimicrob Agents Chemother, 55: 2092-7.

Rees, J. R., R. W. Pinner, R. A. Hajjeh, M. E. Brandt, and A. L. Reingold. 1998. 'The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992-1993: results of population-based laboratory active surveillance', Clin Infect Dis, 27: 1138-47.

Ren, J., X. Jin, Q. Zhang, Y. Zheng, D. Lin, and Y. Yu. 2017. 'Fungicides induced triazole-resistance in Aspergillus fumigatus associated with mutations of TR46/Y121F/-T289A and its appearance in agricultural fields', J Hazard Mater, 326: 54-60.

Resendiz Sharpe, A., K. Lagrou, J. F. Meis, A. Chowdhary, S. R. Lockhart, P. E. Verweij, and Isham Ecmm Aspergillus Resistance Surveillance working group. 2018. 'Triazole resistance surveillance in Aspergillus fumigatus', Med Mycol, 56: 83-92.

Ribas, E. Ribas A. D., P. Spolti, E. M. Del Ponte, K. Z. Donato, H. Schrekker, and A. M. Fuentefria. 2016. 'Is the emergence of fungal resistance to medical triazoles related to their use in the agroecosystems? A mini review', Braz J Microbiol, 47: 793-99.

Rodriguez-Tudela, J. L., L. Alcazar-Fuoli, E. Mellado, A. Alastruey-Izquierdo, A. Monzon, and M. Cuenca-Estrella. 2008. 'Epidemiological cutoffs and cross-resistance to azole drugs in Aspergillus fumigatus', Antimicrobial Agents and Chemotherapy, 52: 2468-72.

Rose, H. D. 1972. 'Mechanical control of hospital ventilation and Aspergillus infections', Am Rev Respir Dis, 105: 306-7. Rybak, J. M., J. R. Fortwendel, and P. D. Rogers. 2019.

'Emerging threat of triazole-resistant Aspergillus fumigatus', J Antimicrob Chemother, 74: 835-42.

Rybak, J. M., W. Ge, N. P. Wiederhold, J. E. Parker, S. L. Kelly, P. D. Rogers, and J. R. Fortwendel. 2019. 'Mutations in hmg1, Challenging the Paradigm of Clinical Triazole Resistance in Aspergillus fumigatus', MBio, 10.

Schoustra, S. E., A. J. M. Debets, Ajmm Rijs, J. Zhang, E. Snelders, P. C. Leendertse, W. J. G. Melchers, A. G. Rietveld, B. J. Zwaan, and P. E. Verweij. 2019. 'Environmental Hotspots for Azole Resistance Selection of Aspergillus fumigatus, the Netherlands', Emerg Infect Dis, 25: 1347-53.

Selmecki, A. M., K. Dulmage, L. E. Cowen, J. B. Anderson, and J. Berman. 2009. 'Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance', PLoS Genet, 5: e1000705.

Seyedmousavi, S., J. Guillot, P. Arne, G. S. de Hoog, J. W. Mouton, W. J. Melchers, and P. E. Verweij. 2015. 'Aspergillus and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease', Med Mycol, 53: 765-97.

Shah, A., and C. Panjabi. 2016. 'Allergic Bronchopulmonary Aspergillosis: A Perplexing Clinical Entity', Allergy Asthma Immunol Res, 8: 282-97.

Slaven, John W., Michael J. Anderson, Dominique Sanglard, Graham K. Dixon, Jacques Bille, Ian S. Roberts, and David W. Denning. 2002. 'Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate', Fungal Genetics and Biology, 36: 199-206.

Snelders, E., S. M. Camps, A. Karawajczyk, A. J. Rijs, J. Zoll, P. E. Verweij, and W. J. Melchers. 2015. 'Genotype-phenotype complexity of the TR46/Y121F/T289A cyp51A azole resistance mechanism in Aspergillus fumigatus', Fungal Genet Biol, 82: 129-35.

Snelders, E., S. M. Camps, A. Karawajczyk, G. Schaftenaar, G. H. Kema, H. A. van der Lee, C. H. Klaassen, W. J. Melchers, and P. E. Verweij. 2012. 'Triazole fungicides can induce cross-resistance to medical triazoles in Aspergillus fumigatus', PLoS One, 7: e31801.

Snelders, E., R. A. Huis In 't Veld, A. J. Rijs, G. H. Kema, W. J. Melchers, and P. E. Verweij. 2009. 'Possible environmental origin of resistance of Aspergillus fumigatus to medical triazoles', Appl Environ Microbiol, 75: 4053-7.

Snelders, E., A. Karawajczyk, G. Schaftenaar, P. E. Verweij, and W. J. Melchers. 2010. 'Azole resistance profile of amino acid changes in Aspergillus fumigatus CYP51A based on protein homology modeling', Antimicrob Agents Chemother, 54: 2425-30.

Snelders, E., A. Karawajczyk, R. J. Verhoeven, H. Venselaar, G. Schaftenaar, P. E. Verweij, and W. J. Melchers. 2011. 'The structure-function relationship of the Aspergillus

fumigatuscyp51A L98H conversion by site-directed mutagenesis: the mechanism of L98H azole resistance', Fungal Genet Biol, 48: 1062-70.

Snelders, E., H. A. van der Lee, J. Kuijpers, A. J. Rijs, J. Varga, R. A. Samson, E. Mellado, A. R. Donders, W. J. Melchers, and P. E. Verweij. 2008. 'Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism', PLoS Med, 5: e219.

Sniegowski, P. D., and P. J. Gerrish. 2010. 'Beneficial mutations and the dynamics of adaptation in asexual populations', Philos Trans R Soc Lond B Biol Sci, 365: 1255-63.

Staff, KING 5. 2019. 'King County to make Aspergillus mold infections reportable to public health'. https://www.king5.com/article/news/health/seattle-childrens-aspergillus-mold-reporting/281-6ae6b740-e479-4334-a9e3-78c80f3494f1.

Steinbach, W. J., K. A. Marr, E. J. Anaissie, N. Azie, S. P. Quan, H. U. Meier-Kriesche, S. Apewokin, and D. L. Horn. 2012. 'Clinical epidemiology of 960 patients with invasive aspergillosis from the PATH Alliance registry', J Infect, 65: 453-64.

Stensvold, Christen Rune, Lise Nistrup Jørgensen, and Maiken Cavling Arendrup. 2012. 'Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture', Current Fungal Infection Reports, 6: 178-91.

Streifel, A. J., J. L. Lauer, D. Vesley, B. Juni, and F. S. Rhame. 1983. 'Aspergillus fumigatus and other thermotolerant fungi generated by hospital building demolition', Appl Environ Microbiol, 46: 375-8.

Tashiro, M., and K. Izumikawa. 2016. 'The Current Status of Drug-resistant Aspergillus', Med Mycol J, 57: J103-12.

Tekaia, F., and J. P. Latge. 2005. 'Aspergillus fumigatus: saprophyte or pathogen?', Curr Opin Microbiol, 8: 385-92.

Tobin, M. B., R. B. Peery, and P. L. Skatrud. 1997. 'Genes encoding multiple drug resistance-like proteins in Aspergillus fumigatus and Aspergillus flavus', Gene, 200: 11-23.

Valdes, I. D., J. van den Berg, A. Haagsman, N. Escobar, J. F. Meis, F. Hagen, P. J. Haas, J. Houbraken, H. A. B. Wosten, and H. de Cock. 2018. 'Comparative genotyping and phenotyping of Aspergillus fumigatus isolates from humans, dogs and the environment', BMC Microbiol, 18: 118.

Vallabhaneni, S., K. Benedict, G. Derado, and R. K. Mody. 2017. 'Trends in Hospitalizations Related to Invasive Aspergillosis and Mucormycosis in the United States, 2000-2013', Open Forum Infect Dis, 4: ofw268.

van der Linden, J. W., M. C. Arendrup, A. Warris, K. Lagrou, H. Pelloux, P. M. Hauser, E. Chryssanthou, E. Mellado, S. E. Kidd, A. M. Tortorano, E. Dannaoui, P. Gaustad,

J. W. Baddley, A. Uekotter, C. Lass-Florl, N. Klimko, C. B. Moore, D. W. Denning, A. C. Pasqualotto, C. Kibbler, S. Arikan-Akdagli, D. Andes, J. Meletiadis, L. Naumiuk, M. Nucci, W. J. Melchers, and P. E. Verweij. 2015. 'Prospective multicenter international surveillance of azole resistance in Aspergillus fumigatus', Emerg Infect Dis, 21: 1041-4.

van der Linden, J. W., S. M. Camps, G. A. Kampinga, J. P. Arends, Y. J. Debets-Ossenkopp, P. J. Haas, B. J. Rijnders, E. J. Kuijper, F. H. van Tiel, J. Varga, A. Karawajczyk, J. Zoll, W. J. Melchers, and P. E. Verweij. 2013. 'Aspergillosis due to voriconazole highly resistant Aspergillus fumigatus and recovery of genetically related resistant isolates from domiciles', Clin Infect Dis, 57: 513-20.

van der Linden, J. W., E. Snelders, G. A. Kampinga, B. J. Rijnders, E. Mattsson, Y. J. Debets-Ossenkopp, E. J. Kuijper, F. H. Van Tiel, W. J. Melchers, and P. E. Verweij. 2011. 'Clinical implications of azole resistance in Aspergillus fumigatus, The Netherlands, 2007-2009', Emerg Infect Dis, 17: 1846-54.

van der Linden, J. W., A. Warris, J. W. van der Meer, D. Bresters, W. J. Melchers, and P. E. Verweij. 2009. '[Azole-resistant invasive aspergillosis]', Ned Tijdschr Geneeskd, 153: A765.

van Paassen, J., A. Russcher, A. W. In 't Veld-van Wingerden, P. E. Verweij, and E. J. Kuijper. 2016. 'Emerging aspergillosis by azole-resistant Aspergillus fumigatus at an intensive care unit in the Netherlands, 2010 to 2013', Euro Surveill, 21.

Verweij, P. E., A. Chowdhary, W. J. Melchers, and J. F. Meis. 2016. 'Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles?', Clin Infect Dis, 62: 362-8.

Verweij, P. E., S. J. Howard, W. J. Melchers, and D. W. Denning. 2009. 'Azole-resistance in Aspergillus: proposed nomenclature and breakpoints', Drug Resist Updat, 12: 141-7.

Verweij, P. E., G. H. Kema, B. Zwaan, and W. J. Melchers. 2013. 'Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould Aspergillus fumigatus', Pest Manag Sci, 69: 165-70.

Verweij, P. E., E. Mellado, and W. J. Melchers. 2007. 'Multiple-triazole-resistant aspergillosis', N Engl J Med, 356: 1481-3.

Verweij, P. E., E. Snelders, G. H. Kema, E. Mellado, and W. J. Melchers. 2009. 'Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use?', Lancet Infect Dis, 9: 789-95.

Verweij, P. E., D. T. Te Dorsthorst, A. J. Rijs, H. G. De Vries-Hospers, and J. F. Meis. 2002. 'Nationwide survey of in vitro activities of itraconazole and voriconazole against

clinical Aspergillus fumigatus isolates cultured between 1945 and 1998', J Clin Microbiol, 40: 2648-50.

Verweij, P. E., J. Zhang, A. J. M. Debets, J. F. Meis, F. L. van de Veerdonk, S. E. Schoustra, B. J. Zwaan, and W. J. G. Melchers. 2016. 'In-host adaptation and acquired triazole resistance in Aspergillus fumigatus: a dilemma for clinical management', Lancet Infect Dis, 16: e251-e60.

Walker, T. A., S. R. Lockhart, S. E. Beekmann, P. M. Polgreen, S. Santibanez, R. K. Mody, K. D. Beer, T. M. Chiller, and B. R. Jackson. 2018. 'Recognition of Azole-Resistant Aspergillosis by Physicians Specializing in Infectious Diseases, United States', Emerg Infect Dis, 24.

Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, T. F. Patterson, and America Infectious Diseases Society of. 2008. 'Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America', Clin Infect Dis, 46: 327-60.

Wang, H. C., J. C. Huang, Y. H. Lin, Y. H. Chen, M. I. Hsieh, P. C. Choi, H. J. Lo, W. L. Liu, C. S. Hsu, H. I. Shih, C. J. Wu, and Y. C. Chen. 2018. 'Prevalence, mechanisms and genetic relatedness of the human pathogenic fungus Aspergillus fumigatus exhibiting resistance to medical azoles in the environment of Taiwan', Environ Microbiol, 20: 270-80.

Webb, B. J., J. P. Ferraro, S. Rea, S. Kaufusi, B. E. Goodman, and J. Spalding. 2018. 'Epidemiology and Clinical Features of Invasive Fungal Infection in a US Health Care Network', Open Forum Infect Dis, 5: ofy187.

Wiederhold, N. P., V. G. Gil, F. Gutierrez, J. R. Lindner, M. T. Albataineh, D. I. McCarthy, C. Sanders, H. Fan, A. W. Fothergill, and D. A. Sutton. 2016. 'First Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in Aspergillus fumigatus Isolates in the United States', J Clin Microbiol, 54: 168-71.

Wiederhold, N. P., and P. E. Verweij. 2020. 'Aspergillus fumigatus and pan-azole resistance: who should be concerned?', Curr Opin Infect Dis, 33: 290-97.

Winters, B., J. Custer, S. M. Galvagno, Jr., E. Colantuoni, S. G. Kapoor, H. Lee, V. Goode, K. Robinson, A. Nakhasi, P. Pronovost, and D. Newman-Toker. 2012. 'Diagnostic errors in the intensive care unit: a systematic review of autopsy studies', BMJ Qual Saf, 21: 894-902.

Xiao, L., V. Madison, A. S. Chau, D. Loebenberg, R. E. Palermo, and P. M. McNicholas. 2004. 'Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from Aspergillus fumigatus and Candida albicans provide insights into posaconazole binding', Antimicrob Agents Chemother, 48: 568-74.

Yona, A. H., I. Frumkin, and Y. Pilpel. 2015. 'A relay race on the evolutionary adaptation spectrum', Cell, 163: 549-59.

Zhang, J., J. van den Heuvel, A. J. M. Debets, P. E. Verweij, W. J. G. Melchers, B. J. Zwaan, and S. E. Schoustra. 2017. 'Evolution of cross-resistance to medical triazoles in Aspergillus fumigatus through selection pressure of environmental fungicides', Proc Biol Sci, 284.

Zilberberg, M. D., B. H. Nathanson, R. Harrington, J. R. Spalding, and A. F. Shorr. 2018. 'Epidemiology and Outcomes of Hospitalizations With Invasive Aspergillosis in the United States, 2009-2013', Clin Infect Dis, 67: 727-35.

## Figure Legends

**Figure 1 (A.1) Illustration of genetic circuitry enabling enhanced *cyp51A* expression in isolates possessing one of two representative azole resistance mechanisms (A)** The *wild type* configuration of the *cyp51A* promoter is shown. Under normal regulation, proper *cyp51A* expression levels are orchestrated by at least two transcriptional regulators. The activator, SrbA, binds to upstream sterol regulatory elements (SRE) to induce *cyp51A* expression, whereas the multi-subunit CCAAT-Binding Complex (CBC) binds to a conserved CGAAT motif downstream of the SREs to inhibit *cyp51A* expression. **(B)** Tandem repeat mutations of the *cyp51A* promoter wherein the SREs are duplicated, as occurs in isolates bearing the TR34 and TR46 mutations, lead to increased binding of SrbA and activation of gene expression. Although the CBC binding motif is also duplicated in these mutations, physical blockage of additional binding of the CBC complex occurs by the increased presence of the SrbA activator. (C) An illustration of a target gene-independent clinical triazole resistance mechanism involving a single mutation within the gene encoding a member of the heterotrimeric HAP complex, HapE. This mutation reduces binding of the entire CBC to its regulatory binding site in the *cyp51A* promoter. Note that, as in (B), reduced binding of the CBC complex results in loss of repression and consequently increased gene expression. Both representative mechanisms ultimately enable increased transcription of *cyp51A* mRNA, thus increasing the level of Cyp51A protein produced within fungi and enabling the biosynthesis of ergosterol.

**Figure 2. (A.2) Protein model illustrating impact of *cyp51A* mutations on binding of the frontline anti-*Aspergillus* triazole, voriconazole.** PyMOL 2 was used to create three dimensional models of the *A. fumigatus* Cyp51A protein using the previously resolved crystal structure of the *A. fumigatus* Cyp51B as a scaffold. **(A)** Three-dimensional ribbon model of Cyp51A. Inset depicts the Y121 residue (iii) which stabilizes the interaction between voriconazole (iv) and heme (v). **(B)** Three-dimensional model of the surface of the Cyp51A protein. Inset depicts the ligand access channel and entrance to the Cyp51A catalytic site which is occupied by both heme and voriconazole (i). Also depicted is the G54W amino acid substitution (ii) predicted to interfere with the binding of the long lipophilic sidechains of itraconazole and posaconazole.

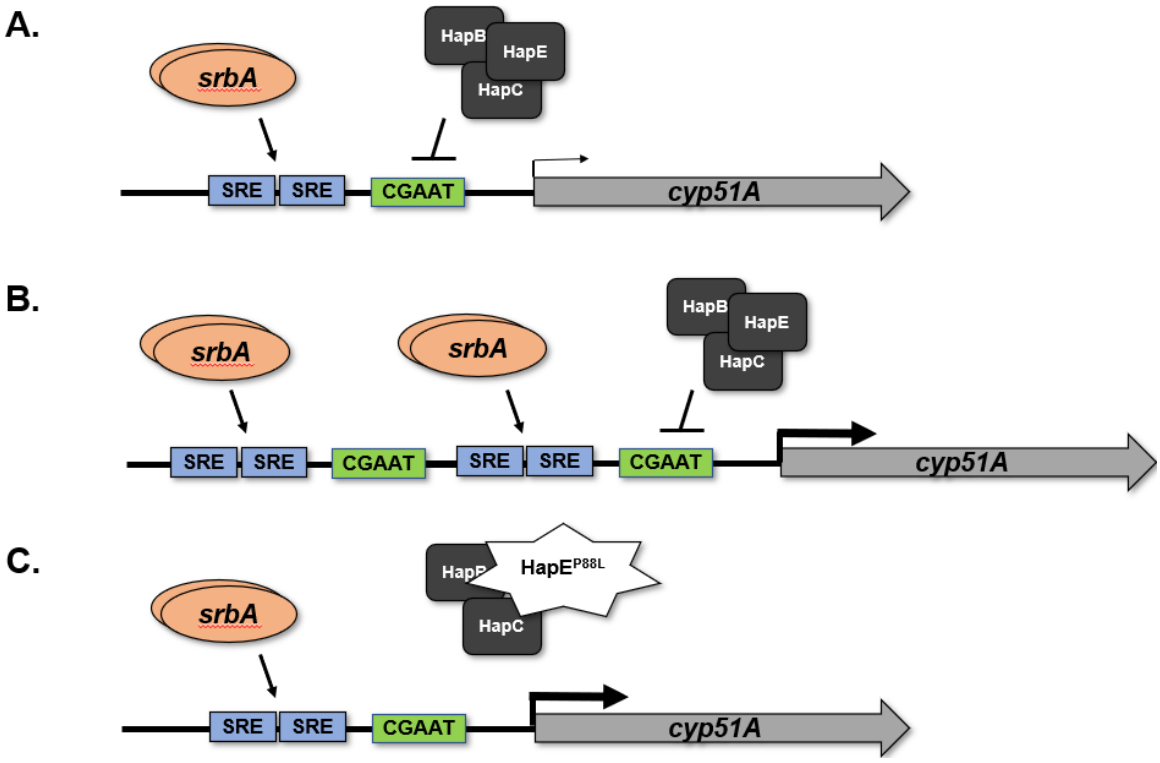


**Figure A.1: Illustration of Genetic Circuitry Enabling Enhanced *cyp51A* Expression in Isolates Possessing One of Two Representative Azole Resistance Mechanisms**

## Figures

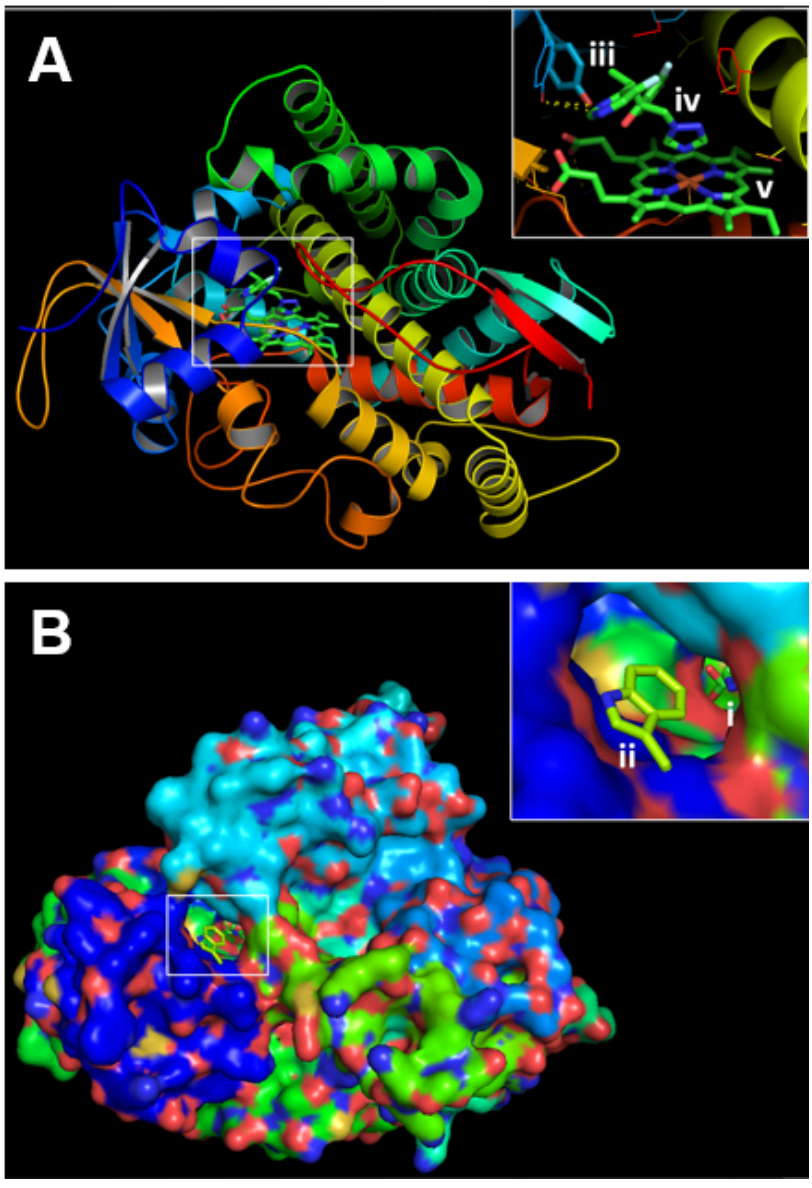


**Figure A.2: Protein Model Illustrating Impact of *cyp51A* Mutations on Binding of the Frontline Anti-*Aspergillus* Triazole, Voriconazole**

**Table A.1:** List of Mechanisms Associated with Clinical Triazole Resistance in Isolates of *A. fumigatus*

| Gene Name | Systematic Name<br>Description | Alteration | Reference |
|---|---|---|---|
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | TR34/L98H | Mellado, E., et al., 2007<br>Snelders, E., et al., 2008<br>Gsaller, F., et al., 2016 |
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | TR46/Y121F/T289A | Chowdhary, A., et al., 2014<br>Snelders, E., et al., 2015 |
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | G54 | Diaz-Guerra, T.M., et al., 2003 |
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | G138 | Manavathu, E.K., et al., 2000<br>Xiao, L., et al., 2004 |
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | M220 | Mellado, E., et al., 2004<br>Howard, S.J., et al., 2013 |
| *cyp51A* | Afu4g06890<br>14-alpha sterol demethylase | G448S | Krishnan Natesan, S., et al., 2012<br>Bellete, B., et al., 2010 |
| *hapE* | Afu2g14720<br>Sequence-specific CCAAT DNA binding transcription factor | P88L | Camps, S.M., et al., 2012 |
| *hmg1* | Afu2g03700<br>Hydroxymethylglutaryl-CoA (HMG-CoA) reductase | S269F | Hagiwara, D., et al., 2018 |
| *hmg1* | Afu2g03700<br>Hydroxymethylglutaryl-CoA (HMG-CoA) reductase | F262 deletion | Rybak, J.M., et al., 2019 |
| *hmg1* | Afu2g03700<br>Hydroxymethylglutaryl-CoA (HMG-CoA) reductase | S305P | Rybak, J.M., et al., 2019 |
| *hmg1* | Afu2g03700<br>Hydroxymethylglutaryl-CoA (HMG-CoA) reductase | I412S | Rybak, J.M., et al., 2019 |
| *cdr1B/ abcC* | Afu1g14330<br>Putative ABC transporter | Overexpression | Fraczek, M.G., et al., 2013<br>Hagiwara, D., et al., 2016 |
| *atrF* | Afu6g04360<br>Putative ABC transporter | Overexpression | Slaven, J.W., et al., 2002 |
| *mdr1* | Afu5g06070<br>ABC multidrug transporter | Overexpression | Fraczek, M.G., et al., 2013<br>Nascimento, A.M., et al., 2003<br>Rajendran, R., et al., 2011 |
| *mdr2* | Afu4g10000<br>ABC multidrug transporter | Overexpression | Fraczek, M.G., et al., 2013<br>Nascimento, A.M., et al., 2003<br>Rajendran, R., et al., 2011 |
| *Mdr3* | Afu3g03500<br>Putative multidrug resistance protein | Overexpression | Nascimento, A.M., et al., 2003 |
| *Mdr4* | Afu1g12690<br>ABC multidrug transporter | Overexpression | Nascimento, A.M., et al., 2003 |

# Appendix B

# Chapter 3 Article

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Ctrl/Alt+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command+Right Arrow, respectively. See the Preface for further details.

## B.1 Introduction

Final submission reproduced with open access permission. A. C. O. Souza* A. Martin-Vicente* (*Co-first authors) A. V. Nywening et al. (2021). "Loss of Septation Initiation Network (SIN) kinases blocks tissue invasion and unlocks echinocandin cidal activity against Aspergillus fumigatus". In: *PLoS Pathog* 17.8, e1009806. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1009806. URL: https://www.ncbi.nlm.nih.gov/pubmed/34370772. *Equally contributing authors: A. C. O. Souza and A. Martin-Vicente. **Published Article:**

https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1009806

This appendix provides the pre-print final author manuscript submission of the published article. This manuscript is included as an appendix as it introduces prior data and knowledge which informed the hypothesis and aims described within the body of this ETD. This publication provides a clear and detailed overview of the construction of a collection of gene disruption mutants in a *wild type* background strain of *A. fumigatus*. The article contains a clear and detailed summary of the process and protocol required for accurate gene editing in this species using our modified CRISPR/Cas9 technique. The publication also provides detailed descriptions of many of the experimental methods and procedures utilized to test the hypothesis and address specific questions for this project. Therefore, the content of this article are directly relevant to the premise, rationale, experimental design, interpretation of the data and results, and conclusions presented within the body chapters of this ETD. The main figures from the article and their legends have been included in

this document, reproduced in the Appendix as they appear in the original Open Access published article. **Note:** Supplemental files for this article are available to view in ProQuest, or at the original source publication if desired, but have not been included in the reproduced document included in this appendix. Though references occur within the article to these supplemental datasets and images, these were not included as content of the published article, but were provided at as separate files with the publication online available to be downloaded if desired.

## B.2 Article

### Loss of Septation Initiation Network (SIN) kinases blocks tissue invasion and unlocks echinocandin cidal activity against *Aspergillus fumigatus*

Ana Camila Oliveira Souza [1#], Adela Martin-Vicente [1#], Ashley V. Nywening [2], Wenbo Ge [1], David J. Lowes [1], Brian M. Peters [1,3], Jarrod R. Fortwendel [1,3]

[1]Department of Clinical Pharmacy and Translational Science, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, [2]Integrated Program in Biomedical Sciences, College of Graduate Health Sciences, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, [3]Department of Microbiology, Immunology, and Biochemistry, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America #These authors contributed equally to this work. jfortwen@uthsc.edu

## Abstract

Although considered effective treatment for many yeast fungi, the therapeutic efficacy of the echinocandin class of antifungals for invasive aspergillosis (IA) is limited. Recent studies suggest intense kinase- and phosphatase-mediated echinocandin adaptation in *A. fumigatus*. To identify *A. fumigatus* protein kinases required for survival under echinocandin stress, we employed CRISPR/Cas9-mediated gene targeting to generate a protein kinase disruption mutant library in a *wild type* genetic background. Cell wall and echinocandin stress screening of the 118 disruption mutants comprising the library identified only five protein kinase disruption mutants displaying greater than 4-fold decreased echinocandin minimum effective concentrations (MEC) compared to the parental strain. Two of these mutated genes, the previously uncharacterized *A. fumigatus sepL* and *sidB* genes, were predicted to encode protein kinases functioning as core components of the Septation Initiation Network (SIN), a tripartite kinase cascade that is necessary for septation in fungi. As the *A. fumigatus* SIN is

completely uncharacterized, we sought to explore these network components as effectors of echinocandin stress survival. Our data show that mutation of any single SIN kinase gene caused complete loss of hyphal septation and increased susceptibility to cell wall stress, as well as widespread hyphal damage and loss of viability in response to echinocandin stress. Strikingly, mutation of each SIN kinase gene also resulted in a profound loss of virulence characterized by lack of tissue invasive growth. Through the deletion of multiple novel regulators of hyphal septation, we show that the non-invasive growth phenotype is not SIN-kinase dependent, but likely due to hyphal septation deficiency. Finally, we also find major antifungal drug classes are currently in use. Although effective as treatment for other fungal diseases, the echinocandin class of antifungals have limited usefulness against IA. Our overall goal is to identify novel fungal proteins that, if targeted for inhibition as part of a co-therapeutic approach, could improve the anti-*Aspergillus* activity of echinocandins and therefore lead to better patient outcomes. Here, we sought to identify *A. fumigatus* genes required for fungal survival under echinocandin stress by generating and screening an *A. fumigatus* mutant library composed of disruptions in genes encoding putative protein kinases. We found that protein kinases required for hyphal septation are essential for survival in the presence of echinocandins and, surprisingly, for the ability of *A. fumigatus* to invade lung tissue. Our results suggest that novel septation inhibitors could enhance echinocandin activity while simultaneously limiting *A. fumigatus* virulence.

## Introduction

*Aspergillus fumigatus* is among the most common causes of human invasive fungal infections in immunocompromised individuals, including solid organ transplant recipients, those undergoing hematopoietic stem cell transplant, and patients receiving highly immunosuppressive chemotherapies [1–3]. If untreated, these infections are almost always fatal and, even with proper diagnosis and treatment, are associated with an overall 50% mortality rate [4]. Furthermore, the estimated annual cost of *Aspergillus* infections in the U.S. approaches $1 billion [5]. The most life-threatening *Aspergillus* infection occurs typically in the setting of profound and prolonged immune suppression and is known as invasive aspergillosis (IA). IA is initiated by the inhalation of *A. fumigatus* conidia from the environment [6]. In the immune compromised host, these conidia undergo a process of germination characterized by an initial phase of isotropic swelling followed by a switch to highly polarized growth leading to the formation of a germ tube. These germ tubes continue to extend through focused growth at the cell apex to generate the invasive hyphal forms that can invade surrounding tissue in search of nutrients, eventually reaching the pulmonary microvasculature system to disseminate [6]. Although decades of research have focused on *A. fumigatus* conidial adherence to and nutrient utilization in the host lung environment, as well as on the cellular and molecular processes essential for subsequent hyphal formation and invasion, our understanding of these processes remain incomplete.

Therapy of invasive aspergillosis is limited to three currently available classes of antifungal compounds. The polyene class, of which Amphotericin B is the only member used for invasive disease, can be associated with acute infusion-related toxicities as well as nephrotoxicity with prolonged administration [7]. The triazole class are the frontline treatment for *Aspergillus* infections, with voriconazole considered the treatment of choice for this indication [8]. Treatment of aspergilloses is often prolonged, and mold-active antifungal prophylaxis employing triazole drugs is now common [9–13], both of which increase the potential for the development of drug-resistant organisms. Since the 1990s, triazole resistance in clinical isolates of this fungal pathogen has been increasing worldwide and is now the subject of significant research in the US and abroad [14–20]. Therefore, clinical use of the polyene and triazole classes is limited by patient toxicity and threatened by resistance, respectively.

The third major class of antifungals with anti-*Aspergillus* activity are the echinocandins, including caspofungin, micafungin and anidulafungin. These compounds are generally well-tolerated and are often used in salvage therapy for invasive infections [21]. Echinocandins are specific inhibitors of cell wall biosynthesis in fungi, as they inhibit the activity of the $\beta$-1,3-glucan synthase enzyme. This enzyme is encoded by the *fksA* gene in *A. fumigatus* and is the sole protein driving synthesis of the major cell wall component, $\beta$-1,3-glucan [22]. Whereas the activity of the echinocandins is fungicidal for the major yeast pathogens of the Candida genus, they are considered fungistatic against the *Aspergilli* [21,23]. Treatment of *A. fumigatus* with echinocandins causes lysis of hyphal tips and blunting of hyphal growth, but viability is maintained [24]. In addition, a caspofungin paradoxical effect (CPE) of growth inhibition has been described for caspofungin both *in vitro* and *in vivo* and is characterized by decreasing effectiveness of drug with increasing concentrations [21,25]. Current research suggests that the CPE is the result of the induction of tolerance mechanisms within hyphal compartments that survive caspofungin therapy. These mechanisms include remodeling of the cell wall, upregulation of cell wall integrity machinery and the induction of calcium-regulated stress pathways [21,25]. Although not conclusive, multiple studies using models of invasive aspergillosis have suggested that the CPE is not merely an *in vitro* phenomenon and may be an issue underlying treatment failure during caspofungin therapy of invasive aspergillosis in specific cases [25]. Likely underpinned by the fungistatic nature of the echinocandins against Aspergilli, breakthrough infections during echinocandin prophylaxis have been reported to be as high as 28% [26] and one study has identified echinocandin prophylaxis as an independent risk factor for breakthrough infections when compared with triazole prophylaxis [27]. Therefore, the echinocandins are mostly utilized where triazole therapy is contraindicated or has failed for invasive aspergillosis.

Recent studies have shown that the phospho-proteome of Aspergilli is highly responsive to echinocandin-induced stress, implying extensive kinase- and phosphatase-mediated

re-wiring of cellular physiology for survival during inhibition of $\beta$-1,3-glucan biosynthesis [28–30]. Further, multiple studies in *A. fumigatus* have implicated protein kinase and protein phosphatase activity as important to cell wall stress imposed by echinocandins [31–36]. Together, these reports suggest that the further study of phospho-regulatory events required for survival during echinocandin-induced stress could uncover novel avenues for combination therapies directed at enhancing echinocandin activity against Aspergilli and other human pathogenic fungi. Here, we utilized a CRISPR/Cas9-based rapid gene disruption technique to generate a protein kinase gene disruption library in a *wild type* genetic background of *A. fumigatus*. Screening of this library for cell wall stress and echinocandin sensitivity phenotypes uncovered multiple protein kinases contributing to growth under each condition. In addition to the previously characterized cell wall integrity pathway and cAMP-mediated signaling protein kinases, our screens identified orthologs of the Septation Initiation Network (SIN) kinases as essential for growth under echinocandin-induced stress [37,38].

As septa are considered essential for the limitation of cell wall damage to filamentous fungal hyphae and the putative SIN is unstudied in *A. fumigatus*, we sought to further characterize the importance of each *A. fumigatus* SIN kinase to survival under echinocandin-induced stress both *in vitro* and during invasive disease. Our data indicate that each SIN kinase is essential for septum formation and for survival under echinocandin-induced cell wall stress. Strikingly, each of the SIN kinase disruption mutants were avirulent in a corticosteroid model of invasive aspergillosis (IA) and two of the three mutants were also avirulent in a chemotherapeutic model of IA. This lack of virulence was characterized by loss of tissue invasion and inability to accumulate fungal burden. Nevertheless, using culture-based residual fungal tissue burden as a gold-standard determination of fungicidal activity, we show that echinocandin therapy was enhanced in mice infected with SIN kinase mutants. Further, we show that loss of additional regulators of septation also results in avirulence characterized by lack of tissue invasion and loss of viability under echinocandin stress, suggesting that our phenotypes are likely due to loss of septation and not septation-independent functions of the *A. fumigatus* SIN.

## Results

### Generation of an *A. fumigatus* protein kinase disruption mutant library

To identify protein kinase-driven pathways important for survival under echinocandin stress in *A. fumigatus*, we first generated a protein kinase disruption library in the A1163 (CEA10) *wild type* genetic background through coupling of CRISPR/Cas9-based gene targeting with a miniaturized protoplast transformation technique. Putative protein kinase genes were first identified through BLAST searches of the *A. fumigatus* A1163 (CEA10) genome database at FungiDB (fungidb.org) using the previously published known protein kinases of *Aspergillus nidulans* [39]. This search yielded 148 putative protein kinases representing 10

different protein kinase classes, as well as putative kinases falling into no known kinase class (**Supplemental File 1**). Of these 148 putative kinase genes, 142 were found to be encoded in the genomes of both sequenced laboratory strains, A1163 (CEA10) and Af293, and were therefore selected for disruption (**Supplemental File 1**). For library construction, we employed a miniaturized version of a CRISPR/Cas9-based gene editing technique, previously adapted in our lab, that provides up to 90% gene targeting efficiency in A. fumigatus [40,41]. Protospacer adjacent motif (PAM) sites for Cas9-induced double strand breaks and integration of hygromycin repair (HygR) templates were selected using the Eukaryotic Pathogen CRISPR Guide RNA/DNA Design Tool (EuPaGDT, grna.ctegd.uga.edu) through batch upload analysis of all protein kinase coding sequences. Each PAM site was selected to direct double-strand DNA breaks near the putative transcriptional start site of each gene and integration of repair templates designed to disrupt readthrough of the first exon, when possible (**Supplemental File 1**). Cas9-ribonucleotides (RNPs) for gene targeting were assembled in vitro using custom designed guide RNAs (gRNAs) and commercially available Cas9 enzyme, as previously described [40]. Transformations were miniaturized into single wells of 96-well plates with a final well volume of 200 µl (**Figure B.1A**). After transformation, total contents of individual wells were plated to osmotically stabilized agar and overlayed with hygromycin-containing top agar for selection (**Figure B.1B**). Individual transformants were isolated to secondary selection plates and subsequently screened by multiple PCR reactions to confirm correct integration of the hygromycin repair template (**Figure B.1C**). After three rounds of transformations, successful disruption of 118 protein kinase genes were confirmed (**Supplemental File 1**). The remaining 24 protein kinases for which disruptions were not achieved are largely orthologs of putatively essential genes in *A. nidulans*, suggesting conserved essentiality in *A. fumigatus* [39].

## Protein kinase-mediated regulation of *A. fumigatus* growth and asexual development

Of the 118 disrupted protein kinase mutant strains generated in this study, twenty-six were unable to grow at the same rate as the parental strain when cultured on standard laboratory minimal media (**Figure B.1**).

> **Figure 1. (B.1) Construction of a protein kinase disruption library in *A. fumigatus* by CRISPR/Cas9-mediated gene editing. A)** Miniaturized protoplast transformations were carried out in 96-well plates, with a final total volume of 200 µl per well, and each well representing an attempted disruption of a single protein kinase gene. **B)** After the transformation process, the entire contents of each well were spread onto individual sorbitol minimal medium (SMM) agar plates and allowed to recover overnight at room temperature before overlaying with hygromycin-containing top agar for selection. Following these transformation procedures, typically 10 to 30 transformant colonies were evident on each selection plate after 3 to 4 days of incubation at 37°C. However, due to

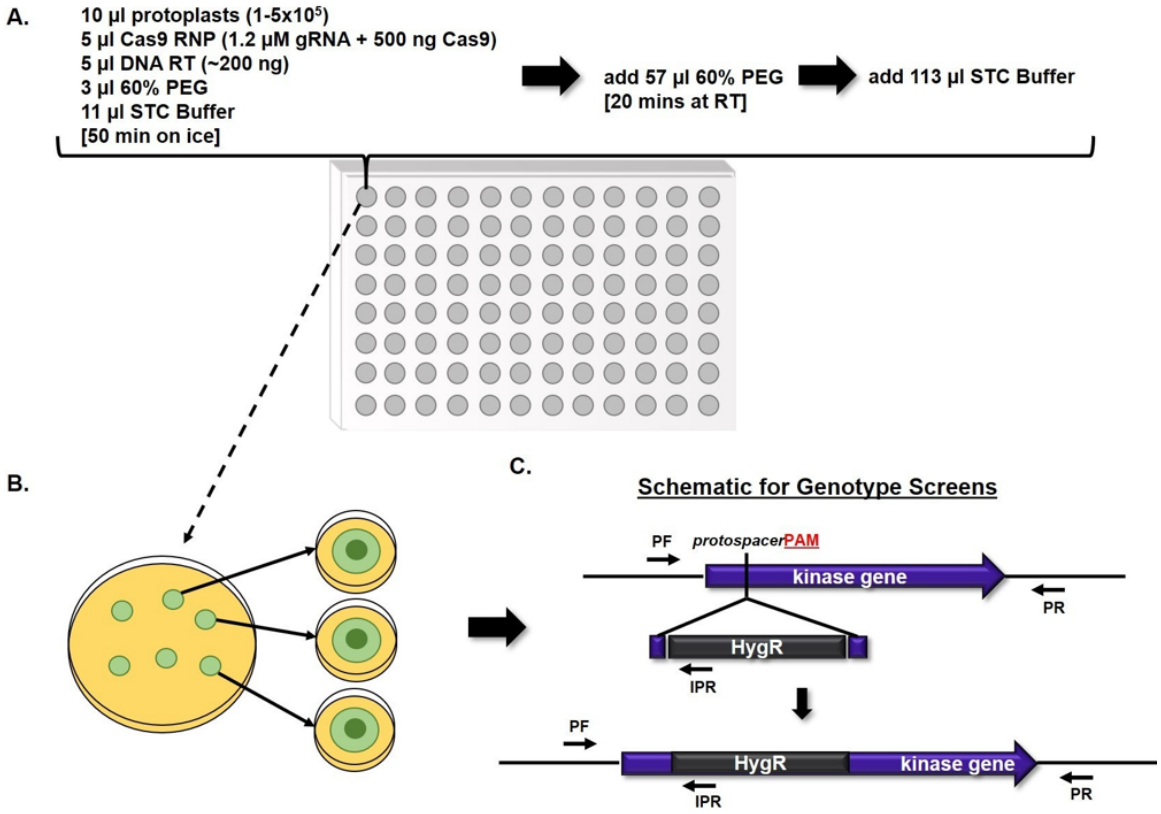


**Figure B.1: Figure 1. Construction of a protein kinase disruption library in *A. fumigatus* by CRISPR/Cas9-mediated gene editing.**

the high efficiency of gene targeting with the CRISPR/Cas9 system, only 3 to 4 colonies per transformation were required to be isolated for genotypic screening. **C)** Putative transformants were subjected to genotypic analyses by PCR to confirm proper integration of the repair template for gene disruption. These PCR analyses included screens with allele specific primer sets PF/PR and PF/IPR, pictured above. PF = Forward screening primer. PR = Reverse screening primer. IPR = Internal reverse screening primer complementary to HygR sequence. HygR = Hygromycin Resistance cassette, utilized as the repair template for gene disruption. All kinase genes were targeted for disruption at the 5′ end of the gene (within the first exon, where possible), as indicated by the placement of the protospacer and protospacer adjacent motif (PAM, red bold font) above.

Disruption of seven different protein kinases resulted in a reduction of growth of greater than 50% when compared to the parent strain, generating compact colonies that were

unable to expand radially on minimal media (**Figure B.2A** and **Supplementary Figure 1A**). Among these kinase disruptions were the cell wall integrity mitogen-activated protein kinase (MAPK), *mpkA* (AFUB_070630), and the upstream MAPK kinase (MAPKK), *mkkA* (AFUB_006190). Loss of either of these kinases has been previously shown to result in compact colony morphology [35]. Although deletion of the cell wall integrity MAPKK kinase (MAPKKK), *bck1* (AFUB_038060), was previously shown to result in reduced growth as well, disruption of this kinase in our library was associated with only a mild reduction in colony growth (**Figure B.2B** and **Supplementary Figure 1A**). In addition, significantly reduced growth was generated by disruption of the catalytic subunit of Protein Kinase A, *pkaC1* (AFUB_027890), or the PAK-kinase, *cla4* (AFUB_053440) (**Figure B.2A** and **Supplementary Figure 1A**). Both kinases were also previously characterized as important for vegetative growth in *A. fumigatus* [42,43]. Previously uncharacterized *A. fumigatus* protein kinases causing >50% growth reduction upon disruption included orthologs of the eukaryotic LAMMER kinase (*lkh1*; AFUB_016170), an *S. cerevisiae* kinase regulating the actin cytoskeleton (*prk1*; AFUB_006320), and a cyclin-dependent protein kinase (*sgv1*; AFUB_053070) (**Figure B.2A** and **Supplementary Figure 1A**).

Eighteen additional protein kinase disruptions resulted in mild-to-moderate growth reductions ranging between 10–50% of the parental strain (**Figure B.2B** and **Supplementary Figure 1A**). Of the previously characterized kinase genes fitting into this category, we identified slow growth in disrupted orthologs of a phosphorelay sensor kinase (*tscB*; AFUB_017740) [44], the CrossPathway Control kinase (*cpcC*; AFUB_054310) [45], a p21-Activated Kinase (PAK) family protein (*ste20/pakA*; AFUB_021710) [32], a cyclin-dependent protein kinase (*ssn3*; AFUB_035220) [46], and the cell wall integrity MAP kinase kinase kinase (*bck1*; AFUB_038060) [35] (**Figure B.2B** and **Supplementary Figure 1A**). Thirteen mutant strains also displayed a significant impairment in asexual differentiation, as evidenced by significantly reduced conidia production, with nine of these kinase disruptions resulting in severe loss of conidiation when compared to CEA10 (**Figure B.2C** and **Supplementary Figure 1B**). Six of these kinases have been previously characterized as required for conidiation, including three that comprise an asexual developmental kinase cascade in *A. fumigatus* (*steC*: AFUB_053960), *ste7/mkkB*; AFUB_043130, and *mpkB*; AFUB_078810) [47]. The remaining previously characterized kinases were *pkaC1*, *mpkA*, and *cla4*, each of which negatively impact asexual development upon deletion [35,42,43]. The finding that disruption of previously characterized growth-mediating kinases resulted in growth retardation of strains in our library supported the validity of our gene disruption approach. Therefore, these initial studies identified multiple novel protein kinases regulating *A. fumigatus* growth and development.

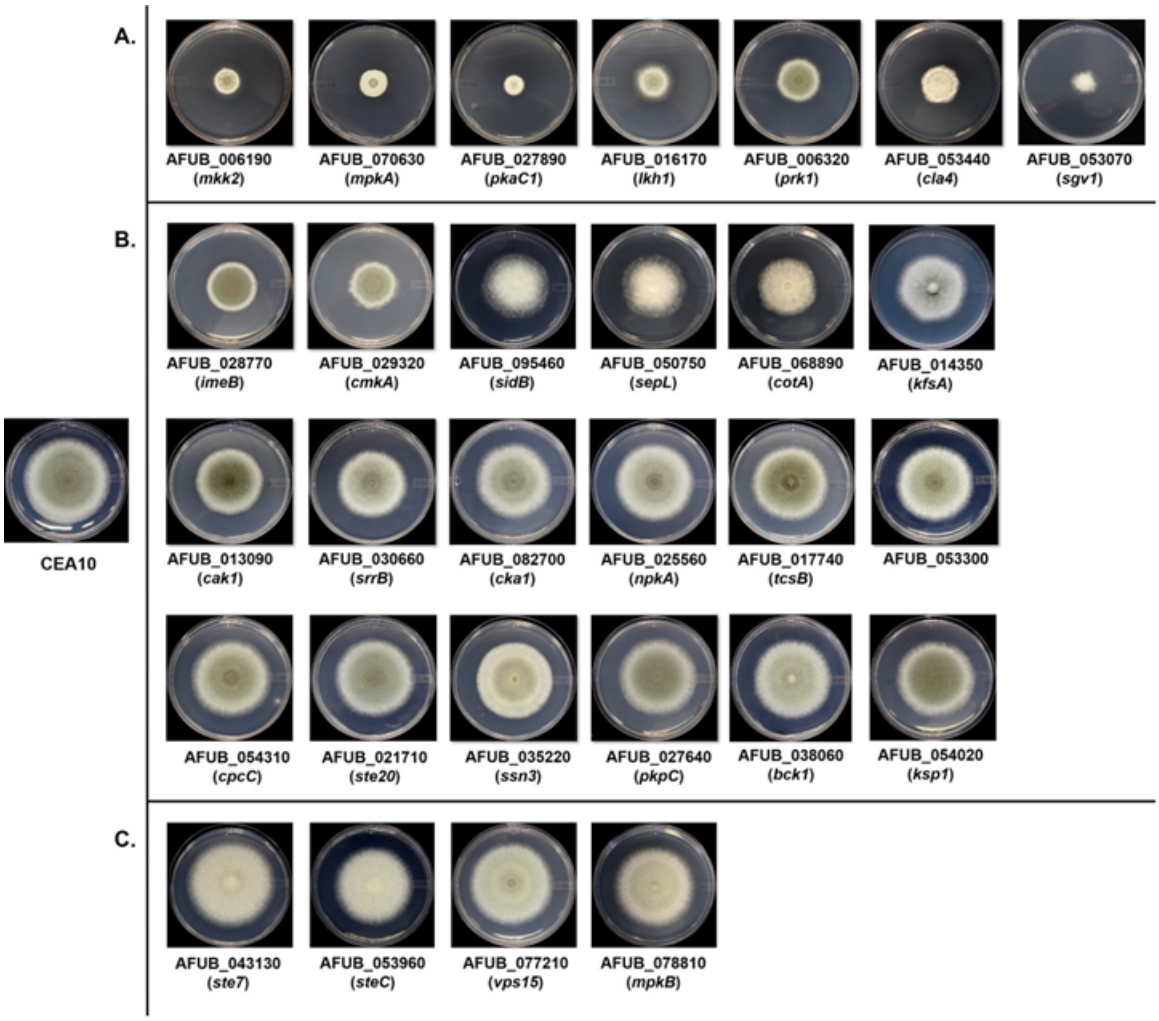


**Figure B.2: Figure 2. Colony morphologies of selected *A. fumigatus* protein kinase disruption mutants.** 96-hr colony morphologies of severely **(A)** and moderately **(B)** growth restricted protein kinase disruption mutants, as well as colony morphologies of mutants that are not growth restricted but display reduced conidiation **(C)**. Ten thousand conidia from each strain were point inoculated onto the center of minimal media agar and cultured for 96 hrs at 37°C.

## Multiple protein kinases are required for growth under cell wall and echinocandin stress

To identify protein kinases required for cell wall stress tolerance, we performed spot-dilution assays in the presence of the common cell wall stress agents calcofluor white

(CFW) and congo red (CR) on minimal media (MM), as well as caspofungin minimum effective concentration (MEC) assays, for all 118 viable kinase disruption mutants. Whereas caspofungin is an echinocandin-class antifungal that directly inhibits the fungal $\beta$-glucan synthase, CFW and CR are known to interfere with cell wall assembly by interacting with nascent chitin chains to prevent crosslinking of chitin to glucan moieties [48]. Spot-dilution assays identified seven protein kinase disruptions that displayed increased susceptibility to both CFW and CR, and an additional seven mutants that were hypersusceptible to only CR (**Figure B.3A** and **B**). Among those 14 mutants found to be hypersusceptible to either cell wall active compound were the disruptions of the cell wall integrity kinases *mkkA* and *mpkA* (**Figure B.2A**) which have previously been shown to be required for survival under various forms of cell wall stress [35]. Interestingly, the MAPKKK at the head of the *A. fumigatus* cell wall integrity pathway, Bck1, was again not identified by our assays as producing a cell wall stress hypersensitivity phenotype upon disruption. This finding, coupled with the lack of a severe growth restriction phenotype in **Figure B.2** for the *bck1* disruption mutant, indicated that some mutations in our library may be either non-disruptive or only partially disruptive to gene function. Our cell wall stress screens also uncovered protein kinases whose disruption generated resistance to either CFW (*pkaC1*) or to CR (*kfsA*, *cmkA*, and *stk22*), as evidenced by the increased ability to sustain colony formation under stress (**Figure B.2C**).

To see if the CFW and CR susceptibility phenotypes correlated with echinocandin susceptibility, modified caspofungin MEC analyses were performed by broth microdilution (BMD) [49]. Whereas only five protein kinase disruption mutants were identified to display 4-fold increased susceptibility to caspofungin (i.e., at least two dilution shift), an additional 44 mutants displayed a 2-fold (one dilution) increase in caspofungin susceptibility (**Supplemental File 1**). Of those mutants that we previously identified as hypersusceptible to CFW, CR, or both, only the AFUB_087120, AFUB_013090 (*cak1*), and AFUB_018600 (*pom1*) disruption mutants showed no shift in caspofungin MEC values. All other cell wall stress susceptible mutants displayed at least a 2-fold reduction in caspofungin MEC values (**Supplemental File 1**). Interestingly, the disruption mutants for AFUB_09320 (*cmkA*), AFUB_014350 (*kfsA*), and AFUB_027890 (*pkaC1*) which displayed increased resistance to either CFW or CR, also displayed increased susceptibility to caspofungin (2-fold reduced MEC). Importantly, among the caspofungin hypersusceptible kinase mutants that displayed 4-fold decreased MEC values were those known to be involved in cell wall integrity signaling (*mpkA* and *mkk2*) and the cAMP-activated protein kinase (pkaC1), of which *mpkA* and *pkaC1* have been previously characterized as necessary for response to echinocandin stress [50,51].

Mutation of two additional kinases that are not members of the cell wall integrity pathway, AFUB_095460 (*sidB*) and AFUB_05070 (*sepL*), also displayed high levels of susceptibility in both of our cell wall stress and caspofungin MEC assays (**Figure B.3A** and **Supplemental File 1**).

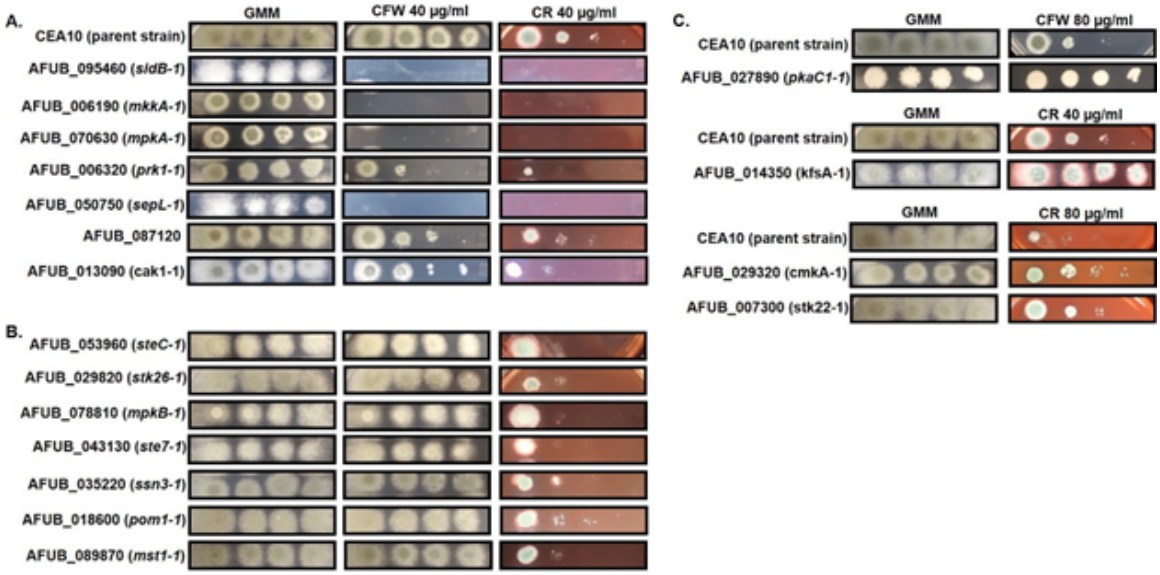


**Figure B.3: Figure 3. Multiple protein kinases contribute to cell wall integrity in *A. fumigatus* A)** Protein kinase gene disruption mutants displaying increased susceptibility to both cell wall disrupting agents, calcofluor white (CFW) and congo red (CR), by spot-dilution assay when compared to the *wild type* parent (CEA10). **B)** Protein kinase gene disruption mutants displaying hyper-susceptibility to only CR when compared to the parent strain. **C)** Protein kinase gene disruptants displaying increased resistance to CFW (*pkaC1*-1) or to varying concentrations of CR (*kfsA*-1, *cmkA*-1, and *stk22*-1). For each target protein kinase gene, the systematic name is listed with the strain name given in parentheses. Strain names were designed using either the previously published or putative (based on homology to *Aspergillus nidulans*) gene names with the addition of "-1" to indicate a disruption mutation of that gene. GMM = glucose minimal media with no CFW or CR added. For all assays, conidial inocula were applied at $10^4$, $10^3$, $10^2$, and $10^1$ total conidia and plates were incubated at 37°C for 72 hrs.

These putative *A. fumigatus* kinases are orthologous to the *A. nidulans* SepL and SidB kinases that function as members of the Septation Initiation Network (SIN) kinase cascade. The core of the *A. nidulans* SIN pathway is composed of three kinases: the SteK-class proteins, SepH and SepL, and the AGC-class kinase, SidB (**Figure B.4A**) [39]. Deletion of any single *A. nidulans* SIN kinase gene results in aseptate hyphae and reduction of conidiation whereas analysis of the *Neurospora crassa* SIN kinase orthologs has found only two of the

three conserved kinases to be essential for the process of hyphal septation [39,52]. As the SIN pathway is completely uncharacterized in *A. fumigatus*, we sought to examine the importance of each SIN kinase to hyphal septation and to protection against echinocandin damage both in vitro and in vivo during invasive aspergillosis.

## *A. fumigatus* SIN kinases are required for septation and for survival in response to echinocandins

Although a disruption of the *sepH* gene was generated as part of our initial library construction, the *sepH*-1 mutant did not show the same colony growth, conidiation, cell wall stress or echinocandin stress phenotypes as the *sepL*-1 or *sidB*-1 mutants. To see if our *sepH*-1 mutant actually represented a loss of gene function, we also generated a complete gene deletion of *sepH* by CRISPR/Cas9 gene targeting (**Supplementary Figure 2A** and **Supplementary Figure 2B**) and found that this mutant (Δ*sepH*) phenocopied the other SIN kinase disruption mutants with respect to colony phenotype and cell wall stress imposed by CFW and CR (**Figure B.4B** and **C**).

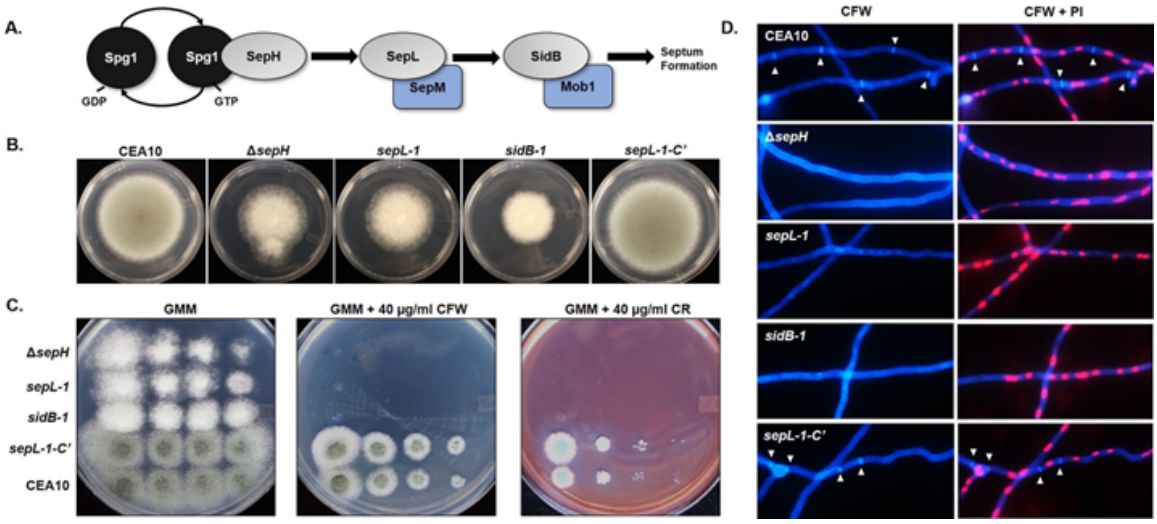


**Figure B.4: Figure 4. The Septation Initiation Network (SIN) kinases are each required for hyphal septation and protection against cell wall damage in *A. fumigatus*.**

**Figure 4. (B.4) The Septation Initiation Network (SIN) kinases are each required for hyphal septation and protection against cell wall damage in *A. fumigatus*** **A)** The putative core SIN pathway in *A. fumigatus* based on signal transduction models constructed for *Schizosaccharomyces pombe* and *Aspergillus nidulans*. A protein kinase cascade, initiated by activation of the SepH kinase through interaction with the GTP-bound GTPase, Spg1, leads to downstream activation of the SepL and SidB kinases to eventually promote initiation of septation. SepL and SidB are shown with their putative regulatory binding partners, SepM and Mob1, respectively. **B)** Deletion of *sepH* (Δ*sepH*) phenocopies *sepL* and *sidB* disruption (*sepL*-1 and *sidB*-1, respectively) as evidenced by restricted colony size and loss of conidiation (i.e., white colony formation). Complementation of SIN activity in the *sepL*-1 disruption mutant by gene replacement (*sepL*-1-C′) results in full growth recovery and conidiation. Ten thousand conidia from each strain were spot-inoculated onto the center of a GMM agar plate and cultured for 96 hrs at 37°C. **C)** Loss of any single SIN kinase results in absence of growth in the presence of the cell wall destabilizing compounds CFW or CR. Conidia from each strain were spot inoculated in descending concentrations onto GMM alone or GMM containing either 40 μg/ml CFW or CR. **D)** Loss of any single SIN kinase results in the absence of septa in mature hyphae. Conidia from each strain were cultured to mature hyphae (16 hrs at 37°C) and subsequently stained with calcofluor white (CFW) and propidium iodide (PI) to visualize septa and nuclei, respectively. White arrowheads indicate septa in the CEA10 (parent) and *sepL*-1 complemented (*sepL*-1-C′) strains. No septa were evident in the Δ*sepH*, *sepL*-1 or *sidB*-1 mutants.

This finding indicated that, as for the *bck1*-1 mutant, disruption of *sepH* using our approach likely only partially impacted function. For that reason, the Δ*sepH* deletion mutant and not the *sepH*-1 disruption mutant was used moving forward. Inspection of mature hyphae from each mutant using CFW fluorescence staining to highlight the cell wall revealed that septation was completely ablated upon loss of any single SIN kinase (**Figure B.4D**). Nuclear morphology and positioning in the each of SIN kinase mutants was grossly normal (**Figure B.4D**), indicating that septation is not required for this aspect of *A. fumigatus* hyphal biology. This finding is in line with data reported for other septate filamentous fungi in which the cell cycle and cytokinesis (i.e., septation) are linked but not essentially coupled, as in yeast organisms [53]. Repair of SIN kinase pathway activity through complementation of the *sepL*-1 disruption by re-integration of the *sepL wild type* allele into the native locus (**Supplementary Figure 2C** and **Supplementary Figure 2D**) resulted in complete recovery of all growth and cell wall stress phenotypes (**Figure B.4B** and **C**). In addition, this *sepL*-1 complement strain (*sepL*-1-C′) displayed a complete recovery of septum formation (**Figure B.4D**).

We next performed both E-test and fluorescence-based quantitative killing assays

comparing the CEA10 parent and each SIN kinase mutant. E-test assays were performed utilizing both caspofungin (CAS) and micafungin (MFG) embedded strips and the production of a zone-of-clearance recorded after 48 hours of culture. In keeping with the fact that echinocandins are fungistatic against *Aspergilli*, the CEA10 parental strain displayed no zone-of-clearance surrounding E-test strips from either echinocandin (**Figure B.5A** and **B**). Only an elliptical zone of depressed growth was evident for CEA10 and agar cores taken from within this zone grew normally when supplanted to agar containing no echinocandin (**Figure B.5A** and **B**, inset). In contrast, each of the SIN kinase mutants developed a visible zone-of-clearance for both echinocandins suggesting significantly inhibited growth (**Figure B.5A** and **B**). The only exception to this was found on the Δ*sepH* E-test plates for both echinocandins where a small number of compact microcolonies were able to form in the zone-of-clearance (**Figure B.5A** and **B**, Δ*sepH* panels). Agar cores removed from the zone-of-clearance from each SIN kinase mutant plate were inviable when supplanted to drug-free agar. When specifically selected for sub-culture to drug free agar, the Δ*sepH* zone-of-clearance microcolonies generated viable colonies but maintained hypersensitivity to both echinocandins on retest. These findings implied that the zone-of clearance generated on the SIN kinase mutant E-test plates represented fungicidal activity of both echinocandins. In contrast, E-test and broth microdilution assays revealed no differences in voriconazole antifungal drug susceptibility among the SIN kinase mutants when compared to the parental strain (MIC = 0.5 μg/ml for all strains).

To more quantitatively measure SIN kinase mutant death in response to echinocandin stress, we also employed fluorescence-based assays using the live-cell stain 5-carboxyfluorescein diacetate (CFDA). CFDA is a cell-permeable esterase substrate that has been previously used as a viability indicator for *A. fumigatus* and other fungi [23]. Conidia from the parent strain and each SIN kinase mutant were grown in the presence or absence of 0.5 μg/ml micafungin and subsequently stained with CFDA to detect germlings and / or microcolonies with live hyphae or hyphal segments. Using fluorescence microscopy, individual microcolonies from each strain were scored as either live (CFDA-positive) or dead (CFDA-negative). In the absence of micafungin, the CEA10 parent and SIN kinase mutants displayed similar levels of CFDA-positive staining after 12 hours of culture, indicating similar baseline viability among the strain set (**Figure B.5C**). Quantitation of CFDA-positivity past the 12-hour timepoint was not possible in the untreated samples, as continued hyphal growth obscured individual microcolonies for all strains. After 12 hours of growth in the presence of micafungin, each of the SIN kinase mutants displayed significant reductions in CFDA positivity, showing between only 40% and 60% positivity (**Figure B.5D**). The CEA10 parent maintained an almost 90% positivity in staining. At the 12-hour timepoint, germlings in the CEA10 parent were noted to be either fully CFDA-positive (**Figure B.5E**, white arrowhead) or to display unstained hyphal tip regions with positively stained subapical segments (**Figure B.5E**, black dotted line arrows). In contrast, each of the SIN kinase mutants displayed either wholly CFDA-stained or -unstained (**Figure B.5E**, black arrowhead) germlings. These findings suggested protection of interseptal hyphal segments of

the CEA10 parent that was lost in the SIN kinase mutants due to lack of septation. Further, after 24 hours of culture in the presence of micafungin, the SIN kinase mutants displayed no CFDA positivity, whereas the CEA10 parent maintained almost 100% positive staining (**Figure B.5D**).

Both the E-test and CFDA-based killing assays described above measured the impacts of echinocandin stress when applied to each strain from the onset of growth (i.e., before conidial germination begins). As septa were completely lacking in mature hyphae of each of the SIN kinase mutants, we also reasoned that damage from mature hyphal tip lysis induced by echinocandins would no longer be confined to the tip compartment, as previously described [24,54].

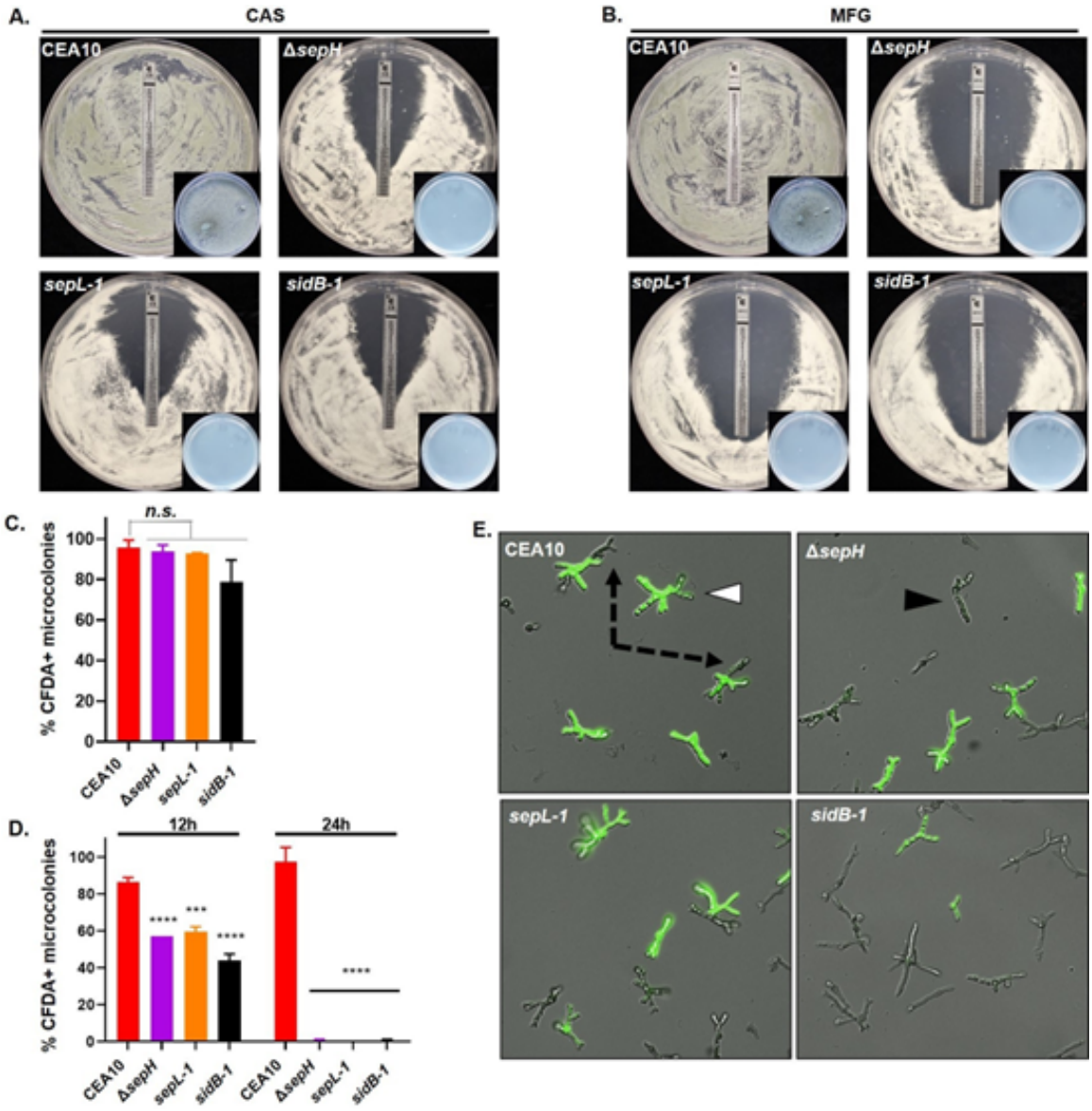


**Figure B.5: Figure 5. The *A. fumigatus* SIN kinases are required for survival under echinocandin stress.**

**Figure 5. (B.5) The *A. fumigatus* SIN kinases are required for survival under echinocandin stress. A, B)** Loss of *sepH*, *sepL*, or *sidB* increases susceptibility to echinocandins in a modified E-test assay. 1 x $10^6$ total conidia in 500 µl sterile water from the *wild type* parent (CEA10), the *sepH* deletion (Δ*sepH*), or the *sepL* (*sepL*-1) or *sidB* (*sidB*-1) disruption strains were spread evenly over GMM agar plates. E-test strips for caspofungin (A) or micafungin (B) were applied and assays incubated for 48 hrs. Note the zone-of clearance with no detectable growth for the Δ*sepH*, *sepL*-1 and *sidB*-1 mutants in the presence of either echinocandin. Insets show representative, drug-free minimal media culture plates onto which a single agar plug from the zone-of-clearance for each assay was sub-cultured. Multiple agar plugs (n = 10), taken from within 1 cm of the E-test strip and between the 32 and 0.25 ug/ml markers, were sub-cultured in the same manner for each assay. Note lack of growth for the SIN kinase mutant subcultures. CAS = caspofungin, MFG = micafungin. **C)** Quantitation of viability by CFDA staining of the CEA10 control and SIN kinase mutants in the absence of echinocandin stress. Conidia from each strain were germinated for 12 hrs and subsequently stained with 5-carboxyfluorescein diacetate (CFDA) to detect live hyphal elements. **D)** Quantitation of viability by CFDA staining of the strain set in the presence of caspofungin. Conidia from each strain were cultured for 12 hrs and 24 hrs at 37°C in the presence of 0.5 µg/ml caspofungin and subsequently stained with CFDA to detect live microcolonies. CFDA positivity was scored for 100 microcolonies in each experiment and all assays were completed in triplicate. Data were averaged for each strain and treatment. One-way ANOVA and Dunnett's multiple comparisons post hoc analyses indicated differences in CFDA staining in the absence of micafungin were not significant (n.s.), whereas the Δ*sepH*, *sepL*-1 and *sidB*-1 mutants were significantly less viable after 12 and 24 hrs growth in the presence of caspofungin. $p<0.0001$; $p = 0.0001$. **E)** Echinocandin stress during early growth stages leads to death of the SIN kinase mutants. White arrowhead denotes example of a microcolony stained positive with CFDA (bright green). Black arrowhead denotes a CFDA-negative microcolony. Dash-lined arrows denote dead (CFDA-negative) hyphal compartments of CFDA-positive microcolonies only seen in the CEA10 control.

To test this, conidia from the parental and each mutant strain were cultured to mature hyphal growth before addition of micafungin (0.5 µg/ml) and hyphal damage was subsequently analyzed by propidium iodide (PI) permeability [55]. In the absence of echinocandin-induced stress, hyphae from each strain showed little-to-no permeability to PI, supporting the CFDA staining results and suggesting that loss of SIN pathway function alone does not significantly impact cell wall integrity (**Figure B.6A**). In contrast, hyphal damage induced by 2 hours of micafungin treatment of the SIN kinase mutants was extensive and widespread when compared to the *wild type* parental control (**Figure B.6B**). This was

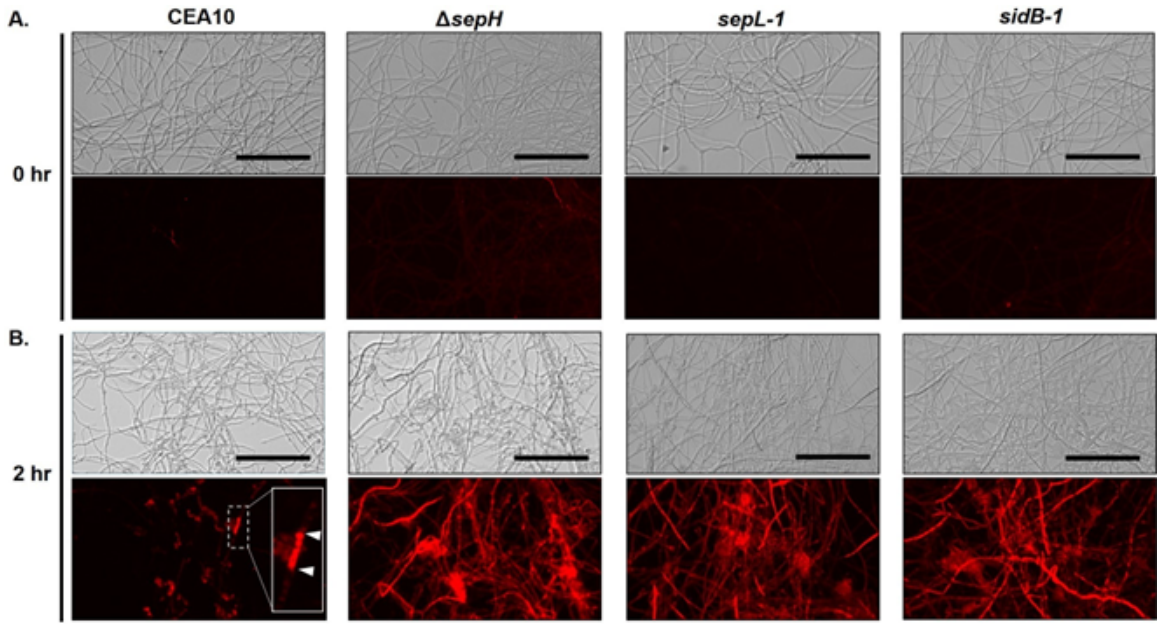


**Figure B.6: Figure 6. Hyphae of SIN kinase mutants exhibit extensive damage in the presence of echinocandin.** Analysis of hyphal integrity using propidium iodide (PI) permeability as a measure of damage in response to echinocandin stress. Mature hyphae from the CEA10, Δ*sepH*, *sepL*-1, and *sidB*-1 strains were stained with PI (12.5 µg/ml) before **(A)** or after 2 hours **(B)** exposure to micafungin (0.5 µg/ml). Upper panels are brightfield images and lower panels are fluorescence acquired. Hyphae from all strains exhibited minimal staining with no exposure to echinocandin, suggesting intact cell walls **(A)**. Limited staining of hyphal compartments was noted in the CEA10 parental strain after 2 hrs micafungin exposure, suggesting cell wall damage limited by the presence of septa (B, lower panel inset, white arrowheads denote hyphal compartment). In contrast, extensive PI staining was induced after micafungin treatment in each of the SIN kinase mutant strains **(B)**. All fluorescence images were acquired at using identical exposure. Scale bar = 100 µm.

appeared to result in cidal anti-*Aspergillus* activity against each of the SIN kinase mutants.

***A. fumigatus* SIN kinases are required for tissue invasive growth** To examine the impacts of hyphal septation loss on virulence, we next compared the CEA10 parent and SIN kinase mutant strains in two well-described mouse models of invasive aspergillosis, representing chemotherapeutic and corticosteroid-induced immune suppression [56]. Mice (n = 8 / arm) were immune suppressed with injections of cyclophosphamide and triamcinolone acetonide or with triamcinolone acetonide alone for the chemotherapeutic and corticosteroid model, respectively. For both models, mice were intranasally inoculated with 1 x $10^5$ conidia and survival was followed for 15 days post-inoculation. Sham treated mice (n = 5 / arm), receiving only intranasal sterile saline inoculations coupled with immune suppressive regimens, resulted in no mortality. For the CEA10 parent strain, mortality began at Day +4 in both models with 100% mortality reached by Day +7 in the chemotherapeutic model and mortality reaching 60% by Day +15 in the corticosteroid model (**Figure B.7A** and **B**). As a measure of virulence in a strain where SIN kinase pathway activity was restored after disruption, the *sepL*-1-C' complement strain induced mortality statistically similar to that of the CEA10 parent in both models (**Figure B.7A** and **B**). Surprisingly, the SIN kinase mutants were avirulent in both models, with the only exception being the *sepL*-1 mutant which induced 50% mortality only in the chemotherapeutic model (**Figure B.7A** and **B**). All SIN kinase mutant-induced mortality levels were significantly reduced, compared to the parent strain. To examine the histopathological impact of hyphal septation loss on infection, we also analyzed hyphal growth in vivo through silver-stained tissue sections of infected lungs from each group. At 4 days post-infection, the *wild type* CEA10 strain had generated large, deeply invasive hyphae (**Figure B.8**). In contrast, each of the SIN kinase mutants had formed small hyphal masses residing only in the open airways at the same timepoint postinfection (**Figure B.8**). No deeply invasive growth was noted for any SIN kinase mutant. A single instance of shallow invasion was noted for the Δ*sepH* mutant and this was associated with what appeared to be a loss-of-polarity phenotype characterized by highly branched hyphal tips (**Figure B.8**, inset). These data suggested that the SIN kinase pathway is essential to virulence through support of invasive tissue growth. To quantitatively analyze SIN kinase mutant fitness and host-pathogen interaction, we next completed qPCR-based fungal burden assays, as previously described [57]. Mice (n = 5 / group) were immune suppressed following the chemotherapeutic protocol and subsequently infected with 1 x $10^6$ conidia from each strain by intranasal inoculation. At Day +4, lungs were aseptically removed and processed for genomic DNA extraction. qPCR-based quantitation of *A. fumigatus* DNA in lung tissues revealed that both the Δ*sepH* and *sidB*-1 mutants accumulated significantly less fungal burden *in vivo* than the CEA10 parent (**Figure B.9A**). Although the *sepL*-1 mutant displayed reduced burden by qPCR when compared to CEA10, this difference was not statistically significant (**Figure B.9A**). This inability to accumulate *wild type* levels of fungal mass *in*

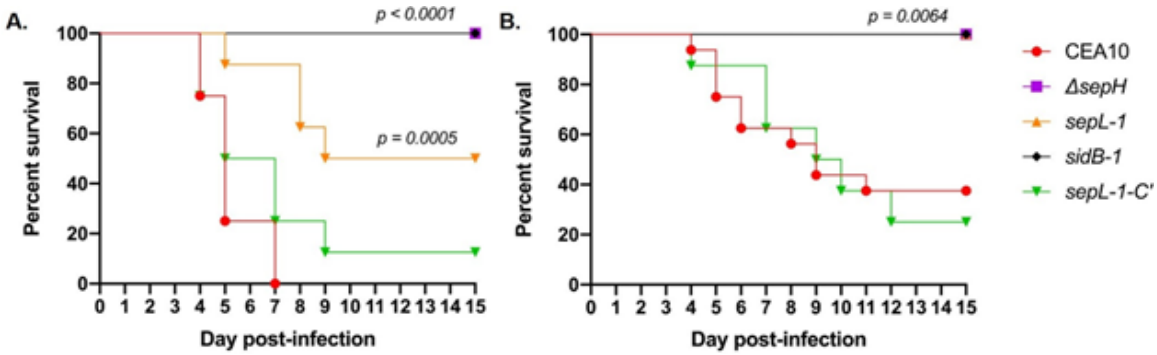


**Figure B.7: Figure 7. SIN kinase activity is required for virulence in mouse models of invasive aspergillosis.** Low- and high-magnification photomicrographs of Gomori methenamine silver (GMS)-stained lung tissue sections from the CEA10, Δ*sepH*, *sepL*-1 and *sidB*-1 at day +4 post-inoculation. Mice were immune suppressed with triamcinolone acetonide and inoculated with each strain as described for the previous survival studies. Hyphae (black stained fungal elements) from the CEA10 strain were noted to invade lung tissue, forming fulminant lesions. In contrast, growth of each SIN kinase mutant was limited to the airways with minimal to no tissue invasion (white arrowheads). Rare tissue invasion was associated with loss of polarity maintenance (Δ*sepH* inset panel). Scale bar = 50 µm.

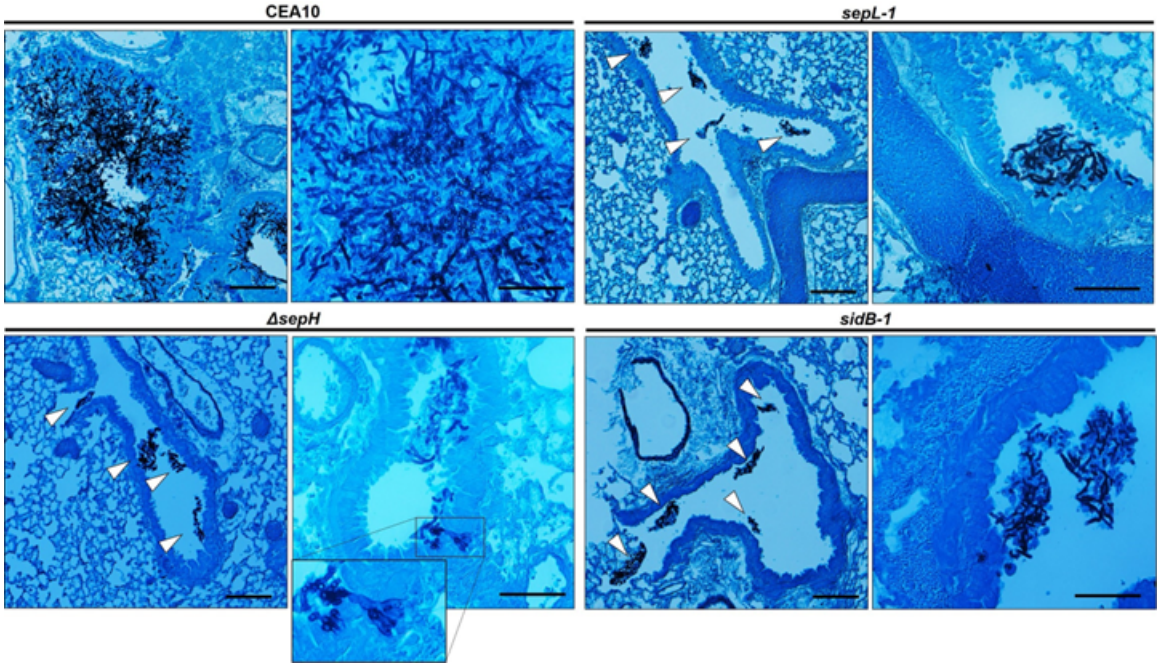


**Figure B.8: Figure 8. Loss of virulence among the SIN kinase mutants is associated with lack of tissue invasion.** Low- and high-magnification photomicrographs of Gomori methenamine silver (GMS)-stained lung tissue sections from the CEA10, Δ*sepH*, *sepL*-1 and *sidB*-1 at day +4 post-inoculation. Mice were immune suppressed with triamcinolone acetonide and inoculated with each strain as described for the previous survival studies. Hyphae (black stained fungal elements) from the CEA10 strain were noted to invade lung tissue, forming fulminant lesions. In contrast, growth of each SIN kinase mutant was limited to the airways with minimal to no tissue invasion (white arrowheads). Rare tissue invasion was associated with loss of polarity maintenance (Δ*sepH* inset panel). Scale bar = 50 µm.

*vivo* was also associated with a diminished ability to induce pro-inflammatory cytokine release *in vivo*. ELISA-based detection of IL-1$\beta$ and TNF$\alpha$ in lung homogenates from the same mice utilized for fungal burden revealed that all SIN kinase mutants induced significantly lower cytokine levels compared to the CEA10 control (**Figure B.9B** and **C**). To test if these findings were simply due to negative impacts on fitness, we compared the abilities of the SIN kinase mutants to induce IL-1$\beta$ release in vitro using the THP-1 macrophage-like cell line. Release of IL-1$\beta$ is a pro-inflammatory response typically induced by *A. fumigatus* cell wall PAMP exposure and is known to be dependent on inflammasome signaling [58,59]. Differentiated THP-1 cells were co-incubated with conidia from the *wild type* CEA10 or SIN kinase mutants (MOI 10:1) for 16 h and supernatants were subsequently analyzed for IL-1$\beta$ release by ELISA. Surprisingly, all SIN kinase mutants were found to induce significantly lower levels of IL-1$\beta$ release when compared to CEA10 (**Figure B.9D**). In addition, IL-1$\beta$ release in our assay was confirmed to be dependent on activation of the NLRP3 inflammasome, as the CEA10 parental strain was unable to induce IL-1$\beta$ release in NLRP3 $-/-$ or ASC $-/-$ cells, components necessary for canonical inflammasome activation and assembly (**Figure B.9E**) [60]. Inflammasome-dependence was further evidenced by the ability to block *Aspergillus*-induced IL-1$\beta$ release using the well-characterized NLRP3 inhibitor, MCC950 (**Figure B.9F**). Together, these data indicate that the SIN kinase pathway is not only important for supporting *A. fumigatus* pathogenic fitness but is also required for normal damage induced immune activation.

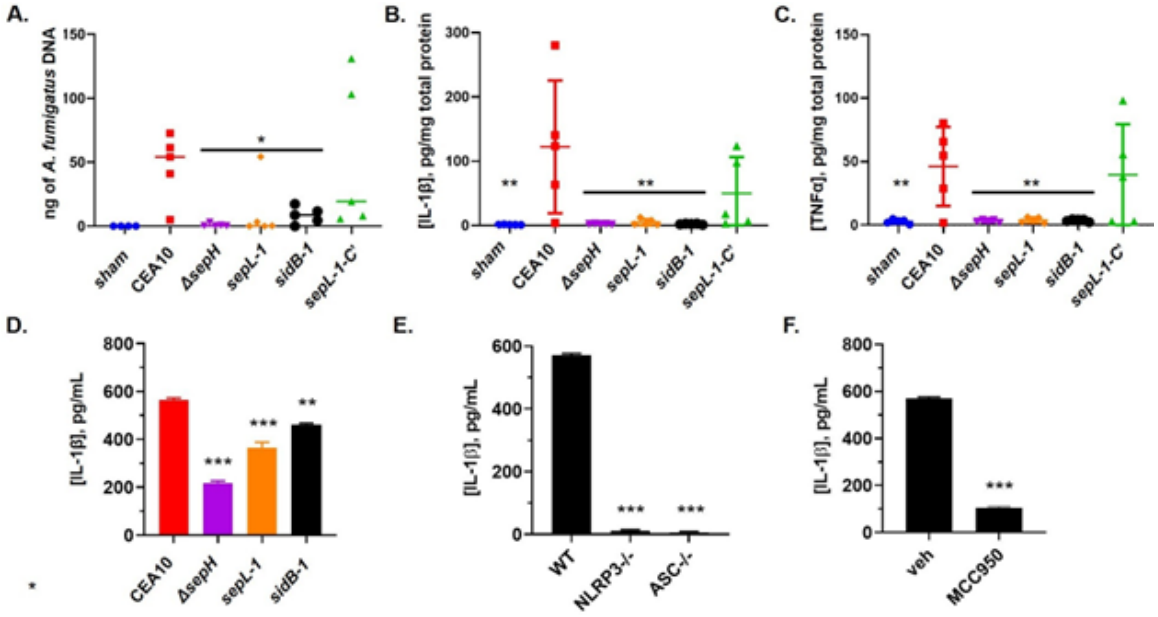


**Figure B.9: Figure 9. Loss of virulence in the SIN kinase mutants is characterized by decreased fungal burden and host response to infection.**

**Figure 9. (B.9) Loss of virulence in the SIN kinase mutants is characterized by decreased fungal burden and host response to infection. A)** Analysis of fungal burden by qPCR at day +4 post inoculation. Mice (n = 5 / group) were immune suppressed with cyclophosphamide and triamcinolone acetonide and inoculated with 1x $10^6$ conidia from each strain. Data are represented as nanograms of *A. fumigatus* specific DNA in 500 ng of total DNA. $p < 0.02$. Quantitation of IL-1$\beta$ **(B)** and TNF$\alpha$ **(C)** revealed decreased host response in SIN kinase mutant infected mice. Mice (n = 5 / group) were immune suppressed as indicated for fungal burden analysis and lung tissue was removed at day +4 post-inoculation, homogenized and analyzed by ELISA. $p = 0.0024$ for (B); $p = 0.0031$ for (C). An in vitro IL-1$\beta$ release assay uncovered decreased induction of inflammasome activation by the SIN kinase mutants. **D)** Conidia from each strain were co-incubated with phorbol 12-myristate 13-acetate (PMA) activated THP-1 cells (MOI 10:1) for 16 hrs and supernatants analyzed by ELISA for IL-1$\beta$ concentration. $p < 0.0001$; $p = 0.0014$. **E)** Inflammasome dependence of IL-1$\beta$ release was established by co-culturing PMA-activated WT (THP1-null), *Nlrp3-/-* (THP1-KO-NLRP3), and *Asc-/-* (THP1-KO-ASC) THP-1 cells with CEA10 conidia (MOI 10:1) as indicated for (D). $p < 0.0001$. **F)** Inflammasome dependence of *Aspergillus* induced IL-1$\beta$ release was further confirmed by repeating this assay in the presence of the inflammasome inhibitor, MCC950 (10 µM). $p < 0.0001$. All experiments were conducted in technical replicates (n = 4) and repeated independently in triplicate. Statistical comparisons in (A), (B), (C), and (D) were made by one-way ANOVA with Dunnett's multiple comparisons test post hoc and represent comparison of each SIN kinase mutant to the CEA10 control. Statistical comparisons in (E) were made by one-way ANOVA with Dunnett's multiple comparisons post hoc and represent the *NLRP3-/-* and *ASC-/-* versus WT control. The statistical comparison of MCC950 versus vehicle in (F) was made by unpaired T-test.

## Loss of hyphal septation improves echinocandin-mediated fungal clearance during invasive disease

Taken together, our *in vitro* and *in vivo* data suggest that *A. fumigatus* mutants lacking septa should be more susceptible to echinocandin therapy during infection. To examine the *in vivo* therapeutic relevance of our *in vitro* findings, we next employed the *wild type* CEA10 parent and the *sepL*-1 strain in a chemotherapeutic mouse model of invasive aspergillosis with and without echinocandin therapy. The *sepL*-1 mutant and chemotherapeutic model were chosen here as this combination was found to result in measurable mortality in our previous experiments. Ten mice per experimental arm were infected with $10^6$ conidia of either the CEA10 or *sepL*-1 strain by intranasal inoculation on day 0. On Days +1, +2 and +3, mice were administered (or not) micafungin therapy (1 mg/kg/day or 2 mg/kg/day) by

intraperitoneal injection (three total doses). Survival at the end of the 14-day infection was 0% for the CEA10 untreated arm and 75% for the *sepL*-1 untreated arm (p = 0.002) (**Figure B.9A**). Strikingly, with micafungin therapy at 2 mg/kg/day, the *sepL*-1 mutant-infected mice exhibited 100% survival whereas the *wild type* strain-infected mice, treated in the same manner, produced 0% survival by day +9

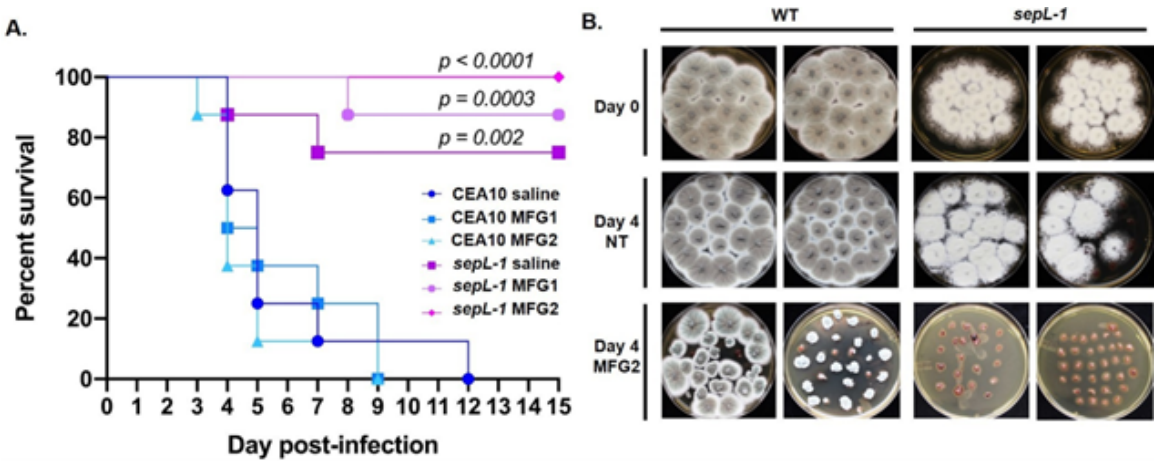


**Figure B.10: Figure 10. Loss of hyphal septation improves echinocandin therapy characterized by clearance of fungal burden from lung tissue. A)** Survival analysis of mice infected with either the CEA10 or *sepL*-1 mutant strain with and without micafungin therapy. All mice were immune suppressed through intraperitoneal injection of cyclophosphamide on days -3, +1, +4, and +7 and a single subcutaneous injection of triamcinolone acetonide on day -1. Mice were inoculated with 1 X $10^6$ conidia of the indicated strain suspended in 20 µl of sterile saline on day 0 and then received three separate intraperitoneal injections of micafungin at either 1 mg/kg (MFG1) or 2 mg/kg (MFG2) on days +1, +2 and +3. Statistical comparisons were made by Mantel-Cox log-rank test and represent each *sepL*-1 mutant experimental arm compared to its CEA10 control (i.e., CEA10 saline vs. *sepL*-1 saline, CEA10 MFG1 vs. *sepL*-1 MFG1, and CEA10 MFG2 vs. *sepL*-1 MFG2). **B)** CEA10 and *sepL*-1 residual lung tissue burden at day 0 and day 4 with and without micafungin 2 mg/kg therapy. Organ cultures are shown from two representative animals from each treatment group. Note that the *sepL*-1 infected mice treated with micafungin 2 mg/kg are culture negative at day 4 post-inoculation. MFG = micafungin.

(p < 0.0001) (B.9A). Although survival differences between treated and untreated CEA10 and *sepL*-1 strains were significant for each therapeutic regimen, we found no significant difference between *sepL*-1 treated and untreated experimental arms (**Figure B.10A**). This

was due to a combination of the low virulence of the *sepL*-1 mutant and the number of mice used per arm. However, in a separate experiment, the ability of micafungin therapy to enhance the reduction of residual tissue burden in *sepL*-1 infected mice was also examined. Mice (n = 8 / arm) were immune suppressed and inoculated identical to survival assays and were provided (or not) micafungin therapy at days +1, 2, and 3. Lungs were removed at day +0 or day +4, sectioned, and cultured on Inhibitory Mold Agar at 37°C for 48 hours. All non-micafungin treated mice infected with either the CEA10 or *sepL*-1 strains produced positive fungal cultures using tissue extracted at day +0 and day +4, indicating live fungus in the lung environment for both strains at this timepoint (**Figure B.10B**). At day +4 with micafungin therapy (2 mg/kg/day), 75% of CEA10-infected mouse lung cultures (6 / 8) were still positive for fungal growth (**Figure B.10B**). Compared to fungal colony morphologies arising from micafungin-free lungs (CEA10 day +0 or day +4, no micafungin), the micafungin-treated mouse lung cultures produced compact colony growth confirming presence of micafungin in tissues during therapy (**Figure B.10B**). In contrast, lungs from *sepL*-1 infected, micafungin-treated mice extracted at day +4 contained no culturable fungal elements (**Figure B.10B**). Together, these data suggest that loss of hyphal septation improves echinocandin therapy by enhancing the ability of this drug class to clear invading *A. fumigatus* hyphae from the lung.

### Gene deletion of additional septation mediators phenocopies the SIN kinase mutants

As the striking loss of virulence and tissue invasion phenotypes of the SIN kinase mutants could be dependent or independent of hyphal septation, we next wanted to test whether these phenotypes could be replicated in a SIN kinase-independent but septation-dependent manner. Therefore, we sought to identify novel genes essential for septation in *A. fumigatus*. In the model filamentous fungus, *A. nidulans*, the *acnA* gene encodes an alpha-actinin protein that is essential for hyphal septation and loss of septation in a strain lacking *acnA* is associated with a complete absence of contractile actin ring assembly [61]. This data mirrors results reported for a Schizosaccharomyces pombe alpha-actinin gene, *ain1*, which is also mediates CAR assembly in the model fission yeast [62]. Additionally, in *Saccharomyces cerevisiae*, the *MLC1* gene encodes for a myosin light chain protein that is essential for regulating myosin heavy chain interactions during contractile actin ring assembly [63]. Loss of *S. cerevisiae MLC1*, or the *MLC1* ortholog in *S. pombe* (*cdc4*), results in lethality for both yeasts due to the inability to complete CAR formation and subsequent cytokinesis [64,65]. Given their conserved roles in septation and the non-essential nature of septation in *A. fumigatus*, we reasoned that we should be able to acquire gene deletions of the *A. fumigatus* orthologs of *acnA* and *MLC1* for further study here. The putative protein sequences for *acnA* and *MLC1* from *A. nidulans* and *S. cerevisiae*, respectively, were utilized for a BLASTp search of the *A. fumigatus* genome (FungiDB). For *MLC1*, this search identified two proteins with significant identity to the Mlc1p sequence. These were proteins encoded by the uncharacterized gene,

AFUB_091530 (42% identity), and an ortholog of the highly conserved calmodulin gene, AFUB_067160 (34% identity). Therefore, AFUB_091530 was chosen as the *A. fumigatus MLC1* ortholog and named *mlcA*. Surprisingly, no orthologs of the *A. nidulans* AcnA protein could be identified in *A. fumigatus*. However, additional BLASTp searches using the S. pombe *ain1* protein sequence identified a single *A. fumigatus* gene, AFUB_055850, with 49% identity to Ain1 and was therefore named *ainA*. This BLASTp search also identified a single Ain1 ortholog in *A. nidulans* (AN7707) that was unique from the aforementioned AcnA protein. Employing CRISPR/Cas9-based gene targeting, single gene deletion and complementation mutants were generated for both genes (**Supplementary Figure 3**).

As was observed for the SIN kinase mutants, CFW staining of mature hyphae revealed loss of septum formation in the Δ*mlcA* and Δ*ainA* mutants. Whereas the parental *wild type* CEA10 displayed fully formed septa after 20 hours of culture in minimal media, the Δ*mlcA* and Δ*ainA* mutants formed completely aseptate hyphae. The Δ*mlcA* mutant was additionally characterized by the presence of brightly stained CFW-positive puncta of cell wall material throughout hyphae (**Figure B.11A**). This finding suggests that, in addition to loss of septum formation, Δ*mlcA* deletion causes abnormal deposition of cell wall material during hyphal growth. E-test assays, employing anidulafungin-embedded strips, resulted in the formation of a zone-of-clearance for both the Δ*mlcA* and Δ*ainA* mutants (**Figure B.11B**). These results were similar to those generated by the SIN kinase mutants (**Figure B.5A** and **B**). These findings were, again, in contrast to the *wild type* CEA10 parent that formed only a zone of depressed growth in response to echinocandin stress by E-test (**Figure B.11B**). When employed for survival analyses in a corticosteroid model of invasive aspergillosis, Δ*mlcA* and Δ*ainA* again phenocopied the SIN kinase mutants. At 15 days post-inoculation, both mutants resulted in no mortality whereas the *wild type* and complement strains performed similarly and produced significantly higher mortality (**Figure B.12A** and **C**). Further, histopathological analysis of silverstained tissue sections from lungs of *mlcA* and *ainA* infected mice revealed growth of fungal elements only in the airways (**Figure B.11B** and **D**). These results again mirrored those for the SIN kinase mutants where a lack of tissue invasion was noted for aseptate hyphae (**Figure B.8**). Together, these findings suggest that deletion or disruption of the signaling pathways or machinery required for septum initiation and formation results in the inability to support invasive growth in the host lung environment.

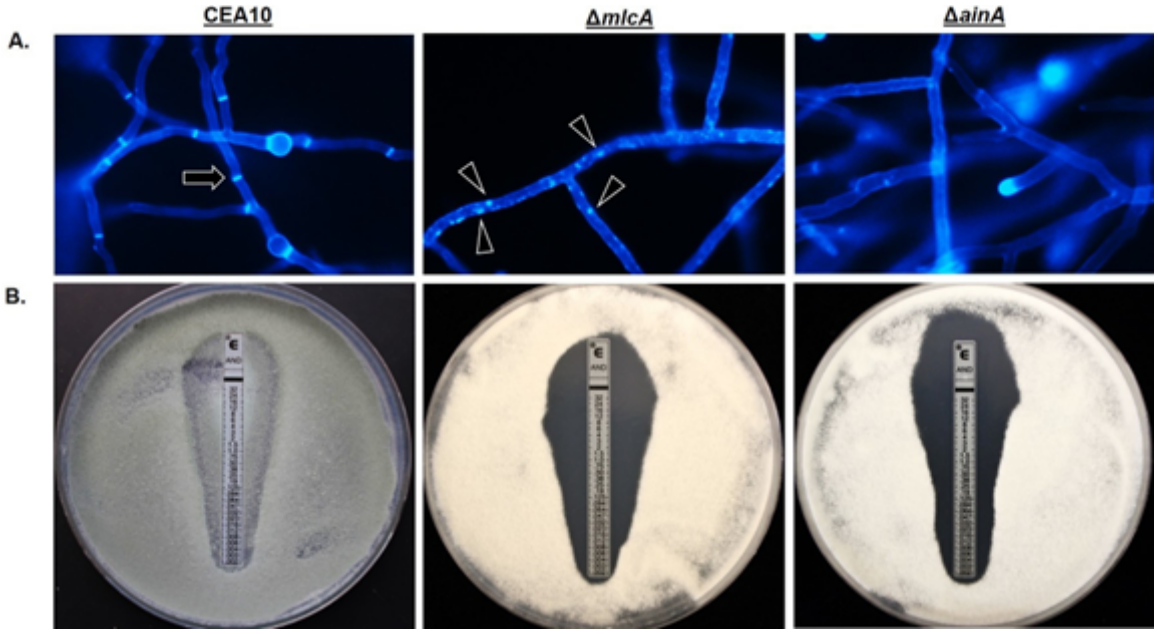


**Figure B.11: Figure 11. The *A. fumigatus* genes encoding myosin light chain (*mlcA*) and alpha-actinin (*ainA*) are required for hyphal septation and echinocandin resistance. A)** Characterization of septation by CFW staining of the *wild type* parental strain (CEA10) and the *mlcA* (Δ*mlcA*) and *ainA* (Δ*ainA*) deletion mutants. Conidia from each strain were cultured to mature mycelial growth on sterile coverslips submerged in minimal media. Microscopic analysis of CFW-stained cultures revealed fully formed, normal septa in the CEA10 control strain (black arrow), whereas Δ*mlcA* formed aseptate hyphae with brightly stained puncta of cell wall material and Δ*ainA* developed only aseptate hyphae. **B)** Anidulafungin E-test assays for each strain. Note the zone-of-clearance of the Δ*mlcA* and Δ*ainA* strains which show the complete absence of growth.

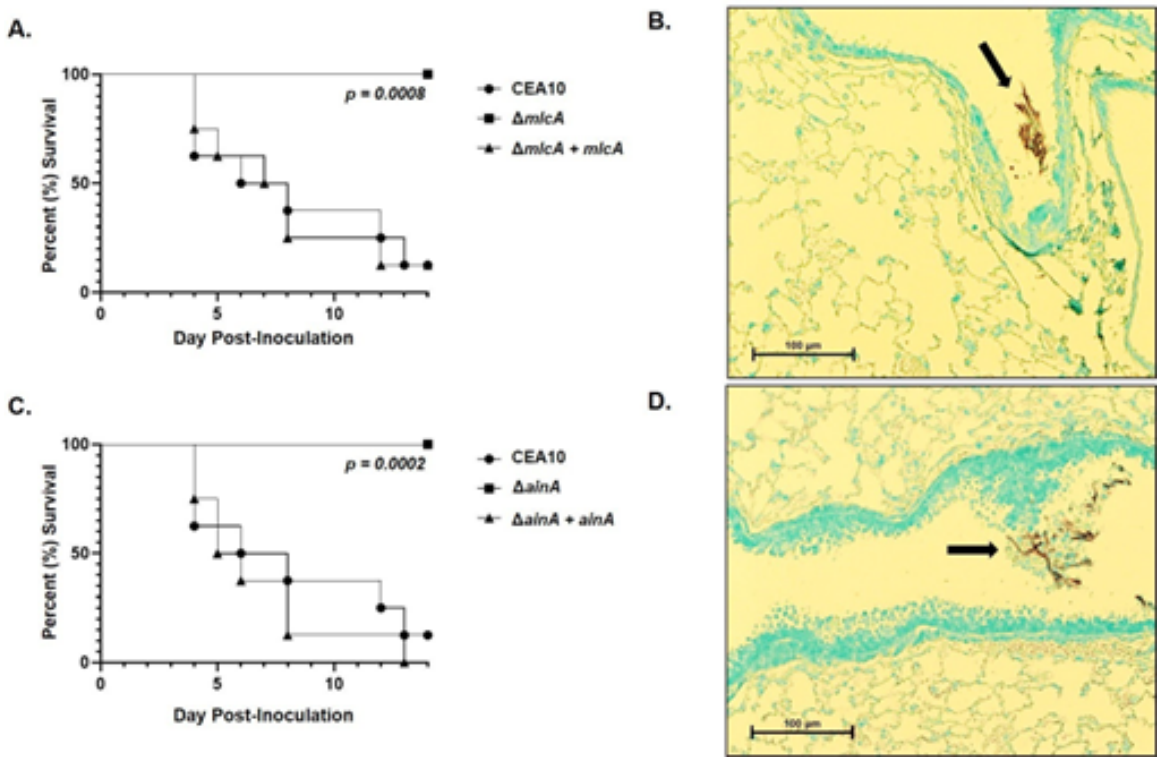


**Figure B.12: Figure 12. Loss of septation caused by deletion of *mlcA* or *ainA* results in avirulence associated with lack of tissue invasion.** Survival analysis and GMS-stained tissue histology from immune suppressed mice infected with the Δ*mlcA* isogenic strain set **(A)** and **(B)** or the Δ*ainA* isogenic strain set **(C)** and **(D)**, respectively. Mice (n = 8 / strain) were immune suppressed with a single, subcutaneous injection of triamcinolone acetonide (40 mg/kg) on Day -1. On Day 0, all mice were intranasally administered 100,000 conidia suspended in 20 µl of sterile saline. No deaths were recorded in sham treated mice (sterile saline alone, n = 8). Statistical comparisons were made by Mantel-Cox log-rank test. Black arrows denote non-invasive fungal growth identified only in the airways of Δ*mlcA* and Δ*ainA* infected mice.

## Discussion

Hyphal septa are the product of incomplete cytokinesis and have long been appreciated to function as a protective barrier to mechanical and chemical stresses that disrupt the cell walls of filamentous fungi. This was the first investigation of the function of the SIN kinases in the human pathogen, *A. fumigatus*. The SIN complex has been studied most intensely in the fission yeast, *Schizosaccharomyces pombe* [66]. Although the SIN-complex shares similarity and encompasses some protein components homologous to those comprising the Mitotic Exit Network (MEN) in other yeast species like *Saccharomyces cerevisiae*, the signaling pathways are distinct [37,38]. The core of the SIN in *S. pombe* is composed of a GTPase protein, Spg1, that is negatively regulated by a two-component GTPase activating protein, Cdc16/Byr4 [37]. Although biochemical evidence is lacking even in the model organisms, this module is thought to signal through a kinase cascade consisting of the Cdc7 –Sid1 –Sid2 protein kinases with the co-regulatory components, Cdc14 and Mob1, binding to and regulating the Sid1 and Sid2 kinases, respectively [37]. The core tripartite kinase cascade is conserved in the model filamentous fungi *A. nidulans* (SepH-SepL-SidB) and *Neurospora crassa* (CDC-7, SID-1, DBF-2) [39,40] and the orthologs of these kinases were the focus of this study in *A. fumigatus*. Although downstream SIN-complex effectors are not verified in *A. fumigatus*, the Rho-type GTPase, Rho4, and the putative Rho Guanine Exchange Factors, Bud3 and Rgf3, are known to regulate septum formation and a *rho4* deletion is the only mutation in *A. fumigatus* previously reported to completely block septation [52,54,67]. Rho4 has been proposed to be a downstream effector of SIN-kinase activity that, in turn, recruits a formin protein (SepA ortholog in *A. fumigatus*) to mediate assembly of the contractile actomyosin ring (CAR) on which the septum is built in *A. nidulans* [68]. The CAR is further built and constriction regulated by bundling of actin filaments through the action of $\alpha$-actinin cross-linking proteins and by interactions with myosin light and heavy chain proteins [69]. Interestingly, although the *A. nidulans* $\alpha$-actinin, AcnA, has been shown to regulate septation, we found this gene to not be conserved in *A. fumigatus*. This finding suggests differences in how *A. fumigatus* and *A. nidulans* regulate septum formation. In contrast, we found that the $\alpha$-actinin gene, *ainA*, as well as the myosin light chain gene, *mlcA*, were conserved among *A. fumigatus*, *A. nidulans* and *S. pombe*. Both genes played essential roles in completion of septation. This event is intimately coupled with the exit from mitosis in *S. pombe* such that SIN-complex mutants are typically inviable in this yeast. In filamentous organisms like the Aspergilli, however, mitosis and septation are not interdependent and, as our data show, aseptate *Aspergillus* mutants are viable under normal growth conditions. The initial publications describing anti-*Aspergillus* echinocandin activity as being characterized by fungal hyphal tip lysis, were also the first to suggest the possibility that hyphal septation likely underpins the fungistatic nature of this drug class against Aspergilli [24]. This conclusion was further supported by subsequently published genetic and pharmacologic evidence. For example, when the echinocandin-target gene *fksA* is deleted in *A. fumigatus*, hyphae are devoid of $\beta$-1,3-glucan and appear to be killed in the presence of compounds that inhibit septation [54]. Additionally, the aseptate $\Delta$rho4 *A. fumigatus* mutant cannot

grow in the presence of echinocandins [52]. Although highly suggestive of an essential role for hyphal septation in protection against echinocandins, the septation inhibitors previously employed, hydroxyurea and diepoxyoctane, potentially have many off-target effects as developmental and cell cycle inhibitors and the *rho4* gene was additionally characterized as directly contributing to modulation of the cell wall [52]. Our data definitively show that loss of *A. fumigatus* hyphal septation imparts fungicidal activity to echinocandins. Thus, septation is a mechanism to limit damage imposed by loss of cell wall integrity via inhibition of $\beta$-1,3-glucan synthesis. When faced with echinocandin stress, the FksA enzyme mislocalizes from its normal position at the hyphal tip and the tip compartment is lysed [70]. However, the interseptal compartments remain viable [24,70]. In many septation-competent fungi, septal pores that remain after septum formation to promote exchange of cytoplasmic materials plug quickly after cell wall damage to protect against cytoplasmic leakage [71,72]. After tip-lysis by echinocandins, new hyphal growth within the lysed hyphal tip compartments, known as "intrahyphal hyphae", can develop from existing septa in the proximal subapical compartment [70]. The FksA enzyme is relocalized to these new sites of apical growth to support the nascent growth axis. Therefore, the septum may act not only as physical barrier to limit hyphal damage to the colony periphery, but also as a scaffold for continued hyphal growth under cell wall stress in the Aspergilli.

Under the conditions we tested, the aseptate SIN kinase mutants were more susceptible to echinocandin-mediated cell wall damage than even the CWI kinase mutants, which represent the core signaling pathway of cell wall stress responses (**Supplementary Figure 4**). The majority of published works identifying cellular mechanisms supporting *A. fumigatus* survival in response to echinocandins have logically focused on adaptive cell wall stress signaling, such as the CWI kinase cascade and cAMP-dependent protein kinase A signaling [21,32,34]. Although they represent potentially powerful targets for future combination therapies with echinocandins, inhibiting many of these pathways leaves the cell septation machinery largely intact and, therefore, provides the pathogen a means for persistence in the host under echinocandin stress. Recent studies suggest that the process of septation may also be stress responsive in fungi. In the yeast pathogen *Candida albicans*, loss of the I chitin synthase gene is lethal due to the central role of the Chs1p enzyme in building the primary septum for successful cell division [73]. However, induction of cell wall stress in a *CHS1* repressed strain causes stress-induced formation of an alternative "salvage septum" by other chitin synthase enzymes, leading to completion of cytokinesis and cell survival in the absence of *CHS1* [74]. Therefore, the enzymes that are typically required for the physical construction of the septum can be re-wired under cell wall stress to promote survival in *C. albicans*. In *A. nidulans*, a recent phosphoproteomics analysis of micafungin-stressed hyphae identified the SIN network ortholog, SidB, as being hyperphosphorylated in a manner likely dependent on the CWI MAPK, MpkA [30]. Further *in vitro* growth analyses analyzing septum formation in micafungin-stressed and micafungin-free samples revealed that the rate of septation increases during echinocandin-induced cell wall stress [30]. Together, these data argue that formation of this important protective barrier in filamentous fungi may

be both a fundamental structural cellular component and stress responsive. A plethora of previous studies have shown that neutrophil activity is required for clearance of hyphae and hyphal fragments during infection [75]. Patients most at-risk for invasive aspergillosis are profoundly neutropenic and, as such, viable hyphal fragments remaining after unsuccessful therapy or under sub-therapeutic levels of drug are potentially especially problematic. Our *in vivo* virulence data suggests that blockade of septation could negatively impact *A. fumigatus* persistence by promoting echinocandin-induced hyphal death, likely before effective adaptive responses can be upregulated. The nearly complete lack of virulence of the SIN kinase mutant strain was a surprising finding and is the first description of a connection between hyphal septation and virulence in a filamentous human pathogen. The molecular and cellular mechanisms for why septation is required for *A. fumigatus* virulence are unknown. However, loss of hyphal septation has been shown to block virulence of the smut fungus, *Ustilago maydis*, due to loss of needed turgor pressure to build the appressorium [76]. Although human infectious fungi do not utilize special infection structures like appressoria for invasion, the inability of *A. fumigatus* aseptate hyphae to invade murine lung tissue could be from lack of turgor pressure in the tip compartment required for physical invasion. A recent study exploring the connections between hyphal growth and polarity maintenance has found filamentous fungal organisms with fast growing hyphal tips and high turgor pressure to often lose polarity when undergoing invasive growth (i.e., penetration into small spaces) [77]. in contrast, slow growing fungi with lower turgor pressure are able to maintain a single polarized growth axes under the same conditions. Therefore, a trade-off exists between growth rate and morphological plasticity for these fungi that is especially important during invasive growth into substrates. As we noted loss of polarity in our SIN kinase mutants during attempted tissue invasive growth (**Figure B.8**), it may be possible that septation is involved in imposing such a tradeoff between growth rate, turgor pressure and maintenance of hyphal morphogenesis for *A. fumigatus*. Although we utilize multiple septation-deficient mutants in our study, our data do not strictly rule out the possibility that hypersusceptibility of the SIN kinase mutants to cell wall stress could be at least partially septum-independent. For example, the hyphal septation machinery may be required to maintain cell wall stability or may crosstalk with cell wall biosynthesis pathways for support of overall hyphal structure during tissue invasive growth. This explanation would suggest a regulatory link between septation and cell wall integrity / biosynthesis networks. In support of this possibility, our finding that the SIN kinase mutants displayed reduced stimulation of IL-1$\beta$ release *in vitro* may argue that cell wall PAMP exposure and / or cell wall structure are impacted by loss of septation. Further in-depth studies on SIN kinase signaling and cell wall integrity pathways will fully delineate the septation-dependent and -independent mechanisms underpinning the tissue invasion phenotypes resorted here. In conclusion, we report for the first time that hyphal septation in *A. fumigatus* is required for virulence of this important human pathogen. We also show, definitively, that loss of hyphal septation generates cidal activity of echinocandins against *A. fumigatus* and enhances *in vivo* echinocandin activity by promoting clearance of viable residual tissue burden. Together,

our findings suggest that inhibitors of septation could enhance echinocandin-mediated killing while simultaneously limiting the invasive potential of *A. fumigatus* hyphae.

## Materials and methods

### Ethics statement

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center under protocol number 19–0067.

### *A. fumigatus* strains and growth conditions

The *wild type* strain, CEA10, was utilized as the parental strain for all genetic manipulations described herein. All strains generated as part of this study are listed in **Supplemental File 1**. For quantification of colony diameter, five microliters of sterile water containing $10^4$ conidia were inoculated onto the center of glucose minimal media (GMM) agar plates [78] and incubated at 37°C. Colony diameters were measured every 24 hours and pictures were taken after 96 hours incubation. Colony diameters of each mutant were compared to those of the parental strain, CEA10, using two-way ANOVA with Tukey's test for multiple comparisons (GraphPad Prism v8.2.1). To evaluate and quantify the production of conidia, $2x10^4$ conidia of each mutant were inoculated onto the center of GMM agar plates and incubated for 4 days at 37°C. After this time, colony diameters were measured and conidia were harvested in identical volumes of sterile water from each plate. Recovered conidia were counted using a hemocytometer and results were expressed as conidia per mm2 of colony area. Results from the kinase disruption mutants were compared to CEA10 using one-way ANOVA followed by Dunnett's test for multiple comparisons (GraphPad v8.2.1). Conidial quantification and colony diameters were determined at least twice for each mutant.

### Genetic manipulations of *Aspergillus fumigatus*

All putative protein kinase encoding genes were identified via BLAST search of the *A. fumigatus* genome database (FungiDB.org) using the known protein kinases of *Aspergillus nidulans* [39]. In total, 142 putative protein kinase encoding genes were identified and are listed in **Supplemental File 1**. Each putative protein kinase gene was targeted for disruption in a *wild type* genetic background (CEA10) using a CRISPR-Cas9 gene editing technique previously described by our laboratory [41]. To increase throughput of mutant generation, standard *A. fumigatus* protoplast-mediated transformation protocols were adapted to a miniaturized, 96-well plate system. Briefly, CRISPR RNAs (crRNAs) and primers used to amplify hygromycin resistance cassette repair templates, engineered to incorporate 40 bp micro-homology regions at both 5′ and 3′ ends, were designed using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) from the University of Georgia

(http://grna.ctegd.uga.edu/). This tool allows users to identify Protospacer Adjacent Motifs (PAM) sites and protospacers in desired regions of the genome while predicting off-target sites. Protospacer regions were selected within the first exon of each gene, when possible. Repair templates were designed to delete 5 nucleotides of the Open Reading Frame (ORF) upon integration in an attempt to generate a frameshift and, consequently, disrupt gene function in the event of read-through during transcription. Forward and reverse primers for generation of repair templates were purchased in arrayed 96-well plates and utilized for PCR reactions also carried out in 96-well plates. Five microliters of each PCR reaction was utilized for gel electrophoresis to ensure a single band of appropriate size was generated for each reaction. After confirmation of successful PCR, five microliters of the unpurified PCR reaction, containing about 200 ng of amplified repair template, was utilized for transformation. All primers utilized for repair template generation are listed in **Supplemental File 1**. Guide RNAs (gRNAs) and Ribonucleoprotein (RNP) complexes for each gene were built *in vitro* using commercially available tracrRNA and Cas9 enzyme, as previously described [41]. To reduce the cost of each transformation, as well as the time required for library construction, we adapted a traditional *A. fumigatus* protoplast transformation procedure to a miniaturized system, which allowed the performance of each gene disruption in a final total volume of 200 µl. The transformations were carried out in 96-well plates (**Figure B.1**), which allowed 96 different transformations in a single day. Briefly, 96-well plates with each well containing a transformation mixture composed of protoplasts (1–5 x $10^5$), Cas9 RNP complex (1.2 µM gRNA + 500 ng Cas9), repair template ( 200 ng), 60% polyethylene glycol 3350 (PEG 3350), and 11 µl of STC Buffer [79] were incubated on ice for 50 min. After this time, 57 µl of 60% PEG were added to each well and incubated at RT for 20 min. Finally, the volume of each well was brought to 200 µl using STC buffer and the entire contents of each well were plated onto a single Sorbitol Minimal Medium [79] agar plate. The protoplasts were allowed to recover by incubating the culture plates at RT overnight, and top agar (SMM with 0.75% agar) containing 450 µg / ml of hygromycin was added the next day. The plates were then incubated at 37°C until colonies were observed. After 3–4 days, single colonies were transferred to new GMM plates supplemented with 150 µg / ml of hygromycin, single spored and genotypically screened by multiple PCRs, (as seen in **Figure B.1D**). To generate *sepH*, *mlcA*, and *ainA* deletion strains, CRISPR/Cas9-mediated gene targeting was employed, as previously described [41]. To aid in complete gene deletion, two PAM sites, located upstream and downstream of the respective genes, were selected and HygR repair templates were designed to contain microhomology regions targeting areas outside each PAM. Transformation was carried out as described above and positive transformants were screened by PCR. Complementation of the Δ*sepH*, Δ*mlcA*, Δ*ainA*, and *sepL*-1 mutants was also carried out by CRISPR/Cas9-mediated gene targeting. The complete ORF for each gene was amplified from CEA10 genomic DNA to contain regions homologous to the target sequence (5′ end) as well as to a phleomycin resistance cassette (3′ end). The phleomycin resistance cassette was amplified from the plasmid pAGRP [80] and primers deigned to incorporate sequence complimentary

to the 3′ end of the *sepL* locus. As such, an overlapping region between the two fragments was generated. New PAM sites outside of the targeted loci were selected for Cas9 RNP targeting and both repair template fragments were mixed during transformation. Positive transformant colonies were confirmed for proper integration as described above.

**Cell wall stress and echinocandin susceptibility assays**

Protein kinase disruption mutants were screened for cell wall sensitivity by monitoring their growth in the presence of cell wall disrupting agents. Qualitative primary screens were performed with at least two biological replicates of each mutant and the parental strain by arraying strains (5 µl of a $10^4$ conidia / ml suspension) onto GMM agar plates containing either 40 µg / ml or 80 µg / ml of either congo red (CR) or calcofluor white (CFW). To select positive hits from the primary screen, colony development was examined after incubation at 37°C for 72 hours and strains displaying decreased or increased colony size versus the parental isolate were chose for secondary analysis. GMM agar plates without CR or CFW were used as a growth control. For secondary analyses of strains selected from primary screens, quantitative spot dilution assays were conducted. In brief, fresh conidial suspensions were prepared from single spore isolates of each mutant and 5 µl of 10-fold serial dilutions ranging from $10^6$ to $10^3$ conidia / ml were inoculated onto GMM agar plates supplemented with 40 µg / ml or 80 µg / ml of either CR or CFW. Plates were again incubated at 37°C for 72 hours and GMM agar plates containing no compounds were used for growth control. The *in vitro* activity of caspofungin was determined using a broth microdilution assay [50]. Briefly, ten two-fold dilutions of caspofungin, ranging from 4 to 0.0075 µg / ml, were prepared in Roswell Park Memorial Institute-1640 medium (RPMI-1640) and placed in round bottomed 96-well plates. Then, each well was inoculated with $2x10^4$ conidia and incubated at 35°C. Caspofungin minimal effective concentration (MEC) was read after 24 hours, with the aid of an inverted mirror. The strain *Candida krusei* ATCC 6258 was used as a quality control to ensure accurate activity of the tested drug. The assays were repeated at least twice for each mutant. For those mutants showing reduced or increased susceptibility by at least one two-fold dilution in comparison to the parental strain, the antifungal activity was also evaluated by a spot dilution assay. Briefly, GMM agar plates containing 0.06–0.5 µg / ml of caspofungin, were inoculated with serial dilutions of conidia suspensions ranging from $10^4$ to 10 conidia. The plates were incubated at 37°C for 72 hours and the growth of each mutant was recorded every 24 hours and compared to CEA10. Antifungal susceptibility was also assessed using concentration gradient strips (Etest), using modification of a previously described protocol [81]. A suspension containing $10^6$ conidia in 0.5 ml was homogeneously inoculated onto GMM agar plates. Caspofungin (CAS, Biomerieux) or micafungin (MFG, Biomerieux) embedded strips were applied onto the agar and plates were incubated at 37°C. The production of a zone-of-clearance was recorded after 48 hours of culture.

**CFW and propidium iodide (PI) staining**

CFW and PI staining were performed as previously described [82]. Briefly, one thousand conidia were cultured in coverslips submerged in liquid GMM. After 16 hours at 37°C, the coverslips with adherent hyphae were washed with 50 mM morpholinepropanesulfonic acid (MOPS) buffer solution, adjusted to pH 6.7 and then submerged in fixative solution (8% formaldehyde, 25mM EGTA, 5mM MgSO4, 5% DMSO and 0.2% Triton) for one hour at room temperature (RT). Coverslips were again washed twice with 50 mM PIPES for 10 min and treated with 100 µg / ml of RNAase A for one hour at 37°C. All samples were then washed twice with MOPS buffer and stained with 12.5 µg / ml of PI and 1 µg / ml of CFW for 5 min at RT. Finally, the coverslips were washed twice more with MOPS buffer, mounted and analyzed immediately by fluorescence microscopy using a Nikon Ni-U upright microscope equipped with TRITC and DAPI filters. Images were acquired using Nikon Elements software package.

**Quantitation of viability by CFDA**

Viability in the presence of caspofungin was analyzed using 5,(6)-Carboxyfluorescein Diacetate (CFDA) staining, as previously described [23]. Conidia ($8x10^4$) were inoculated into 4 ml of GMM broth with or without 0.5 µg / ml of caspofungin and poured onto sterile coverslips in 35mm petri dishes. Cultures were then incubated at 37°C to allow conidia to germinate and adhere to coverslips. At the indicated times, culture supernatants were discarded and coverslips were stained with a solution of 50 µg / ml CFDA (Invitrogen) in 0.1M MOPS buffer (pH 3) for 1 hour at 37°C and 250rpm. Coverslips were washed once in 0.1M MOPS buffer and mounted for microscopy. Fluorescence microscopy was performed on a Nikon NiU microscope equipped with a Nikon DS-Qi1Mc camera using GFP filter settings. The percentage of CFDA stained microcolonies was determined by manual counts and images were captured using Nikon Elements software (v4.0).

**Animal studies**

For survival studies, two different models of invasive pulmonary aspergillosis were employed, as previously described [56]. Each model utilized CF-1 female mice weighing approximately 25 g. For the corticosteroid model, mice were immunosuppressed with 40 mg / kg of triamcinolone acetonide (TA) (Kenalog, Bristol-Myers Squibb, Princeton, NJ, USA), given subcutaneously the day prior to the infection. For the chemotherapeutic model, mice were immune suppressed by the intraperitoneal administration of 150 mg / kg of cyclophosphamide, on days -3, +1, +4 and +7, in addition to the TA injection on day -1. On the day of the infection, mice were transiently anesthetized by the inhalation of isoflurane in an induction box (primary and secondary flow rate set at 0.5 liters / minute, 2.5% isoflurane) and inoculated by intranasal instillation with a suspension of $10^5$ (initial survival studies) or $10^6$ conidia (echinocandin therapy experiments) in 20 µl of saline solution. Survival and

health status of the mice were monitored at least twice a day during a period of 15 days. Those mice showing severe signs of distress or disease were humanely euthanized by anoxia with $CO_2$ followed by cervical dislocation. In order to prevent bacterial infections, mice were given a mixture of sulfamethoxazole and trimethoprim in the drinking water, starting 3 days before the inoculation. To determine the *in vivo* susceptibility to echinocandins in selected mutants, mice were immunosuppressed and inoculated as described above and, in addition, were treated with 1 or 2 mg / kg of micafungin (Mycamine, Astellas Pharma Inc., Northbrook, IL, USA) intraperitoneally, once a day. Treatments started one day post-infection (day +1) and lasted 3 days (day +4). Mock groups were given saline alone. All echinocandin treatments were based on previously published studies [83]. Viable residual tissue burden in lungs of mice untreated or treated with micafungin was examined 6 hours (day 0) and 4 days (day +4) after infection. Mice were euthanized at the indicated time, lungs were aseptically harvested, sectioned into small pieces, and cultured on yeast peptone dextrose (YPD) agar for 48 hours at 37°C. In addition to the survival studies, histopathology analyses were performed. Two mice per group were immunosuppressed and infected as described above and euthanized 4 days after the inoculation. Lungs were inflated by intratracheal perfusion with 10% buffered formalin and subsequently embedded in paraffin. Finally, multiple 5 µm sections from the superior, middle and inferior lobes were stained with Grocott's methenamine silver for visualization of fungal elements.

**Lung fungal burden and *in vivo* cytokine secretion**

Lung fungal burden by quantitative PCR (qPCR) and measurement of *in vivo* cytokine secretion by ELISA were performed using slight modifications of previously described protocols [57,84]. Mice immune suppressed following the chemotherapeutic model described above were intranasally inoculated with $10^6$ conidia. After 4 days of infection, mice were euthanized and lungs were harvested and homogenized in 1 ml of sterile PBS using the gentleMACS dissociator (Miltenyi Biotec). Approximately 700 µl of lung homogenate were lyophilized and processed for DNA extraction using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek) according to manufacturer instructions. qPCR analyses were performed in technical duplicate for each sample, using the PrimeTime Gene Expression Master Mix and qPCR Probe Assays (Integrated DNA Technologies) containing the primers to amplify a region of the *A. fumigatus* 18S rRNA gene, as previously described [85]. For each sample, 500 ng of total DNA was used as template, and a standard curve containing 100, 10, 1, 0.1 and 0.01 ng of CEA10 genomic DNA was also included in the assay so that the amounts of *A. fumigatus* specific DNA could be determined. qPCR was conducted on a Bio-Rad CFX96 Real-Time PCR system running the Bio-Rad CFX Maestro 1.0 software (v4.0). Data are represented as nanograms of *A. fumigatus* specific DNA in 500 ng of total DNA. For analysis of cytokine secretion in the lungs, 300 µl of lung homogenate were mixed with 2x Protease Inhibitor Cocktail containing AEBSF, Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A (Sigma) and centrifuged at 14000 rpm for 10 minutes. Resulting supernatants were kept

at -80°C until employed for further measurements using mouse TNF$\alpha$ and IL-1$\beta$ ELISA kits (Invitrogen), according to manufacturer instructions. Total protein quantification was performed using Quick Start Bradford Protein Assay (Bio-Rad) and used for cytokine level normalization.

**Analysis of cytokine release in THP-1 cells**

WT (THP1-null), *Nlrp3-/-* (THP1-KO-NLRP3), and *Asc-/-* (THP1-KO-ASC) THP-1 cells (Invivogen) were utilized. Cytokine release from THP-1 cells was performed as previously described, with minor modifications [59,86]. Briefly, cells were cultured in RPMI-1640 medium containing 25 mM HEPES supplemented with 10% heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin-streptomycin, and 100 µg/mL normocin as described previously. THP-1 cells were assessed for viability by exclusionary Trypan Blue staining and plated at a density of $10^5$ cells/well in 96-well microtier plates using similar medium lacking normocin. Phorbol 12-myristate 13-acetate (PMA) was added at 100 nM final concentration and cells incubated for 24h to adopt a macrophage phenotype. Supernatants were discarded and replaced with 180 µl of fresh RPMI (without phenol red) and 20 µl of distilled water containing $10^6$ *A. fumigatus* conidia (MOI 10:1) added. In some cases, the inflammasome inhibitor MCC950 (10 µM, Invivogen) was added to the THP-1 cells concomitantly with the conidia. THP-1 cells and conidia were co-cultured for 16h and supernatants were collected and analyzed by ELISA for IL-1$\beta$ concentration following manufacturer instructions.

## Supporting information

**Supplementary Figure 1. Disruption of multiple protein kinase genes results in reduced colony growth and conidiation of *A. fumigatus*. A)** Quantitation of colony diameters for the *wild type* parent strain (CEA10) and disruption mutants displaying minimal, moderate or severe growth restriction on minimal media. 10,000 conidia from each strain were point-inoculated onto minimal media and cultured for 96 hrs at 37°C. Colony diameters form triplicate cultures for each strain were measured (mm) and averaged. Statistical comparisons were made by ANOVA and all comparisons generated a p 0.0371. Disruption mutants not shown generated colony diameters that were similar to CEA10. **B)** Quantitation of conidiation for the parent strain (CEA10) and multiple protein kinase gene disruption mutants. Conidia (2 x $10^4$) from each strain were cultured as in (A). Colony area was calculated and conidia were harvested in 10 ml of sterile water before filtration and quantitation using a hemocytometer. Each strain was assayed in triplicate and data were averaged. Statistical comparisons were made by one-way ANOVA with Dunnett's multiple comparisons post hoc and all comparisons generated a p 0.0036. Disruption mutants not included here for colony diameter or conidiation analyses were not significantly different from the parental strain (CEA10). (TIF)

**Supplementary Figure 2. Gene deletion of *sepH* and gene reconstitution of the *sepL* disruption (*sepL*-1).** Schematics for deletion of *sepH* **(A)** and for complementation of the *sepL*-1 disruption mutant **(C)**. Genetic manipulations were carried out using CRISPR/Cas9 gene editing (see Materials and Methods). For each locus targeted, the 20-nucleotide protospacer (black font) and the 3-nucleotide protospacer adjacent motif (PAM, underlined red font) are displayed. Each manipulation utilized Cas9-mediated double strand breaks generated 5′ and 3′ of the targeted gene. Repair templates (HygR = hygromycin resistance cassette; PhleoR = phleomycin resistance cassette) were PCR amplified from plasmids using primers that incorporated 40-basepair microhomology arms for targeting. Correct integration of repair templates was confirmed by PCR using primers P1 and P2 for *sepH* deletion **(B)** and primers P3 and P4 for *sepL*-1 complementation **(D)**. (TIF)

**Supplementary Figure 3. Deletion and complementation of *A. fumigatus* genes AFUB_067160, encoding myosin light chain (*mlcA*), and AFUB_055850, encoding alpha-actinin (*ainA*).** Schematics for deletion (A and D) and complementation (**B** and **E**) of *mlcA* and *ainA*, respectively. Genetic manipulations were carried out using CRISPR/Cas9 gene editing (see Materials and Methods). For each locus targeted, the 20-nucleotide protospacer (black font) and the 3-nucleotide protospacer adjacent motif (PAM, underlined red font) are displayed. Each manipulation utilized Cas9-mediated double strand breaks generated 5′ and 3′ of the targeted gene. Repair templates (HygR = hygromycin resistance cassette) were PCR-amplified from plasmids using primers that incorporated 40-basepair microhomology arms for targeting. Correct integration of repair templates and gene complementations were confirmed by PCR using primers P5 and P6 for *mlcA* (**A** and **B**) and primers P7 and P8 for *ainA* (**E** and **F**). (TIF)

**Supplementary Figure 4. Gene disruption of *A. fumigatus* cell wall integrity kinases (*mkk2* and *mpkA*) and the protein kinase A catalytic subunit (*pkaC1*) generates increased susceptibility to echinocandins.** Modified E-test assays for the *mkk2*-1, *mpkA*-1, and *pkaC1*-1 disruption mutants using minimal media (see Materials and Methods). Note the residual growth in the zone-of clearance for both *mkk2*-1 and *mpkA*-1 mutants indicating lack of echinocandin cidal activity. Insets show representative, drug-free minimal media culture plates onto which a single agar plug from the zone-of-clearance for each assay was sub-cultured. Multiple agar plugs (n = 10), taken from within 1 cm of the E-test strip and between the 32 and 0.25 μg/ml markers, were sub-cultured in the same manner for each assay. CAS = caspofungin; MFG = micafungin. (TIF)

**Supplemental File 1** (XLSX)

## Author Contributions

**Conceptualization:** Brian M. Peters, Jarrod R. Fortwendel.

**Formal analysis:** Ana Camila Oliveira Souza, Adela Martin-Vicente, Jarrod R. Fortwendel.

**Funding acquisition:** Jarrod R. Fortwendel.

**Investigation:** Ana Camila Oliveira Souza, Adela Martin-Vicente, Ashley V. Nywening, Wenbo Ge, David J. Lowes, Brian M. Peters, Jarrod R. Fortwendel.

**Methodology:** Ana Camila Oliveira Souza, Adela Martin-Vicente, Ashley V. Nywening, Wenbo Ge, David J. Lowes, Brian M. Peters, Jarrod R. Fortwendel.

**Project administration:** Jarrod R. Fortwendel.

**Supervision:** Jarrod R. Fortwendel.

**Writing – original draft:** Ana Camila Oliveira Souza, Adela Martin-Vicente, Brian M. Peters, Jarrod R. Fortwendel.

## References

1. Steinbach WJ, Marr KA, Anaissie EJ, Azie N, Quan S-P, Meier-Kriesche H-U, et al. Clinical epidemiology of 960 patients with invasive aspergillosis from the PATH Alliance registry. Journal of Infection. 2012; 65(5):453–64. https://doi.org/10.1016/j.jinf.2012.08.003 PMID: 22898389

2. Lo´pez-Medrano F, Ferna´ndez-Ruiz M, Silva JT, Carver PL, van Delden C, Merino E, et al. Clinical Presentation and Determinants of Mortality of Invasive Pulmonary Aspergillosis in Kidney Transplant Recipients: A Multinational Cohort Study. Am J Transplant. 2016; 16(11):3220–34. Epub 2016/10/28. https://doi.org/10.1111/ajt.13837 PMID: 27105907.

3. Koehler P, Hamprecht A, Bader O, Bekeredjian-Ding I, Buchheidt D, Doelken G, et al. Epidemiology of invasive aspergillosis and azole resistance in patients with acute leukaemia: the SEPIA Study. Int J Antimicrob Agents. 2017; 49(2):218–23. Epub 2016/12/19. https://doi.org/10.1016/j.ijantimicag.2016. 10.019 PMID: 27989379.

4. Maertens JA, Raad II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, et al. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. The Lancet. 2016; 387(10020):760–9. https://doi.org/10.1016/S0140-6736(15)01159-9 PMID: 26684607

5. Kim A, Nicolau DP, Kuti JL. Hospital costs and outcomes among intravenous antifungal therapies for patients with invasive aspergillosis in the United States. Mycoses. 2011; 54(5):e301–12. Epub 2010/06/ 19. https://doi.org/10.1111/j.1439-0507.2010.01903.x PMID: 20557463.

6. Latge´ J-P, Chamilos G. Aspergillus fumigatus and Aspergillosis in 2019. Clinical Microbiology Reviews. 2019; 33(1):e00140–18. https://doi.org/10.1128/CMR.00140-18 PMID: 31722890

7. Laniado-Laborı´n R, Cabrales-Vargas MN. Amphotericin B: side effects and toxicity. Rev Iberoam Micol. 2009; 26(4):223–7. Epub 2009/10/20. https://doi.org/10.1016/j.riam.2009.06.003 PMID: 19836985.

8. Patterson TF, Thompson GR III, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clinical Infectious Diseases. 2016; 63(4):e1–e60. https://doi.org/10.1093/cid/ ciw326 PMID: 27365388

9. Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med. 2007; 356(4):348–59. Epub 2007/01/26. https://doi.org/10.1056/NEJMoa061094 PMID: 17251531.

10. Pinney MF, Rosenberg AF, Hampp C, Schain D, Akindipe O, Baz M. Invasive fungal infections in lung transplant recipients not receiving routine systemic antifungal prophylaxis: 12-year experience at a university lung transplant center. Pharmacotherapy. 2011; 31(6):537–45. Epub 2011/09/20. https://doi. org/10.1592/phco.31.6.537 PMID: 21923437.

11. Ullmann AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, et al. Posaconazole or Fluconazole for Prophylaxis in Severe Graft-versus-Host Disease. New England Journal of Medicine. 2007; 356(4):335–47. https://doi.org/10.1056/NEJMoa061098 PMID: 17251530.

12. Burgel P-R, Baixench M-T, Amsellem M, Audureau E, Chapron J, Kanaan R, et al. High prevalence of azole-resistant Aspergillus fumigatus in adults with cystic fibrosis exposed to itraconazole. Antimicrobial agents and chemotherapy. 2012; 56(2):869–74. Epub 2011/11/28. https://doi.org/10.1128/AAC.0507711 PMID: 22123701.

13. Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D. Multiple-azole-resistant Aspergillus fumigatus osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. Med Mycol. 2009; 47(2):217–20. Epub 2008/12/ 23. https://doi.org/10.1080/13693780802545600 PMID: 19101840.

14. van Paassen J, Russcher A, In 't Veld-van Wingerden AW, Verweij PE, Kuijper EJ. Emerging aspergillosis by azole-resistant Aspergillus fumigatus at an intensive care unit in the Netherlands, 2010 to 2013. Euro Surveill. 2016; 21(30). Epub 2016/08/20. https://doi.org/10.2807/1560-7917.ES.2016.21.30. 30300 PMID: 27541498.

15. Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, et al. First Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in Aspergillus fumigatus Isolates in the United States. Journal of Clinical Microbiology. 2016; 54(1):168–71. https://doi.org/10.1128/JCM.02478-15 PMID: 26491179

16. Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? Clin Infect Dis. 2016; 62(3):362–8. Epub 2015/10/22. https://doi.org/10.1093/cid/civ885 PMID: 26486705; PubMed Central PMCID: PMC4706635.

17. Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. Azole Resistance in Aspergillus fumigatus Isolates from the ARTEMIS Global Surveillance Study Is Primarily Due to the TR/L98H Mutation in the cyp51A Gene. Antimicrobial Agents and Chemotherapy. 2011; 55(9):4465–8. https:// doi.org/10.1128/AAC.00185-11 PMC3165364. PMID: 21690285

18. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, et al. Azole antifungal resistance in Aspergillus fumigatus: 2008 and 2009. Journal of Antimicrobial Chemotherapy. 2010; 65 (10):2116–8. https://doi.org/10.1093/jac/ dkq279 PMID: 20729241

19. Susan JH, Dasa C, Michael JA, Ahmed A, Matthew CF, Alessandro CP, et al. Frequency and Evolution of Azole Resistance in Aspergillus fumigatus Associated with Treatment Failure. Emerging Infectious Disease journal. 2009; 15(7):1068. https://doi.org/10.320 1 /eid1507.090043 PMID: 19624922

20. Walker TA, Lockhart SR, Beekmann SE, Polgreen PM, Santibanez S, Mody RK, et al. Recognition of Azole-Resistant Aspergillosis by Physicians Specializing in Infectious Diseases, United States. Emerg Infect Dis. 2018; 24(1):111–3. Epub 2017/12/21. https://doi.org/10.3201/eid2401.170971 PMID: 29261092; PubMed Central PMCID: PMC57 49442.

21. Aruanno M, Glampedakis E, Lamoth F. Echinocandins for the Treatment of Invasive Aspergillosis: from Laboratory to Bedside. Antimicrob Agents Chemother. 2019; 63(8). Epub 2019/05/30. https://doi.org/ 10.1128/aac.00399-19 PMID: 31138565; PubMed Central PMCID: PMC6658788.

22. Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, Latge´ JP. Glucan synthase complex of Aspergillus fumigatus. Journal of bacteriology. 2001; 183(7):2273–9. https://doi.org/10.1128/ JB.183.7. 2273-2279.2001 PMID: 11244067.

23. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, et al. The Antifungal Echinocandin Caspofungin Acetate Kills Growing Cells of Aspergillus fumigatus In Vitro. Antimicrob Agents Chemother. 2002; 46(9):3001–12. https://doi.org/10.1128/AA C.46.9.3001-3012.2002 PMID: 12183260

24. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, et al. The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro. Antimicrobial agents and chemotherapy. 2002; 46(9):3001–12. https://doi.org/10.1128/AAC.4 6.9.3001-3012.2002 PMID: 12183260.

25. Wagener J, Loiko V. Recent Insights into the Paradoxical Effect of Echinocandins. J Fungi (Basel). 2017; 4(1):5. https://doi.org/10.3390/jof4010005 PMID: 29371498.

26. Lionakis MS, Lewis RE, Kontoyiannis DP. Breakthrough Invasive Mold Infections in the Hematology Patient: Current Concepts and Future Directions. Clin Infect Dis. 2018; 67(10):1621–30. Epub 2018/06/ 04. https://doi.org/10.1093/cid/ciy473 PMID: 29860307; PubMed Central PMCID: PMC6206100.

27. Gomes MZ, Jiang Y, Mulanovich VE, Lewis RE, Kontoyiannis DP. Effectiveness of primary anti-Aspergillus prophylaxis during remission induction chemotherapy of acute myeloid leukemia. Antimicrob Agents Chemother. 2014; 58(5):2775–80. Epub 2014/03/05. https://doi.org/10.1128/AAC.01527-13 PMID: 24590477; PubMed Central PMCID: PMC3993271.

28. Mattos EC, Palmisano G, Goldman GH. Phosphoproteomics of Aspergillus fumigatus Exposed to the Antifungal Drug Caspofungin. mSphere. 2020; 5(3):e00365–20. https://doi.org/10.1128/mSphere. 00365-20 PMID: 32461274

29. Mattos EC, Silva LP, Valero C, de Castro PA, dos Reis TF, Ribeiro LFC, et al. The Aspergillus fumigatus Phosphoproteome Reveals Roles of High-Osmolarity Glycerol Mitogen-Activated Protein Kinases in Promoting Cell Wall Damage and Caspofungin Tolerance. mBio. 2020; 11(1):e02962–19. https://doi. org/10.1128/mBio.02962-19 PMID: 32019798

30. Chelius C, Huso W, Reese S, Doan A, Lincoln S, Lawson K, et al. Dynamic Transcriptomic and Phosphoproteomic Analysis During Cell Wall Stress in Aspergillus nidulans. Molecular & Cellular Proteomics. 2020; 19(8):1310–29. https://doi.org/10.1074/mcp.RA119.001769 PMID: 32430394

31. Silva LP, Frawley D, Assis LJd, Tierney C, Fleming AB, Bayram O, et al. Putative Membrane Receptors Contribute to Activation and Efficient Signaling of Mitogen-Activated Protein Kinase Cascades during Adaptation of Aspergillus fumigatus to Different Stressors and Carbon Sources. mSphere. 2020; 5(5): e00818–20. https://doi.org/10.1128/mSphere.00818-20 PMID: 32938702

32. Manfiolli AO, Mattos EC, de Assis LJ, Silva LP, Ulaş M, Brown NA, et al. Aspergillus fumigatus High Osmolarity Glycerol Mitogen Activated Protein Kinases SakA and MpkC Physically Interact During Osmotic and Cell Wall Stresses. Frontiers in Microbiology. 2019; 10(918). https://doi.org/10.3389/ fmicb.2019.00918 PMID: 31134001

33. Juvvadi PR, Cole DC, Falloon K, Waitt G, Soderblom EJ, Moseley MA, et al. Kin1 kinase localizes at the hyphal septum and is dephosphorylated by calcineurin but is dispensable for septation and virulence in the human pathogen Aspergillus fumigatus. Biochemical and Biophysical Research Communications. 2018; 505(3):740–6. https://doi.org/10.1016/j.bbrc.2018.09.186 PMID: 30292408

34. Altwasser R, Baldin C, Weber J, Guthke R, Kniemeyer O, Brakhage AA, et al. Network Modeling Reveals Cross Talk of MAP Kinases during Adaptation to Caspofungin Stress in Aspergillus fumigatus. PLOS ONE. 2015; 10(9):e0136932. https://doi.org/10.1371/journal.pone.0136932 PMID: 26356475

35. Valiante V, Jain R, Heinekamp T, Brakhage AA. The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in Aspergillus fumigatus .Fungal Genetics and Biology. 2009; 46(12):909–18. https://doi.org/10.1016/j.fgb.2009.08.005 PMID: 19715768

36. Steinbach WJ, Cramer RA Jr., Perfect BZ, Henn C, Nielsen K, Heitman J, et al. Calcineurin Inhibition or Mutation Enhances Cell Wall Inhibitors against Aspergillus fumigatus. Antimicrob Agents Chemother. 2007; 51(8):2979–81. https://doi.org/10.1128/AAC.01394-06 PMID: 17502415

37. Simanis V. The mitotic exit and septation initiation networks. Journal of Cell Science. 2003; 116 (21):4261–2. https://doi.org/10.1242/jcs.00777 PMID: 14514881

38. Bardin AJ, Amon A. MEN and SIN: what's the difference? Nature Reviews Molecular Cell Biology. 2001; 2(11):815–26. https://doi.org/10.1038/35099020 PMID: 11715048

39. De Souza CP, Hashmi SB, Osmani AH, Andrews P, Ringelberg CS, Dunlap JC, et al. Functional Analysis of the Aspergillus nidulans Kinome. PLOS ONE. 2013; 8(3):e58008. https://doi.org/10.1371/journal. pone.0058008 PMID: 23505451

40. Al Abdallah Q, Ge W, Fortwendel JR. A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates. mSphere. 2017; 2(6). https://doi.org/10.1128/mSphere.00446-17 PMID: 29202040

41. Al Abdallah Q, Souza ACO, Martin-Vicente A, Ge W, Fortwendel JR. Whole-genome sequencing reveals highly specific gene targeting by in vitro assembled Cas9-ribonucleoprotein complexes in Aspergillus fumigatus. Fungal Biol Biotechnol. 2018; 5:11. Epub 2018/07/12. https://doi.org/10.1186/ s40694-018-0057-2 PMID: 29992034; PubMed Central PMCID: PMC5987418.

42. Fuller KK, Richie DL, Feng X, Krishnan K, Stephens TJ, Wikenheiser-Brokamp KA, et al. Divergent Protein Kinase A isoforms co-ordinately regulate conidial germination,

carbohydrate metabolism and virulence in Aspergillus fumigatus. Molecular Microbiology. 2011; 79(4):1045–62. https://doi.org/10.1111/j. 1365-2958.2010.07509.x PMID: 21210869

43. Vargas-Muñiz JM, Renshaw H, Richards AD, Waitt G, Soderblom EJ, Moseley MA, et al. Dephosphorylation of the Core Septin, AspB, in a Protein Phosphatase 2A-Dependent Manner Impacts Its Localization and Function in the Fungal Pathogen Aspergillus fumigatus. Frontiers in Microbiology. 2016; 7(997). https://doi.org/10.3389/fmicb.2016.00997 PMID: 27446037

44. Du C, Sarfati J, Latge JP, Calderone R. The role of the sakA (Hog1) and tcsB (sln1) genes in the oxidant adaptation of Aspergillus fumigatus. Medical Mycology. 2006; 44(3):211–8. https://doi.org/10.1080/ 13693780500338886 PMID: 16702099

45. Sasse C, Bignell EM, Hasenberg M, Haynes K, Gunzer M, Braus GH, et al. Basal expression of the Aspergillus fumigatus transcriptional activator CpcA is sufficient to support pulmonary aspergillosis. Fungal Genetics and Biology. 2008; 45(5):693–704. https://doi.org/10.1016/j.fgb.2007.12.008 PMID: 18249572

46. Long N, Zeng L, Qiao S, Li L, Zhong G. Aspergillus fumigatus Afssn3-Afssn8 Pair Reverse Regulates Azole Resistance by Conferring Extracellular Polysaccharide, Sphingolipid Pathway Intermediates, and Efflux Pumps to Biofilm. Antimicrobial Agents and Chemotherapy. 2018; 62(3):e01978–17. https://doi. org/10.1128/AAC.01978-17 PMID: 29311083

47. Frawley D, Stroe MC, Oakley BR, Heinekamp T, Straßburger M, Fleming AB, et al. The Pheromone Module SteC-MkkB-MpkB-SteD-HamE Regulates Development, Stress Responses and Secondary Metabolism in Aspergillus fumigatus. Frontiers in Microbiology. 2020; 11(811). https://doi.org/10.3389/ fmicb.2020.00811 PMID: 32457716

48. Ram AFJ, Klis FM. Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. Nature Protocols. 2006; 1(5):2253–6. https://doi.org/10.1038/nprot.2006. 397 PMID: 17406464

49. CLSI. Reference Method for broth microdilution antifungal susceptibility testing of filamentous fungi. 3rd. ed. CLSI standard M38 Wayne, PA: Clinical and Laboratory Standards Institute. 2017.

50. Shwab EK, Juvvadi PR, Waitt G, Soderblom EJ, Moseley MA, Nicely NI, et al. A Novel Phosphoregulatory Switch Controls the Activity and Function of the Major Catalytic Subunit of Protein Kinase A in Aspergillus fumigatus. mBio. 2017; 8(1):e02319–16. https://doi.org/10.1128/mBio.02319-16 PMID: 28174315

51. Dichtl K, Helmschrott C, Dirr F, Wagener J. Deciphering cell wall integrity signalling in Aspergillus fumigatus: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol Microbiol. 2012; 83(3):506–19. Epub 2012/01/10. https://doi.org/10.1111/j.1365-2958. 2011.07946.x PMID: 22220813.

52. Heilig Y, Schmitt K, Seiler S. Phospho-Regulation of the Neurospora crassa Septation Initiation Network. PLOS ONE. 2013; 8(10):e79464. https://doi.org/10.1371/journal.pone.0079464 PMID: 24205386

53. Kim J-M, Zeng CJT, Nayak T, Shao R, Huang A-C, Oakley BR, et al. Timely Septation Requires SNADdependent Spindle Pole Body Localization of the Septation Initiation Network Components in the Filamentous Fungus Aspergillus nidulans. Molecular Biology of the Cell. 2009; 20(12):2874–84. https://doi. org/10.1091/mbc.e08-12-1177 PMID: 19386763.

54. Dichtl K, Samantaray S, Aimanianda V, Zhu Z, Prevost M-C, Latge´ JP, et al. Aspergillus fumigatus devoid of cell wall B-1,3-glucan is viable, massively shed galactomannan and is killed by septum formation inhibitors. Mol Microbiol. 2014; 95(3):458–71. https://doi.org/10.1111/mmi.12877 PMID: 25425041

55. Ingham CJ, Schneeberger PM. Microcolony Imaging of Aspergillus fumigatus Treated with Echinocandins Reveals Both Fungistatic and Fungicidal Activities. PLOS ONE. 2012; 7(4):e35478. https://doi.org/ 10.1371/journal.pone.0035478 PMID: 22536390

56. Souza ACO, Al Abdallah Q, DeJarnette K, Martin-Vicente A, Nywening AV, DeJarnette C, et al. Differential requirements of protein geranylgeranylation for the virulence of human pathogenic fungi. Virulence. 2019; 10(1):511–26. https://doi.org/10.1080/21505594.2019.1620063 PMID: 31131706

57. Norton TS, Al Abdallah Q, Hill AM, Lovingood RV, Fortwendel JR. The Aspergillus fumigatus farnesyltransferase β-subunit, RamA, mediates growth, virulence, and antifungal susceptibility. Virulence. 2017:1–16. https://doi.org/10.1080/21505594.2017.1328343 PMID: 28489963

58. Briard B, Fontaine T, Samir P, Place DE, Muszkieta L, Malireddi RKS, et al. Galactosaminogalactan activates the inflammasome to provide host protection. Nature. 2020; 588(7839):688–92. https://doi. org/10.1038/s41586-020-2996-z PMID: 33268895

59. Saïd-Sadier N, Padilla E, Langsley G, Ojcius DM. Aspergillus fumigatus Stimulates the NLRP3 Inflammasome through a Pathway Requiring ROS Production and the Syk Tyrosine Kinase. PLOS ONE. 2010; 5(4):e10008. https://doi.org/10.1371/journal.pone.0010008 PMID: 20368800

60. Malik A, Kanneganti T-D. Inflammasome activation and assembly at a glance. Journal of Cell Science. 2017; 130(23):3955–63. https://doi.org/10.1242/jcs.207365 PMID: 29196474

61. Wang J, Hu H, Wang S, Shi J, Chen S, Wei H, et al. The important role of actinin-like protein (AcnA) in cytokinesis and apical dominance of hyphal cells in Aspergillus nidulans. Microbiology. 2009; 155 (8):2714–25. https://doi.org/10.1099/mic.0.029215-0.

62. Li Y, Christensen JR, Homa KE, Hocky GM, Fok A, Sees JA, et al. The F-actin bundler α-actinin Ain1 is tailored for ring assembly and constriction during cytokinesis in fission yeast. Molecular Biology of the Cell. 2016; 27(11):1821–33. https://doi.org/10.1091/mbc.E16-01-0010 PMID: 27075176.

63. Meitinger F, Palani S. Actomyosin ring driven cytokinesis in budding yeast. Seminars in Cell & Developmental Biology. 2016; 53:19–27. https://doi.org/10.1016/j.semcdb.2016.01.043.

64. Stevens RC, Davis TN. Mlc1p is a light chain for the unconventional myosin Myo2p in Saccharomyces cerevisiae. J Cell Biol. 1998; 142(3):711–22. https://doi.org/10.1083/jcb.142.3.711 PMID: 9700160

65. McCollum D, Balasubramanian MK, Pelcher LE, Hemmingsen SM, Gould KL. Schizosaccharomyces pombe cdc4+ gene encodes a novel EF-hand protein essential for cytokinesis. Journal of Cell Biology. 1995; 130(3):651–60. https://doi.org/10.1083/jcb.130.3.651 PMID: 7622565

66. Krapp A, Simanis V. An overview of the fission yeast septation initiation network (SIN). Biochem Soc Trans. 2008; 36(Pt 3):411–5. Epub 2008/05/17. https://doi.org/10.1042/BST0360411 PMID: 18481970.

67. Samantaray S, Neubauer M, Helmschrott C, Wagener J. Role of the guanine nucleotide exchange factor Rom2 in cell wall integrity maintenance of Aspergillus fumigatus. Eukaryotic cell. 2013; 12(2):288– 98. Epub 2012/12/21. https://doi.org/10.1128/EC.00246-12 PMID: 23264643.

68. Si H, Justa-Schuch D, Seiler S, Harris SD. Regulation of septum formation by the Bud3-Rho4 GTPase module in Aspergillus nidulans. Genetics. 2010; 185(1):165–76. Epub 2010/02/22. https://doi.org/10. 1534/genetics.110.114165 PMID: 20176976.

69. Laporte D, Zhao R, Wu J-Q. Mechanisms of contractile-ring assembly in fission yeast and beyond. Seminars in Cell & Developmental Biology. 2010; 21(9):892–8. https://doi.org/10.1016/j.semcdb.2010. 08.004 PMID: 20708088

70. Moreno-Vela´squez SD, Seidel C, Juvvadi PR, Steinbach WJ, Read ND. Caspofungin-Mediated Growth Inhibition and Paradoxical Growth in Aspergillus fumigatus Involve Fungicidal Hyphal Tip Lysis Coupled with Regenerative Intrahyphal Growth and Dynamic Changes in β-1,3-Glucan Synthase Localization. Antimicrobial Agents and Chemotherapy. 2017; 61(10):e00710–17. https://doi.org/10.1128/AAC. 00710-17 PMID: 28760907

71. Markham P. Occlusions of septal pores in filamentous fungi. Mycological Research. 1994; 98 (10):1089–106. https://doi.org/10.1016/S0953-7562(09)80195-0.

72. Steinberg G, Harmer NJ, Schuster M, Kilaru S. Woronin body-based sealing of septal pores. Fungal Genetics and Biology. 2017; 109:53–5. https://doi.org/10.1016/j.fgb.2017.10.006 PMID: 29107012

73. Munro CA, Winter K, Buchan A, Henry K, Becker JM, Brown AJP, et al. Chs1 of Candida albicans is an essential chitin synthase required for synthesis of the septum and for cell integrity. Molecular Microbiology. 2001; 39(5):1414–26. https://doi.org/10.1046/j.1365-2958.2001.02347.x PMID: 11251855

74. Walker LA, Lenardon MD, Preechasuth K, Munro CA, Gow NAR. Cell wall stress induces alternative fungal cytokinesis and septation strategies. Journal of Cell Science. 2013; 126(12):2668–77. https://doi. org/10.1242/jcs.118885 PMID: 23606739

75. Hohl TM. Immune responses to invasive aspergillosis: new understanding and therapeutic opportunities. Curr Opin Infect Dis. 2017; 30(4):364–71. https://doi.org/10.1097/QCO.0000000000000381 PMID: 28509673.

76. Freitag J, Lanver D, Bo¨hmer C, Schink KO, Bo¨lker M, Sandrock B. Septation of Infectious Hyphae Is Critical for Appressoria Formation and Virulence in the Smut Fungus Ustilago Maydis. PLOS Pathogens. 2011; 7(5):e1002044. https://doi.org/10.1371/journal.ppat.1002044 PMID: 21625538

77. Fukuda S, Yamamoto R, Yanagisawa N, Takaya N, Sato Y, Riquelme M, et al. Trade-off between Plasticity and Velocity in Mycelial Growth. mBio. 2021; 12(2):e03196–20. https://doi.org/10.1128/mBio. 03196-20 PMID: 33727355

78. Fortwendel JR, Zhao W, Bhabhra R, Park S, Perlin DS, Askew DS, et al. A Fungus-Specific Ras Homolog Contributes to the Hyphal Growth and Virulence of Aspergillus fumigatus. Eukaryotic Cell. 2005; 4 (12):1982–9. https://doi.org/10.1128/EC.4.12.1982-1989.2005 PMID: 16339716

79. He Z-M, Price MS, Obrian GR, Georgianna DR, Payne GA. Improved protocols for functional analysis in the pathogenic fungus Aspergillus flavus. BMC Microbiology. 2007; 7(1):104. https://doi.org/10.1186/ 1471-2180-7-104 PMID: 18039373

80. Fortwendel JR, Juvvadi PR, Rogg LE, Asfaw YG, Burns KA, Randell SH, et al. Plasma membrane localization is required for RasA-mediated polarized morphogenesis and virulence of Aspergillus fumigatus. Eukaryotic Cell. 2012; 11(8):966–77. https://doi.org/10.1128/EC.00091-12 PMID: 22562470

81. Espinel-Ingroff A, Rezusta A. E-Test Method for Testing Susceptibilities of Aspergillus spp. to the New Triazoles Voriconazole and Posaconazole and to Established Antifungal Agents: Comparison with NCCLS Broth Microdilution Method. Journal of Clinical Microbiology. 2002; 40(6):2101–7. https://doi. org/10.1128/JCM.40.6.2101-2107.2002 PMID: 12037072

82. Fortwendel JR, Fuller KK, Stephens TJ, Bacon WC, Askew DS, Rhodes JC. Aspergillus fumigatus RasA regulates asexual development and cell wall integrity. Euk Cell. 2008; 7(9):1530–9. https://doi. org/10.1128/EC.00080-08 PMID: 18606827

83. Lewis RE, Albert ND, Kontoyiannis DP. Comparison of the dose-dependent activity and paradoxical effect of caspofungin and micafungin in a neutropenic murine model of invasive pulmonary aspergillosis. Journal of Antimicrobial Chemotherapy. 2008; 61(5):1140–4. https://doi.org/10.1093/jac/dkn069 PMID: 18305201

84. Caffrey AK, Lehmann MM, Zickovich JM, Espinosa V, Shepardson KM, Watschke CP, et al. IL-1α Signaling Is Critical for Leukocyte Recruitment after Pulmonary Aspergillus fumigatus Challenge. PLOS Pathogens. 2015; 11(1):e1004625. https://doi.org/10.1371/journal.ppat.1004625 PMID: 25629406

85. Bowman JC, Abruzzo GK, Anderson JW, Flattery AM, Gill CJ, Pikounis VB, et al. Quantitative PCR Assay To Measure Aspergillus fumigatus Burden in a Murine Model of Disseminated Aspergillosis: Demonstration of Efficacy of Caspofungin Acetate. Antimicrobial Agents and Chemotherapy. 2001; 45 (12):3474–81. https://doi.org/10.1128/AAC.45.12.3474-3481.2001 PMID: 11709327

86. Willems HME, Lowes DJ, Barker KS, Palmer GE, Peters BM. Comparative Analysis of the Capacity of the Candida Species To Elicit Vaginal Immunopathology. Infection and Immunity. 2018; 86(12): e00527–18. https://doi.org/10.1128/IAI.00527-18 PMID: 30249743

# Appendix C

# Chapter 4 Article

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Ctrl/Alt+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command+Right Arrow, respectively. See the Preface for further details.

## C.1 Introduction

Prepared for publication author manuscript reproduced with permission from all contributing authors: A. V. Nywening, H. Thorn, J. Xie, A. Martin-Vicente, X. Guruceaga Sierra, W. Ge, J. G. Gibbons, and J. R. Fortwendel. (2024). "Loss of the SAC components SldA kinase or SldB or *in vitro* adaptation to triazole produces aneuploidy associated with triazole heteroresistance in *Aspergillus fumigatus*".

This manuscript is included as an appendix as it contains the bulk of the methods, results, conclusions and discussion of my graduate student research project. This article was in the late stages of preparation for submission to a journal for publication while this ETD was under construction. Multiple authors contributed to the manuscript's content and construction. Consequently, to remain compliant with the CGHS ETD guidelines, the article has been reproduced from the original manuscript and placed in this appendix. This work is briefly summarized and referenced in Chapter 4 of this ETD.

## C.2 Article

# Loss of the SAC components SldA kinase or SldB or *in vitro* adaptation to triazole produces aneuploidy associated with triazole heteroresistance in *Aspergillus fumigatus*

Ashley V. Nywening [1,2] (ORCID ID is 0000-0002-4969-4192), Harrison Thorn [1,3] (ORCID ID is 0009-0003-2546-8400), Jinhong Xie [1,3] (ORCID ID is 0000-0002-8994-6606), Adela Martin-Vicente [1] (ORCID ID is 0000-0003-0446-8906), Xabier Guruceaga Sierra [1] (ORCID ID is 0000-0003-3258-2482), Wenbo Ge, John G. Gibbons [4], and Jarrod R. Fortwendel [1] (ORCID ID is 0000-0003-2301-4272)

[1]Department of Clinical Pharmacy and Translational Science, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, USA, [2]Integrated Program in Biomedical Sciences, College of Graduate Health Sciences, University of Tennessee Health Science Center, Memphis, Tennessee, USA, [3]Graduate Program in Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, USA, [4] Department of Food Science, College of Natural Sciences, University of Massachusetts Amherst, Amherst, MA, USA

### Contact Address:

Jarrod R. Fortwendel, PhD University of Tennessee Health Science Center College of Pharmacy 881 Madison Avenue, Rm 343 Memphis, TN 38163

### Key Words

Aspergillus, triazole resistance, antifungal susceptibility, antifungal tolerance, ergosterol biosynthesis, spindle assembly checkpoint, aneuploidy

### Abstract

*Aspergillus fumigatus* represents the chief causative agent of human invasive filamentous fungal infections. Triazoles, the primary therapeutic options to combat invasive aspergillosis (IA), target the fungal biosynthesis of ergosterol, a vital component of the fungal cell membrane. Unfortunately, resistance to this class of medical therapeutic has arisen globally and now threatens the future usefulness of these compounds for antifungal treatment. Infection with *A. fumigatus* that has acquired triazole resistance increases an already high associated mortality rate and reduces the limited arsenal of therapeutic options to combat IA. How this specific fungal pathogen obtains resistance remains poorly understood. In this study, we show that loss of the previously uncharacterized *A. fumigatus* Spindle Assembly Checkpoint components, SldA or SldB, resulted in a tolerance/heteroresistance phenotype

to multiple mold active medical triazoles as well as to compounds inhibiting ergosterol biosynthesis at points upstream of the triazole target, Cyp51A. Consistent with conserved roles in mitotic fidelity, loss of either component resulted in production of aneuploid conidia. Repetitive exposure to voriconazole through laboratory experimental adaptation resulted in increases in both MIC and sub populations of these aneuploid spores for both *sldA* mutant and *wild type* lineages. In the absence of triazole, most adapted lineages of both strains showed partial to total reversion of MIC with simultaneous reduced production of aneuploid conidia. WGS analysis for indications of copy number variation revealed four of six total adapted lineages selected for a specific duplication in chromosome two. Together, our findings reveal that *A. fumigatus* develops transient mechanisms of adaptation and aneuploidy as a natural response to triazole stress and a specific chromosome two duplication may provide a fitness benefit contributing to triazole adaptation. Moreover, our work suggests dysregulation of the SAC as a potential mechanism for obtaining such aneuploidy in response ergosterol biosynthesis inhibition. This knowledge helps to inform our understanding of how *A. fumigatus* survives and adapts to the triazole class of medical antifungals.

## Importance

The rising threat of antifungal resistance in *Aspergillus fumigatus*, which remains the chief among the filamentous fungal species regarding invasive human infection, is an increasingly relevant concern to public health worldwide. The mode and mechanism of triazole resistance acquisition in particular remains an understudied issue for this opportunistic pathogen. This work reveals the importance of a functional Spindle Assembly Checkpoint, and more specifically reliability in key Mitotic Checkpoint Complex components, in maintaining antifungal-susceptible status. Moreover, we demonstrate that transient changes in gross genome size constituting aneuploidy may underlie similarly transient resistance acquisition in this species. This information will inform our understanding of acquisition and stability of triazole antifungal resistance within both clinical and environmental isolates of *A. fumigatus*.

## Introduction

*Aspergillus fumigatus* is the leading cause globally of invasive filamentous fungal infections within susceptible human populations (Seyedmousavi, Guillot et al. 2015, Badali, Canete-Gibas et al. 2022). A saprobic organism, *A. fumigatus* grows on a variety of substrates (De Vroey 1979, Latge and Chamilos 2019, Horta, Steenwyk et al. 2022). The species produces asexual reproductive structures called conidia in abundance which are designed primarily to be easily spread by air currents (Dagenais and Keller 2009). The average individual will breathe in about 300 spores every day from this ubiquitous species (Denning, Park et al. 2011, Escobar, Ordonez et al. 2016, Latge and Chamilos 2019). Immunocompetent hosts can clear the spores through phagocytosis by resident innate immune cells (Dagenais

and Keller 2009). However, conidia which are not cleared may germinate and solidify an infection. Invasive aspergillosis (IA) represents the most severe form of infection, producing a high rate of mortality ranging from 30% to approaching 90% for some patient populations (Dagenais and Keller 2009, Denning, Park et al. 2011, Mayr and Lass-Florl 2011, Maertens, Raad et al. 2016, Benedict, Jackson et al. 2019). Cases of IA have increased worldwide in recent history likely due to increases in susceptible populations, such as patients with neutropenia, hematological malignancies, transplant recipients, those on steroids or other immune suppressing medications and with underlying primary disease states such as COPD, cystic fibrosis, tuberculosis, sarcoidosis, or tissue damage (Patterson, Kirkpatrick et al. 2000, Dagenais and Keller 2009, Kosmidis and Denning 2015, Cadena, Thompson et al. 2021, Badali, Canete-Gibas et al. 2022). Cases of IA also occur in patients with viral respiratory tract infections, including the flu and COVID-19 and can occur even in healthy individuals if exposed to a sufficiently high inoculum of fungal spores (Kosmidis and Denning 2015, Koehler, Bassetti et al. 2021, Badali, Canete-Gibas et al. 2022, Mead, de Castro et al. 2023). Aspergilloses are associated with approximately 14,000 hospitalizations and an annual healthcare burden of between 600 million to 1.2 billion dollars in the United states alone (Benedict, Jackson et al. 2019, Zilberberg, Harrington et al. 2019).

Of the limited number of approved therapeutics, the triazoles remain the primary option recommended to combat Aspergillus infections, with voriconazole representing the primary drug of choice for invasive disease (Patterson, Thompson et al. 2016). Only two other classes of antifungals are currently available as alternative or combinatorial treatments. From the polyene class, only the drug Amphotericin B is approved for treating invasive disease, but is often avoided due to known nephrotoxicity and other intravenous infusion-related drug toxicity resulting from its use (Laniado-Laborín and Cabrales-Vargas 2009). The only other antifungal class available are the echinocandins. However, while tolerated much better than Amphotericin B, these compounds have fungistatic rather than fungicidal activity against species of *Aspergilli* and failure of echinocandin prophylaxis occurs frequently with some reports of breakthrough infection rates as high as 28% (Bowman, Hicks et al. 2002, Lionakis, Lewis et al. 2018, Aruanno, Glampedakis et al. 2019). The triazole antifungals however are fungicidal against *A. fumigatus*, can be administered orally rather than intravenously, and can be given for months with lower treatment-associated adverse events (Ullmann, Lipton et al. 2007, Patterson, Thompson et al. 2016, Rybak, Fortwendel et al. 2019). In fact, extended triazole therapy, either for prophylaxis to prevent infection or in response to chronic involvements, occurs often (Cornely, Maertens et al. 2007, Ullmann, Lipton et al. 2007). Unfortunately, extended periods of patient treatment with triazole antifungals consequently increases the risk of development of triazole resistance (Hodiamont, Dolman et al. 2009, Burgel, Baixench et al. 2012).

The cost of IA to public health is increased by the involvement of a triazole resistant isolate. Whether acquired from an environmental source or developed within a patient's own system as a consequence of therapy, infection with *A. fumigatus* which has adapted to

be resistant to triazole therapy increases the expected risk of patient mortality (Qiao, Liu et al. 2008, Bradley, Le-Mahajan et al. 2022). Since the first recorded incidence of resistant infection in 1997, encounters with triazole resistant *A. fumigatus* have increased (Rivero-Menendez, Alastruey-Izquierdo et al. 2016, van Paassen, Russcher et al. 2016). Recently, *A. fumigatus* was one of only four species placed in the Critical category, the highest priority tier of the list, alongside *Cryptococcus neoformans*, *Candida auris*, and *Candida albicans* on the WHO fungal priority pathogens list (WHO FPPL), a report on the 19 fungi which represent the greatest threats to public health (Parums 2022, Fisher and Denning 2023).

Triazole resistance in *A. fumigatus* remains poorly understood. Of the mechanisms which are currently known or suspected to contribute to triazole resistance in *A. fumigatus*, the majority involve alterations to the expression of gene transcript for the enzymes targeted by these antifungals, (encoded by *cyp51A* and *cyp51B* in this species), or a mutation which affects the ability of the compounds to associate with the enzyme, both of which reduce the effectiveness of the drug (Lazzarini, Esposto et al. 2016, Badali, Canete-Gibas et al. 2022). Alterations which impact Cyp51A are predominantly associated with resistance, whereas the influence of transcriptional or sequential Cyp51B changes is less certain (Lazzarini, Esposto et al. 2016, Rybak, Fortwendel et al. 2019). Additional mechanisms which are implicated in *A. fumigatus* azole resistance include overexpression of drug efflux pumps that actively remove the drug from fungal cells, as well as mutations in the HMG-CoA Reductase ortholog, Hmg1, or in the CCAAT-binding transcription factor complex subunit, HapE (Camps, Dutilh et al. 2012, Gsaller, Hortschansky et al. 2016, Dudakova, Spiess et al. 2017, Rybak, Ge et al. 2019). However, there are many resistant *A. fumigatus* isolates for which no known mechanism is present (Resendiz Sharpe, Lagrou et al. 2018, Bowyer, Bromley et al. 2020).

In recent years, aneuploidy, defined as changes in the nuclear content of chromosomes or large portions of chromosomes, has been identified as an established source for triazole adaptation and acquisition of triazole resistance in *Candida* and *Cryptococcus* species (Cahill, Lengauer et al. 1998, Selmecki, Forche et al. 2006, Selmecki, Dulmage et al. 2009, Altamirano, Fang et al. 2017, Chang, Khanal Lamichhane et al. 2018, Brimacombe, Burke et al. 2019). For example, isolates which have increased representation of the left arm of chromosome five, which is known to contain the genes encoding the target of triazoles in this species, ERG11, and the transcription factor TAC1 which upregulates drug efflux pump gene expression, can provide resistance to fluconazole (Selmecki, Forche et al. 2006, Selmecki, Gerami-Nejad et al. 2008). Such studies reveal aneuploidy can provide a means for resistance to triazole antifungals in pathogenic fungi. Aneuploidy has recently been associated with triazole adaptations in *Aspergillus fumigatus* and *Aspergillus flavus*, the two most common causative agents of human invasive mold infections, indicating this may be a mechanism that contributes to triazole resistance development in *Aspergillus* species as well (Barda, Sadhasivam et al. 2023, Khateb, Gago et al. 2023). Both of the aforementioned studies detected several chromosomal aneuploidies occurring in triazole-adapted or

triazole-resistant isolates, in each case a duplication affecting chromosome three in one *A. flavus* lineage or chromosome eight in another (Barda, Sadhasivam et al. 2023), or involving chromosome one, three, and four in isolates from one patient with *A. fumigatus* chronic pulmonary aspergillosis (CPA), chromosome two in another patient with *A. fumigatus* CPA, or chromosome five in a single isolate from a third patient with an *A. fumigatus* cerebral aspergilloma (Khateb, Gago et al. 2023). However, information is lacking and much remains uncertain concerning the potential contribution of aneuploidies to triazole adaptation in *A. fumigatus*, including whether strains that are predisposed to aneuploidy as a result of chromosome instability automatically exhibit reduced phenotypic susceptibility to triazoles, whether aneuploidy occurs often or rarely in independent lineages during triazole adaptation, whether aneuploidy in an adapted strain is stable or dissipates when triazole stress is removed, and especially concerning which specific aneuploidies are repeatedly selected for during the process of triazole adaptation, as no chromosomal abnormality was repeated among any of only three triazole resistant *A. fumigatus* patient isolates or two *A. flavus* lineages reported to possess chromosomal aneuploidy at the present. Moreover, the means by which pathogenic fungi acquire aneuploidy at an apparently increased rate in the context of triazole stress has remained largely uncertain and few explanations have been proposed (Selmecki, Dulmage et al. 2009, Todd, Forche et al. 2017). Failure of cell cycle checkpoints controlling chromosome sorting during mitosis would seem a logical origin for chromosomal abnormality. Unfortunately, as with much of the *A. fumigatus* genome, many genes which are predicted to regulate processes like drug stress responses or maintenance of genome stability remain yet uncharacterized and our comprehension of intracellular processes that might impact triazole susceptibility remains limited.

Protein kinases are known to play diverse roles in eukaryotic cellular processes, including responses to antifungal stress, via reversible phosphorylation events (Cowen and Steinbach 2008, Chelius, Huso et al. 2020, Mattos, Palmisano et al. 2020, Mattos, Silva et al. 2020). Here, we screened a library of *A. fumigatus* protein kinase disruption mutants constructed by our lab in a previous study to identify novel signaling events important for response to triazole stress (Souza, Martin-Vicente et al. 2021). Out of 118 mutant strains, only loss of two genes predicted to encode *A. fumigatus* protein kinases altered voriconazole susceptibility by at least four-fold. One of these predicted kinase-encoding genes encodes an ortholog of the Spindle Assembly Checkpoint (SAC) checkpoint kinase, SldA, of the model organism *Aspergillus nidulans*. Our findings show that deletion of the SldA or deletion of one of its binding partners in the mitotic checkpoint complex, SldB, results in production of heteroresistant progeny exhibiting decreased susceptibility to compounds that inhibit ergosterol biosynthesis. Loss of these SAC components also resulted in production of subpopulations of genomically abnormal conidia which generally exhibit increased conidial DNA content, suggesting the mutant is predisposed to chromosome missegregation due to deficient SAC, which is consistent with loss of kinase ortholog function in other species (Bernard, Hardwick et al. 1998, Brimacombe, Burke et al. 2019). While loss of SldA did not appear to increase the adaptability of *A. fumigatus* to triazole stress during laboratory *in*

*vitro* adaptation to voriconazole, we discovered that repeated exposure to triazoles resulted in transient increases in minimum inhibitory concentrations that were associated with similarly transient abnormalities in mean conidial DNA content for both *sldA* deletion and *wild type A. fumigatus*. Finally, *in vitro* adaptation to voriconazole drove the acquisition and selection of a specific chromosome two duplication in the majority of both *wild type* and *sldA* deletion lineages. Taken together, our results indicate that loss of the *A. fumigatus* SAC components SldA or SldB results in defects in chromosome segregation and heteroresistance to both triazole antifungals and other EBI compounds. Moreover, we show that chromosome missegregation resulting in aneuploidy occurs naturally in *wild type A. fumigatus* in response to triazole stress and we propose that directed SAC failure represents a likely mechanism whereby this occurs.

## Results

### Identification of protein kinases regulating triazole susceptibility in *A. fumigatus*

Previous studies have revealed that protein kinases mediate the susceptibility of *A. fumigatus* to the echinocandin class of antifungals (**Figure B.5**)(Souza, Martin-Vicente et al. 2021). To identify molecular mechanisms underpinning triazole stress responses, we screened an *A. fumigatus* putative kinase gene disruption mutant library generated previously in our lab for alterations in voriconazole susceptibility (**Figure B.1**) (Souza, Martin-Vicente et al. 2021). All 118 protein kinase gene disruption mutants were analyzed by CLSI M38-A2 broth microdilution MIC assay (Souza, Martin-Vicente et al. 2021), revealing only two putative kinase gene disruption mutants which altered voriconazole MIC by at least four-fold. Surprisingly, both of these mutant strains exhibited a higher MIC than the parental strain, indicating a reduction in susceptibility to the triazole (**Figure C.1**). One of the identified mutants possessed a disruptive mutation in a previously characterized gene encoding a putative *A. fumigatus* cyclin-dependent kinase, Ssn3. Loss of ssn3 has been shown to decrease triazole susceptibility in *A. fumigatus* (Long, Zeng et al. 2018), validating our MIC assay results. The second disruption mutant identified in our MIC assays possessed a disruption in a previously uncharacterized *A. fumigatus* gene predicted to encode the singular copy of the Spindle Assembly Checkpoint (SAC) kinase, SldA. The predicted *A. fumigatus* SldA protein shares significant sequence similarity to the predicted SAC kinase orthologs from other fungal species, including the single ortholog in *A. nidulans*, SldA (63.04% identity), the *C. albicans* kinase BUB1 (30.54% identity), and the two orthologous proteins in *S. cerevisiae*, BUB1 (29.66% identity) and MAD3 (27.81% identity) (**Supplemental Figure C.15B**) (Altschul, Gish et al. 1990, Basenko, Pulman et al. 2018). While many eukaryotes like *S. cerevisiae* encode two related paralogous orthologs of this kinase, (often with some domains and functions for the SAC shared and others distributed between the two proteins), other species like *A. nidulans* possess one singular protein which must accomplish multiple activities required to ensure correct chromosome separation and segregation during mitosis (Bolanos-Garcia and Blundell 2011, Suijkerbuijk, van Dam et al.

2012, De Souza, Hashmi et al. 2013, Edgerton, Paolillo et al. 2015). These include functions for regulating various components of the kinetochore, including recruiting, associating with as part of the mitotic checkpoint complex (MCC), and regulating components which directly participate in the SAC pathway (**Figure C.3**) (Jin, Bokros et al. 2017, Singh, Pesenti et al. 2021, Carvalhal, Bader et al. 2022, Roy, Han et al. 2022). Based on homology to characterized orthologs, the singular *A. fumigatus* SldA kinase appears to possess all required domains for SAC pathway function (**Supplemental Figure C.15A**). The two (KEN) boxes in *A. fumigatus* SldA, (the site of association with Cdc20 in orthologs), are predicted to be located at amino acids K17–N19 and at K300-N302 within the protein sequence of CEA10/A1163 SldA (Bolanos-Garcia and Blundell 2011, Basenko, Pulman et al. 2018). The BUB1 Homology Region 1/ tetratricopeptide repeat motif (TPR), (the site of association with kinetochore component Knl1/BLINKIN), is predicted to be located from A59-I185 (Elowe 2011, Basenko, Pulman et al. 2018). The BUB1 Homology Region 2/GLEBS motif, (the site of association with SldB/BUB3), is predicted to be located from E324-R403 in the protein sequence, with the actual site of association with SldB at amino acids E388 and E389 (Bolanos-Garcia and Blundell 2011, Basenko, Pulman et al. 2018). The BUB1 Homology Region 3/S/T kinase domain is predicted to be located at amino acids T923-T1113, with the actual kinase active site at amino acids I997-I1009 (Bolanos-Garcia and Blundell 2011, Basenko, Pulman et al. 2018).

**The SAC kinase is not required for normal growth and development in *A. fumigatus***

To determine the impacts of *sldA* mutation on growth and development and to ensure that any phenotypes detected were truly the result of a loss-of-function, we next generated a complete sldA gene deletion mutant via CRISPR/Cas9 gene editing (**Supplemental Figure C.16A**) (Al Abdallah, Ge et al. 2017). Simultaneously, we generated a deletion of the gene encoding the putative SldA-binding protein, SldB (**Supplemental Figure C.16C**) (Basenko, Pulman et al. 2018). The putative *A. fumigatus* SldB (encoded by the gene AFUB_076660 in the strain A1163), is a predicted ortholog of *A. nidulans* SldB (encoded by the gene AN2439 in the reference strain FGSC A4), which is known to bind to the single *A. nidulans* SldA kinase to regulate its function in the MCC complex (**Figure C.3**) (Efimov and Morris 1998). *A. fumigatus* SldB is also a predicted ortholog of the *Saccharomyces cerevisiae* BUB3 (encoded by the gene YOR026W in the reference strain S288C) (Basenko, Pulman et al. 2018). In *S. cerevisiae*, which like many eukaryotes expresses two paralogous SAC kinases known as BUB1 (YGR188C in S288C) and MAD3 (YJL013C in S288C) (**Supplemental Figure C.15B**) (Basenko, Pulman et al. 2018), Bub3p is known to bind to the kinase Mad3p to regulate its function in the MCC complex and from *S. cerevisiae* to humans, BUB3 is known to complex with BUB1 to aid kinetochore interactions in response to checkpoint activation (**Figure C.3**) (Taylor, Ha et al. 1998, Primorac, Weir et al. 2013, Basenko, Pulman et al. 2018). Therefore, loss of the *A. fumigatus* SldB would be expected to impact the SAC similarly to loss of the SldA kinase. Both deletion mutants were then complemented by re-insertion of the entire

sldA or sldB ORF into each native locus (**Supplemental Figure C.16B** and **D**).

Growth assays on minimal media revealed no gross differences in colony morphology for the *sldA* (Δ*sldA*) or *sldB* (Δ*sldB*) deletion strains when compared to the *wild type* parental strain, CEA10 (**Figure C.4A**). Quantitation of colony diameter during the initial 96 hours of culture revealed statistically significant, yet minor, reductions in vegetative growth when comparing mutant and *wild type* strains (**Figure C.4B**). When growth was measured as biomass accumulation in submerged culture, no significant reduction in biomass was noted between mutant and *wild type* strains (**Figure C.4C**). An assay to compare the rate of conidial germination revealed that the strains exhibited germination kinetics and a similar percent (%) germination (**Figure C.4D**). Therefore, SldA and SldB are not required for germination of conidia nor colony development.

**Loss of *sldA* or *sldB* gene results in increased susceptibility to benomyl but does not alter nuclear distribution**

Related to its functional role in the SAC, deletion of the *A. nidulans sldA* gene results in hypersusceptibility to the benzimidazole spindle poison, benomyl (De Souza, Hashmi et al. 2013). To determine if the functions of SldA and SldB for the SAC pathway are likely conserved in *A. fumigatus*, susceptibility to benomyl was determined for both Δ*sldA* and Δ*sldB*. On solid agar, both mutant strains exhibited an increase in susceptibility to benomyl with the Δ*sldA* mutant displaying a more severe phenotype than Δ*sldB* (**Figure C.5A**). Examination of benomyl susceptibility in submerged culture by broth microdilution assay resulted in a similar outcome for both strains (**Figure C.5B**). These results suggest conserved functions for SldA and SldB in *A. fumigatus*, and that microtubule stress in combination with loss of the SldA kinase is more poorly tolerated than in combination with loss of the SldB protein.

To assess if the roles for SldA or SldB in resistance to microtubule stress are associated with basal defects in the progression of mitosis, we next quantified the average number of nuclei present within interseptal hyphal compartments. Observation of the distribution of nuclei throughout hyphae using propidium iodide staining did not reveal gross misdistribution (**Figure C.6A**). Quantitative assessment of the average number of nuclei per interseptal compartment revealed no significant differences between the Δ*sldA* and Δ*sldB* strains and *wild type* (**Figure C.6B**). Therefore, *sldA* and *sldB* do not appear to regulate distribution of nuclei throughout the hyphae.

**Loss of *sldA* or *sldB* specifically impacts susceptibility to inhibitors of ergosterol biosynthesis**

As the *sldA* disruption mutant displayed decreased susceptibility to voriconazole, we next sought to define the impact of *sldA* or *sldB* deletion on susceptibility to a panel of ergosterol biosynthesis inhibitors (EBIs). The EBI antifungal compounds were chosen from

multiple drug classes including the triazoles (voriconazole, itraconazole, posaconazole, and isavuconazole), the allylamines (terbinafine) and the statins (fluvastatin) (**Figure C.7**). Importantly, broth microdilution MIC assays revealed that the reduced voriconazole susceptibility phenotype originally detected in the *sldA* disruption strain from our kinase disruption library was also evident in the Δ*sldA* strain (**Figure C.7D**). This data suggests that our original *sldA* disruption was, as expected, a loss-of-function mutation. Although the average voriconazole MIC increase upon loss of *A. fumigatus sldA* was only four-fold, we also found voriconazole susceptibility of an *Aspergillus nidulans sldA* deletion strain to be reduced, supporting this phenotype as a result of SAC kinase deletion in multiple *Aspergillus* species (**Supplemental Figure C.17**). Further, MIC assays revealed that both Δ*sldA* and Δ*sldB* mutant strains exhibited a consistent pattern of reduced susceptibility to each of the triazole compounds utilized (**Figure C.7D-G**). Interestingly, both Δ*sldA* and Δ*sldB* strains were also less susceptible than *wild type* to fluvastatin and terbinafine, indicating that loss of *sldA* or sldB impacts susceptibility to inhibitors of ergosterol biosynthesis in general (**Figure C.7B** and **C**). However, neither mutant exhibited an altered susceptibility to the polyene compound, amphotericin B, which rather than inhibiting ergosterol biosynthesis, instead targets existing ergosterol within the fungal cell membrane (**Figure C.7H**).

As loss of *sldA* or sldB generated resistance to EBIs in general, we predicted that the mechanisms underlying reduced triazole susceptibility in our mutants were likely not due to simple transcriptional upregulation of previously characterized triazole resistance genes such as the triazole target genes or drug efflux pumps (Lazzarini, Esposto et al. 2016, Badali, Canete-Gibas et al. 2022). In agreement with this, analysis for differential gene expression of the triazole target genes, *cyp51A* and *cyp51B*, and of the drug efflux pumps, *atrF* and *abcC/cdr1B*, revealed no major differences in gene expression, either at baseline or under sub-MIC voriconazole exposure, between Δ*sldA* and CEA10 (**Supplemental Figure C.18**). These findings suggested a potentially uncharacterized mechanism underlying triazole susceptibility loss when *sldA* is deleted.

To determine if the Δ*sldA* or Δ*sldB* mutants also displayed altered susceptibility to non-ergosterol biosynthesis pathway related stress, MIC/MEC assays were repeated with the cell wall-targeting antifungal compound, Caspofungin, the DNA damage-inducer, MMS, with the oxidative stress-inducers, 4-NQO and Paraquat, and with the osmotic stressors NaCl and sucrose. Interestingly, none of the chosen stressors revealed a pattern of altered susceptibility in either the Δ*sldA* or Δ*sldB* mutant when compared to *wild type* (**Figure C.8B-G**). Employing the host niche as a stress-inducing environment, we also found that the Δ*sldA* and Δ*sldB* strains did not display altered pathogenic fitness in a murine model of invasive aspergillosis (**Figure C.8A**). Therefore, loss of *sldA* or sldB was not associated with a general alteration of susceptibility to any non-ergosterol biosynthesis stress applied.

**Conidia from the Δ*sldA* and Δ*sldB* mutants display voriconazole heteroresistance and increased DNA content**

Aneuploidy has been shown to provide either phenotypic resistance to triazoles, or a heteroresistance phenotype wherein only a subset of the strain population exhibits reduced triazole susceptibility (Sionov, Chang et al. 2009, Sionov, Lee et al. 2010, Semighini, Averette et al. 2011, Chang, Khanal Lamichhane et al. 2018, Todd and Selmecki 2020, Todd and Selmecki 2023). To determine whether reduced triazole susceptibility is homogeneously inherited by Δ*sldA* and Δ*sldB* progeny or whether loss of either gene instead produces a heteroresistance phenotype, we observed strain growth within MIC wells (**Figure C.9A-C**). Analysis of growth revealed both Δ*sldA* (**Figure C.9B**) and Δ*sldB* (**Figure C.9C**) mutants produce a subpopulation of spores that exhibit growth in concentrations of triazole which are inhibitory to *wild type* (**Figure C.9A**). The majority of Δ*sldA* and Δ*sldB* mutant conidia show susceptibility that is similar to that of the parental CEA10 strain. However, at concentrations of voriconazole past the MIC of the *wild type*, a subset of the conidia accomplished successful germination and production of sporadic colonies. Moreover, apparent variations in phenotypes were observable among these few colonies. This indicated loss of *sldA* or *sldB* did not impart all offspring with an identical, homogeneous level of resistance to triazole, but rather a subset of progeny received some mechanism imparting reduced voriconazole susceptibility. These observations indicate that Δ*sldA* and Δ*sldB* exhibit a phenotype characteristic of heteroresistance or tolerance. To quantify the impact that loss of *sldA* or *sldB* imparts on viability in the presence of voriconazole, we performed CFU analyses. The Δ*sldA* and Δ*sldB* mutants both exhibited a significant increase in the formation of colonies in comparison to *wild type* at each concentration of drug tested, which at several concentrations appeared to be a meaningful increase in viability (**Figure C.9D**). In an assay to compare the survival of mature hyphae exposed to triazole stress, we performed a modified broth microdilution assay to test voriconazole susceptibility using liquid GMM wherein cored sections of pre-sporulating colonies were utilized as inoculum in place of a set quantum of spores (**Supplemental Figure C.19A**). The Δ*sldA* and Δ*sldB* mutants exhibited a consistent pattern of growth at increased concentrations of voriconazole in comparison to *wild type* (**Supplemental Figure C.19B**). These results indicate that loss of *sldA* or *sldB* provide increased mean survival of both spores and mature hyphae compared to *wild type* in response to voriconazole exposures.

**Aneuploidy in conidial populations is associated with SAC component deletion**

Flow cytometry to measure DNA content is an established method utilized to assess for aneuploidies within populations of fungal cells or dormant fungal spores (J. Ramón De Lucas 1998, Selmecki, Forche et al. 2010, Harrison, Hashemi et al. 2014, Dos Reis, Silva et al. 2018, Todd, Braverman et al. 2018). As correct function of SAC components is required for maintenance of ploidy status in other fungi, we sought to determine whether chromosomal mis-segregation events due to mitotic checkpoint dysfunction resulting in

aneuploidy may contribute to the increased triazole MIC in Δ*sldA* and Δ*sldB*. To do this, we performed flow cytometric analyses of fixed and stained conidia, as previously described (Goto, Mishra et al. 2011, Dos Reis, Silva et al. 2018). To validate the staining protocol, we first analyzed a *wild type* population of triazole susceptible, *wild type* CEA10 conidia alongside stable diploid and haploid strains of the model yeast *Saccharomyces cerevisiae* (**Figure C.10A**). Flow analysis produced the expected fluorescence signal for the control *S. cerevisiae* strains ATCC 204508 and 201390, indicative of a haploid and a diploid genome respectively and which correspond to previously published results for these strains (Dos Reis, Silva et al. 2018). The analysis also produced a clear fluorescence peak representative of a single haploid nuclear content ($G_1$) peak as well as a smaller but also distinct population producing a distinct 2n content, which is also known to occur within *A. fumigatus* conidia flow cytometric analysis (J. Ramón De Lucas 1998, Dos Reis, Silva et al. 2018). To determine if deletion of *sldA* or *sldB* impacts the balanced sorting of chromosomal DNA into conidia at baseline, we then analyzed samples of conidia from Δ*sldA*, Δ*sldB*, and CEA10 grown on GMM without any added stressor. Flow cytometric analysis revealed a clear shift in mean $G_1$ DNA content to the right in both Δ*sldA* and Δ*sldB* conidial populations, indicative of an average skew toward slightly increased mean genome size when compared to the parental CEA10 population (**Figure C.10B**). A visible "shoulder" was also present in the $G_1$ peak fluorescence signal for both mutant strains. This indicates the likely existence of sub-populations of Δ*sldA* and Δ*sldB* conidia which were packaged with an abnormally high DNA content, shifting the total mean $G_1$ peak fluorescence of both SAC mutant strains towards the right.

**Loss of *sldA* does not increase the rate of acquired triazole resistance**

To see if loss of the SAC kinase would alter the dynamics of resistance acquisition, we next performed *in vitro* experimental adaptation of both Δ*sldA* and CEA10 to voriconazole on solid agar over nine successive passages of drug exposure (**Supplemental Figure C.20**). Three independent lineages of Δ*sldA* and CEA10 were repeatedly transferred over voriconazole-embedded media and changes in voriconazole susceptibility of each independent lineage were monitored throughout the process. While at the start of the experiment, the Δ*sldA* lineages showed the characteristically increased MIC in comparison to the *wild type*, the *wild type* lineages rapidly adapted to voriconazole exposure and by transfer +2, all lineages of both parental strains exhibited nearly identical gains in voriconazole MIC (**Figure C.11**). After the final passage, both Δ*sldA* and CEA10 lineages reached a peak MIC of between 16 and 32 µg/ml voriconazole. These results suggest that, while loss of a functional SAC kinase causes increased triazole MIC at baseline, this does not necessarily translate to an enhanced adaptability in comparison to *wild type*.

**Aneuploidy in conidial populations is associated with loss of triazole susceptibility**

To determine whether adaptation to triazole may drive aneuploidy acquisition, we next assayed samples of population conidia collected from each lineage of voriconazole adapted Δ*sldA* and CEA10 from generations +9. The histograms of these conidial populations indicated that the strains which were experimentally adapted to voriconazole exhibited non-*wild type* Mean DNA content (i.e., left or right shift in the Mean $G_1$ peak fluorescence, indicating conidia packaged with abnormally low or high DNA content, respectively) (**Figure C.12A** and **B**) (Harrison, Hashemi et al. 2014). Notably, changes in mean DNA content were seen in both the *wild type* and Δ*sldA* genotype lineages in response to voriconazole adaptation and were, therefore, not dependent on the presence of *sldA*. Instead, accumulation of aneuploid conidia appeared to occur as a natural consequence of triazole exposure in *wild type* CEA10 as well. To quantify the putative shifts in DNA content within each conidial population, we determined the DNA Index by determining the mean $G_1$ peak fluorescence for each experimental sample and dividing the value by the calculated mean $G_1$ peak fluorescence of the *wild type* CEA10 sample from the same experiment. DNA Index (DI) is an established method by which to estimate the degree of aneuploidy within a sample of cells based on flow cytometry analysis for DNA-content (Danielsen, Pradhan et al. 2016). The DNA Index for each strain/lineage was calculated based on the histogram data, revealing DNA Index values which indicated approximately 4% to 19% deviation from a *wild type* $G_1$ peak (**Figure C.12A** and **B**). Based on these results, we conclude that the production and accumulation of aneuploid conidia occurs in *A. fumigatus* following complete deletion of the SAC components SldA kinase or SldB, or can also occur in genetically unmodified *wild type* CEA10 following repetitive exposure to medical triazole antifungal.

**Both aneuploidy and adaptation to triazole were unstable in the absence of triazole stress**

Previous work in *C. neoformans* and *C. albicans* has shown that aneuploidy-mediated triazole resistance/heteroresistance often dissipates in the absence of triazole stress (Sionov, Chang et al. 2009, Sionov, Lee et al. 2010, Semighini, Averette et al. 2011, Chang, Khanal Lamichhane et al. 2018, Todd and Selmecki 2020, Todd and Selmecki 2023). To determine whether the level of production of aneuploid conidia or the voriconazole MIC shows impermanence in any of the voriconazole-adapted lineages after transfer over drug-free media, we continued the process of repetitive transfers except without voriconazole addition. These transfer cycles are referred to as Generations -1 through -5 (**Supplemental Figure C.20**). Voriconazole MIC assays revealed that five of these six previously adapted lineages had developed adaptations which were at least partially transient (**Figure C.11**) and (**Table C.4**). Following transfer five over drug-free medium, all lineages except CEA10 #3 showed at least some reduction of MIC, with CEA10 #1 and CEA10 #2 MIC reduced by half, Δ*sldA* #2 reduced twofold, and Δ*sldA* #1 and Δ*sldA* #3 returning to a susceptibility level consistent with the lowest results for the non-adapted parental strain. To determine whether the

levels of aneuploidy production within conidial populations also changed in the absence of triazole stress, we again assayed each lineage by flow cytometry. Analysis of flow cytometry histogram data revealed changes in the mean DNA content occurred in most lineages (**Figure C.12A** and **B**) and (**Table C.4**). One lineage, CEA10 #1, returned about halfway to a *wild type* DNA Index, and three lineages, CEA10 #2, Δ*sldA* #1, and Δ*sldA* #3, returned to a DI consistent with their non-adapted parental strain. Two lineages, CEA10 #3 and Δ*sldA* #2, generally retained deviant DI results, suggesting CIN continued at least following five transfers in the absence of triazole exposure. Taken together, these results indicated that *in vitro* triazole adaptation drove the acquisition of transient chromosomal instability and similarly transient triazole adaptations in both Δ*sldA* mutant and *wild type A. fumigatus*.

**Few lineages developed mutations in genes previously associated with triazole resistance**

To determine whether *in vitro* adaptation to voriconazole might have driven the acquisition of genomically fixed, mutation-based mechanisms conferring reduced triazole susceptibility, we subjected each lineage to Illumina Whole Genome Sequencing (SeqCenter (Pittsburgh, PA) with added analysis for gene variants. The resulting variant calling files were each analyzed and searched for genes of interest, including genes previously known or suspected to impact triazole susceptibility, as well as genes related to the cell cycle and the SAC. Among our six total voriconazole-adapted lineages, only two developed any mutation that impacted a gene previously known to influence triazole phenotypes. The lineage Δ*sldA*.3 developed a mutation in the sequence for Cyp51A which results in a substitution at position 50 in the sequence which replaces the Threonine with an Isoleucine (T to I) (**Figure C.13A**). Position 50 in the Cyp51A sequence is in a location which is near a hotspot for previously reported triazole-resistance conferring mutations, specifically at positions S52 and G54 (**Figure C.13A**). Moreover, this lineage generally retained a high voriconazole MIC following transfer in the absence of voriconazole stress (**Table C.4**). This may indicate that transient mechanisms accounted for part of voriconazole adaptation in this lineage, while a fixed mutation might account for the remainder of the adaptations developed in this lineage. The lineage CEA10.3 developed a mutation in the sequence for Hmg1 which results in a deletion of G483 (**Figure C.13A**). A search through the literature did not reveal any instances where a mutation at this position was previously associated with a loss of triazole susceptibility. Moreover, this lineage displayed only transient voriconazole adaptations. Following transfer in media without added triazole, this lineage reverted completely to a pre-adaptation level triazole susceptibility (**Table C.4**). This impermanence of phenotype implies that either genetically-fixed, mutation-based mechanisms of adaptation did not contribute to the voriconazole phenotype of the lineage at Generation +9, or, for some reason, progeny with the genomically-fixed mechanisms were selected against during five cycles of transfer in drug-free conditions. Among all six lineages, no other variants were detected among the genes included in our search, except for the expected deletion in the sequence for *sldA* within each of our Δ*sldA* lineages (**Table C.5**). This result, combined

with the observation that most of the lineages show some degree of impermanence in the voriconazole adapted-level MIC, indicated that most of the lineages had developed transient mechanisms of triazole adaptation which contributed to the voriconazole phenotypes. Combined with results from flow cytometric analyses which show each lineage increased accumulation of genomically abnormal conidia during *in vitro* voriconazole adaptation indicated that aneuploidy developed in the majority of the lineages which likely contributed to triazole adaptation.

**Four of six lineages exposed to voriconazole selected for a large duplication in chromosome 2**

Whole genome sequencing followed by analysis for copy number variation is the current gold standard by which to identify specific aneuploidy (or aneuploidies) present within the cells of an organism (Bing, Hu et al. 2020). By comparing copy number of a sample genome back to the *wild type* reference, WGS can reveal regions which exhibit abnormally high or low CN, indicating whether the majority of genomes represented by a sample of extracted DNA selected for specific whole or partial duplication or deletion of a chromosome. Moreover, unlike for basic flow cytometric analysis, analysis for CNV using WGS data is capable to pinpoint where in the genome such large-scale chromosomal changes occurred, which can begin to reveal which regions of the genome may contain genes associated with phenotypic changes. We first assembled the genomes of the 8 samples to compare whether the *sldA* deletion and/or voriconazole exposure results in increased structural variation. The samples had genome assembly sizes between 29.08 – 29.27 Mb and there were no obvious patterns of expansion or contraction in the Δ*sldA* and voriconazole evolved strains. However, to examine patterns of structural variation in more detail, we estimated copy number for each non-overlapping 5kb region of the genome across all 8 samples. This analysis revealed several interesting patterns. First, we did not identify any copy number variants between the CEA10 and Δ*sldA* genomes at baseline, indicating CNV did not occur in Δ*sldA* to the threshold of detection in this analysis. However, CEA10.1 and CEA10.3 displayed overlapping duplications across the first 1 Mb of chromosome two compared to *wild type* CEA10 (CEA10.1 = CP097564.1:1-305,000 and CP097564.1:57,000-1,005,000 and CEA10.3 = chromosome 2:1-1,285,000) **(Figure C.14B** and **D**). Additionally, Δ*sldA*.2 and Δ*sldA*.3 also showed a duplication overlapping the same region of chromosome 2 compared to the Δ*sldA* genome (Δ*sldA*.2 = 1-820,000 and Δ*sldA*.3 = 1-1,055,000) (**Figure C.14F** and **G**). This cumulative region of chromosome 2 includes 351 protein coding genes (encompassing AFUB_017180 through AFUB_020990). Additionally, relative to the CEA10 genome, CEA10.1 and CEA10.3 contained a deletion of the first 40 Kb of chromosome 6 (CP097568.1) overlapping 7 genes from AFUB_102200 to AFUB_080090 (**Figure C.14B** and **D**), and CEA10.1 additionally contained a 60 Kb duplication toward the end of chromosome 7 (CP097569.1:1,715,001-1,785,000) overlapping 14 genes from AFUB_092500 to

AFUB_092500 (**Figure C.14B**). Relative to the Δ*sldA* genome, Δ*sldA*.2 contained an additional 350 Kb duplication on chromosome 4 (CP097566.1:1,280,001-1,630,000) overlapping 109 genes from AFUB_063120 to AFUB_064310, and a 190 Kb deletion of the end of chromosome 6 (CP097568.1:3,700,001-3,890,000), overlapping 67 genes from AFUB_079170 to AFUB_079420 (**Figure C.14F**). Finally, Δ*sldA*.2 also contained a 79 Kb deletion at the end of chromosome 7 (CP097569.1:1,790,001-1,875,000), overlapping 25 genes from AFUB_092600 to AFUB_092820 (**Figure C.14F**).

## Conclusions and Discussion

The eukaryotic cell cycle progresses linearly through four distinct phases: $G_1$, S, $G_2$, and M-phase wherein the cell proceeds through mitosis. Several crucial signaling checkpoints exist throughout these stages to ensure errors do not occur (Novak and Tyson 2021). The key checkpoint of mitosis is the Spindle Assembly Checkpoint (SAC), often simply referred to as the mitotic checkpoint (Chan, Jablonski et al. 1999, Suijkerbuijk, van Osch et al. 2010). This is a conserved surveillance system designed to prevent the unbalanced sorting of DNA during nuclear division in eukaryotic species (Bolanos-Garcia and Blundell 2011, Suijkerbuijk, van Dam et al. 2012). In studies involving eukaryotic species from yeast to humans, defective SAC is consistently associated with chromosome-kinetochore microtubule attachment errors remaining uncorrected leading to missegregation of chromosomes at anaphase, a form of chromosomal instability (CIN) which often result in aneuploidy (Thompson, Bakhoum et al. 2010, Bolanos-Garcia and Blundell 2011, Ricke, Jeganathan et al. 2012, Giam and Rancati 2015). A key protein kinase is required for this checkpoint to occur faithfully which is conserved across eukaryotic species (Bolanos-Garcia and Blundell 2011, Ricke, Jeganathan et al. 2012). Depending on the species involved, this kinase, known by names such as BUB1, BUBR1 (for "budding uninhibited by benzimidazoles", referring to the characteristic lack of mitotic arrest when the proteins are missing or nonfunctional despite nocodazole or benomyl treatment), Mad3 (for "mitotic arrest deficient"), or SldA (for "synthetic lethality with dynein", referring to inviability when both the kinase and the motor protein dynein, which regulates spindle placement and movement of SAC components, are nonfunctional) (Hoyt, Totis et al. 1991, Efimov and Morris 1998, Basu, Bousbaa et al. 1999, Bolanos-Garcia and Blundell 2011, Roy, Han et al. 2022). Representative orthologues and paralogs of this protein kinase carry out vital activities required to sense the progress of chromosomal alignment and to activate the corrective machinery involved when the requirements of the checkpoint are not satisfied (Bolanos-Garcia and Blundell 2011, Ricke, Jeganathan et al. 2012, Jin, Bokros et al. 2017). Kinase orthologs regulate activity of various SAC components including Aurora B, Mad2, the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C), the centromeric histone H2A, and through H2A regulation initiates the localization of Sgo1 and PP2A/Rts1 to the kinetochore (**Figure C.3**) (Kawashima, Yamagishi et al. 2010, Lara-Gonzalez, Westhorpe et al. 2012, Ricke, Jeganathan et al. 2012, Jin, Bokros et al. 2017, Brimacombe, Burke et al. 2019). BUB1/BUBR1/Mad3/SldA inhibits

progression to anaphase until the chromosomes are adequately bi-oriented and attached to kinetochore microtubules emanating from opposing centrioles (or spindle pole bodies in fungi) so that upon anaphase, all chromosomes are equally distributed and inherited by the daughter nuclei (**Figure C.2** and **C.3**) (Heasley, DeLuca et al. 2019). Kinase orthologs also play roles for the SAC in regulation of chromosome congression and preserving inter chromatid cohesion prior to anaphase (Meraldi and Sorger 2005, Fernius and Hardwick 2007, Kawashima, Yamagishi et al. 2010).

Many eukaryotes possess two paralogous orthologs of this kinase which together perform the necessary duties for the cell (Suijkerbuijk, van Dam et al. 2012). Often the separate paralogs split the work so that one remains at the kinetochore upon SAC activation to mediate repair of spindle attachment errors while the other forms a diffusible complex known as the Mitotic Checkpoint Complex (MCC), which inhibits progression to anaphase by inhibiting the association of the Anaphase Promoting Complex/Cyclosome (APC/C) with its coactivator Cdc20 (Suijkerbuijk, van Dam et al. 2012). However, many eukaryotic organisms retain a single protein kinase which possesses all required domains to accomplish the tasks required for the checkpoint to function (Suijkerbuijk, van Dam et al. 2012). *Saccharomyces cerevisiae* for example possesses the two kinase orthologues BUB1 and MAD3, of which only MAD3 participates as part of the MCC, while *Candida albicans* possesses only the kinase BUB1 (**Supplemental Figure C.15B** and **C.3**) (Skrzypek, Binkley et al. 2017, Basenko, Pulman et al. 2018). The model filamentous fungal organism *Aspergillus nidulans* expresses the singular ortholog of BUB1/BubR1/MAD3 known as SldA (De Souza, Hashmi et al. 2013, Basenko, Pulman et al. 2018).

In humans and the budding yeast *S. cerevisiae*, the SAC components BUB1 and BUBR1 have been thoroughly studied and the roles of their representative orthologs are generally conserved across eukaryotes (Jin, Bokros et al. 2017). Kinetochore localization of the BUB1 and BUBR1/Mad3 proteins and the MCC component Mad2 is regulated by the combined activities of kinases Mps1 and Aurora B (Ditchfield, Johnson et al. 2003, Roy, Han et al. 2022). In turn, the BUB1 kinase also regulates the activity of Aurora B (Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012). Until chromosomes are accurately attached to kinetochore microtubules (or in response to incorrect attachments), the kinase BUB1 resides at the kinetochore monitoring the tension in the spindle and promoting kinetochore recruitment of SAC components including Mad2 (**Figure C.3**) (Vossen, Alhosawi et al. 2019). BUB1 phosphorylation of the centromeric histone H2A at threonine 121 signals for recruitment of Sgo1/Shugoshin to the kinetochore and also regulates the activity of the Aurora B kinase, which is responsible for regulating the attachment of microtubule ends to the Ndc80 complex and Knl1/BLINKIN components of the kinetochore (**Figure C.3**) (Ricke, Jeganathan et al. 2012, Liu, Rankin et al. 2013, Jin, Bokros et al. 2017, Carvalhal, Bader et al. 2022). Sgo1 in turn recruits protein phosphatase 2A (PP2A)/Rts1, which protects inter-chromosome cohesion by regulating separase (Esp1) to prevent premature cleaving of cohesin subunit Mcd1/Scc1 (Watanabe and Kitajima 2005, Liu, Rankin et al. 2013, Jin,

Bokros et al. 2017, Lianga, Dore et al. 2018).

Prior to the current study, no ortholog of this kinase had been characterized in the filamentous fungal pathogen *Aspergillus fumigatus*. Neither had the orthologs of SldB (AFUB_076660 in A1163), Cdc20 (AFUB_014280), Mad2 (AFUB_034890), or the majority of components supporting the SAC been studied in this species (Basenko, Pulman et al. 2018). However, in species where the orthologous protein kinase(s) has been characterized, certain important phenotypes are reported which reveal the importance of these conserved proteins for responding to microtubule errors and safeguarding against abnormal chromosomal sorting during nuclear division. Due to a combination of chromosome congression defects (Meraldi and Sorger 2005, Schliekelman, Cowley et al. 2009), unremedied KT-MT attachment errors (Meraldi and Sorger 2005), and premature loss of sister chromatid cohesion (Straight, Belmont et al. 1996, Matsuura, Matsumoto et al. 2006, Yang, Tsuchiya et al. 2015), loss of the BUB1/BUBR1/Mad3/SldA protein can result in formation of anaphase bridges, chromosome missegregation, chromosome lagging, and even fragmentation of the DNA when tension is applied across the mitotic spindle, which can cause structurally abnormal chromosomes and formation of micronuclei (**Table C.1**, **C.2**, and **C.3**) (Basu, Bousbaa et al. 1999, Meraldi and Sorger 2005, Jeganathan, Malureanu et al. 2007, Niikura, Dixit et al. 2007, Ibrahim, Diekmann et al. 2008, Schliekelman, Cowley et al. 2009, Kitagawa and Lee 2015). Chromosome fragmentation and missegregation can result in the formation of daughter cells with aneuploid nuclei and/or the formation of micronuclei containing chromosome fragments that retained a centromere (Jeganathan, Malureanu et al. 2007, Brimacombe, Burke et al. 2019, Barbosa, Sunkel et al. 2022). Due to chromosome instability and fragmentation, organisms deficient in kinase ortholog function are often reported to show increased cell death/reduced viability, synthetic lethality with loss of motor proteins responsible for positioning and maintaining spindle tension or chromosome movement including dynein heavy chain or BimC (Cin8/Kip1), and hypersensitivity to compounds which add further stress to the mitotic apparatus, such as microtubule disrupting benzimidazoles (**Figure C.2**) (Efimov and Morris 1998, Fernius and Hardwick 2007, Niikura, Dixit et al. 2007). Loss of the kinase has also been reported to cause accelerated exit from mitosis due to bypass of the spindle checkpoint and loss of mitotic arrest despite conditions which should halt mitosis (Roberts, Farr et al. 1994, Farr and Hoyt 1998, Taylor, Ha et al. 1998, Basu, Bousbaa et al. 1999). For example, depletion of *bub1* transcript in humans with siRNA abrogates the SAC and prevents arrest of cells in mitosis in the presence of the spindle poison nocodazole (Meraldi and Sorger 2005, Jia, Li et al. 2016). In *Drosophila melanogaster*, loss of Bub1 results in failure of sister chromatid cohesion, lagging chromosomes, formation of chromosome bridges, missegregated chromosomes, chromosome fragmentation and signs of aneuploidy (Basu, Bousbaa et al. 1999). Loss of the proteins BUB1 or its binding partner in the MCC BUB3 in *S. cerevisiae* both have the associated phenotypes of increased susceptibility to benzimidazoles, increased variation in the duration of the cell cycle and also defective maintenance of cohesion between sister chromatids (Straight, Belmont et al. 1996, Yang, Tsuchiya et al. 2015). Deletion of BUB1 or BUB3 in *S. cerevisiae* has also been reported

to produce a slow growth phenotype and premature exit from mitosis despite spindle damage caused by treatment with benzimidazoles (Roberts, Farr et al. 1994, Farr and Hoyt 1998). Deletion of the kinase ortholog in *A. nidulans* has not been tested previously for altered triazole susceptibility, but has the reported phenotypes of increased susceptibility to benzimidazoles, moderate defect in colony growth, and increased susceptibility to osmotic stressors (Efimov and Morris 1998, De Souza, Hashmi et al. 2013). Besides defects impacting BUB1 and BUB3 orthologs, defects in many other components which support the SAC can result in some level of chromosomal instability (**Table C.1**, **C.2**, and **C.3**). For a quick reference, we have included a schematic of the mitotic spindle and the kinetochore with the approximate locations of components which for which a defect results in CIN. Components for which alteration to function or expression also results in reduced triazole susceptibility are marked with a red octagon in the figure legend (**Figure C.2** and **C.3**).

Unfortunately, defects in function or even aberrant levels of gene expression of the human kinase orthologs BUB1 and BUBR1/BUB1B are associated with multiple disease states in humans (Cahill, Lengauer et al. 1998, Hernando, Orlow et al. 2001, Kops, Weaver et al. 2005, Bolanos-Garcia and Blundell 2011, Ricke, Jeganathan et al. 2011). Evidence points to BUB1 errors during oogenesis or during early embryonic development as an explanation for increases in the observed incidence of miscarriage and aneuploidy associated with pregnancies in ageing women (Leland, Nagarajan et al. 2009). Certain germline BUB1 defects also cause microcephaly and developmental delay associated with reduced total levels of protein and/or impaired kinase activity and mitotic errors including insufficient chromosomal cohesion leading to premature separation of chromosomes (Carvalhal, Bader et al. 2022). Mutations in BUB1 or BUBR1 and reduced expression of BUBR1 cause heritable diseases including mosaic variegated aneuploidy (MVA), a disease which predisposes patients to development of cancers, and the related condition premature chromatid separation (PCS) (Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Suijkerbuijk, van Osch et al. 2010, Suijkerbuijk, van Dam et al. 2012). Due to the increased prevalence of aneuploidy in human cancer cells, SAC failure in general and BUB1/BUBR1 errors specifically are expected to contribute to cancer development (Dai, Wang et al. 2004, Kops, Weaver et al. 2005, Myslinski, Gerard et al. 2007, Pinto, Vieira et al. 2008, Bakhoum, Thompson et al. 2009, Thompson, Bakhoum et al. 2010, Bolanos-Garcia and Blundell 2011). Dysregulation of BUB1 and BUBR1 expression in humans is detected in certain types of cancers and has been shown to trigger tumorigenesis spontaneously in mice (Dai, Wang et al. 2004, Jeganathan, Malureanu et al. 2007, Niikura, Dixit et al. 2007, Pinto, Vieira et al. 2008, Ando, Kakeji et al. 2010, Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012). Abnormal expression of BUB1 or BUBR1, either increased or decreased, is sufficient to cause aneuploidy showing that tight regulation of this kinase is essential for the mitotic checkpoint to operate correctly (Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Jeganathan, Malureanu et al. 2007, Suijkerbuijk, van Osch et al. 2010, Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012, Suijkerbuijk, van Dam et al. 2012, Fujibayashi, Isa et al. 2020). Examples of cancer associated with BUB1/BUBR1 defects or abnormal expression in the literature include

pancreatic, colorectal, thyroid, breast, lymph node, kidney, gastric, and lung cancers (Dai, Wang et al. 2004, Niikura, Dixit et al. 2007, Pinto, Vieira et al. 2008, Ando, Kakeji et al. 2010, Bolanos-Garcia and Blundell 2011). Many anticancer drugs consist of microtubule damaging compounds which directly activate the SAC to slow the progression of mitosis in cancer cells (Sakurikar, Eichhorn et al. 2012). Unfortunately, similarly to the characteristic loss of mitotic arrest despite the presence of benzimidazole spindle poisons, BUB1 defects in human cancers have been shown to promote adaptation of cancer cells to become resistant to the effects of anticancer drugs (Ciciro, Ragusa et al. 2024). This is unsurprising, as cancers resistant to common chemotherapeutics are reported to exhibit abnormal levels of mitotic kinase expression, elevated CIN, and aneuploidy associated with poor clinical outcomes (Stopsack, Whittaker et al. 2019, Dhital, Santasusagna et al. 2023). However, compounds which target SAC regulators, including Aurora B which regulates the BUB1 kinase, have shown some promise as combined anticancer and anti-*A. fumigatus* therapeutics (He, Fu et al. 2022).

The most pertinent phenotypes discovered previously in a eukaryote lacking the correct regulation of the SAC by the kinase ortholog may be found from studies of the human pathogenic fungal organism *Candida albicans*. Dysregulation of the SAC pathway at the level of Bub1 phosphoregulation appears sufficient to generate aneuploidy-mediated triazole resistance in this species. Loss of the kinase ortholog Bub1, a downstream target Sgo1, expression of histone H2A which lacks Bub1 phosphorylation site S121, or mutation of the H2A phosphorylation site to alanine, all increase the spontaneous production of known triazole-resistance chromosome 5 aneuploidy in *C. albicans* (Brimacombe, Burke et al. 2019). In fact, certain strains of *C. albicans* have apparently acquired a natural adaptation which leverages SAC failure via Bub1-phosphoregulation deficiencies to enhance both aneuploidy generation and reduce fluconazole susceptibility. These strains possess an allelic variant of the centromere specific histone H2A which lacks a site of phosphorylation by the BUB1 kinase, residue S121. Expression of non-BUB1 regulatable H2A enables the strain to avoid phosphoregulation by Bub1 kinase and bypass this key phosphoregulatory control during mitosis. Mutation of the ortholog of histone H2A has been shown previously to result in aneuploidy detectable as abnormal flow cytometry histogram $G_1$ and $G_2$ fluorescence peaks in *S. cerevisiae* (Pinto and Winston 2000). The levels of chromosomal instability in the *C. albicans* strains expressing non-Bub1 phosphoregulatable H2A (as measured by the frequency of occurrences of chromosome 5 aneuploidy within the progeny) were comparable to those of the Sgo1 deletion mutant, though appear slightly less than those of a Bub1 deletion mutant (Brimacombe, Burke et al. 2019). The strain expressing non-phosphoregulatable H2A produced the resistance-conferring chromosome 5 duplication at a rate six times that of the *wild type* strain. Each of these mutants show reduced fluconazole susceptibility and increased benzimidazole susceptibility, characteristic with SAC defects (Straight, Belmont et al. 1996, Fernius and Hardwick 2007). Moreover, the same study found that *C. albicans* could also promote fluconazole adaptation by regulated depletion of the kinetochore component Cse4 (CENP-A), a variant of the centromere-specific histone H3 which interacts with the

Bub1-target histone H2A and forms part of the platform onto which the kinetochore is built in eukaryotes (Camahort, Shivaraju et al. 2009, Hoffmann, Samel-Pommerencke et al. 2018, Brimacombe, Burke et al. 2019). This causes SAC deficiency likely by imbalancing the levels of CENP-A available to interact with other components that support SAC function (Blower and Karpen 2001, Tomonaga, Matsushita et al. 2003, Heun, Erhardt et al. 2006, Shrestha, Rossi et al. 2021). Mutation of Cse4, the ortholog of CENP-A in *S. cerevisiae*, has been previously shown to cause chromosome missegregation (Stoler, Keith et al. 1995). Abnormal CENP-A/Cse4 expression has also been previously demonstrated to cause chromosome missegregation and aneuploidy development in humans, mice, and *Drosophila* as well, illustrating that abnormal function or expression of this SAC regulator results in SAC failure in eukaryotes from *C. albicans* to humans (**Table C.1**, **C.2**, **C.3**) (Blower and Karpen 2001, Tomonaga, Matsushita et al. 2003, Heun, Erhardt et al. 2006, Shrestha, Rossi et al. 2021). These, therefore, are two examples of mechanisms for acquiring aneuploidy-based triazole resistance in a fungal pathogen, both involving circumventing the SAC to enhance the production of aneuploid progeny in response to triazole stress.

These results are consistent with previous work has shown that changes to the function or expression of components which support the SAC can impact triazole susceptibility in fungi (**Table C.1**, **C.2**, **C.3**). Such changes include inhibition of Hsp90 in *S. cerevisiae*, depletion of Aurora B in *C. neoformans*, (which acts as an upstream regulator of Bub1 and is itself regulated by Bub1), and deletion of the MCC component Mad2 in *C. albicans* (**Figure C.3**) (Chen, Bradford et al. 2012, Varshney and Sanyal 2019, Vossen, Alhosawi et al. 2019). Hsp90 is a chaperone with conserved roles for regulating both SAC and DDR pathway events. This chaperone regulates assembly of the kinetochore on chromosomes and of the sites on the kinetochores to which spindle microtubules attach (Stemmann, Neidig et al. 2002, Davies and Kaplan 2010, Chen, Bradford et al. 2012, Pennisi, Ascenzi et al. 2015). Chemical inhibition of *S. cerevisiae* Hsp90 results in progeny with aneuploidies which confer adaptive advantages to fluconazole, benomyl, and unrelated genotoxic compounds (Chen, Bradford et al. 2012). Loss of Mad2 function in *C. albicans* was shown to increase the incidence of aneuploidy, and the rate of aneuploidy increased further in the context of fluconazole stress, consistent with the previously known tendency of fluconazole to promote aneuploidy detectable by flow cytometry in *Candida* species (Harrison, Hashemi et al. 2014, Vossen, Alhosawi et al. 2019).

The tendency of *C. albicans* to generate aneuploidy at an increased rate in response to triazole stress is well established, and some researchers have previously proposed defects in the cell cycle as the route for these changes to occur (Selmecki, Forche et al. 2010, Hill, Ammar et al. 2013, Harrison, Hashemi et al. 2014, Todd and Selmecki 2020). One study identified an aneuploidy in every lineage evolved *in vitro* to fluconazole whereas no aneuploidies were detected in the unexposed control populations (Selmecki, Dulmage et al. 2009). Each of these aneuploidies involved additional segments of the left arm of chromosome 5, which were found to provide a beneficial copy number variation that provided resistance

to triazole by upregulating the *C. albicans* genes encoding TAC1 and ERG11 (Selmecki, Gerami-Nejad et al. 2008, Selmecki, Dulmage et al. 2009). The gain of the aneuploidy under triazole stress conditions and loss of the aneuploidy in the absence of triazole were directly correlated with the subsequent gain and loss of fluconazole resistance, (though after many cycles of sustained azole exposure, the aneuploidy often eventually became a fixed characteristic in several of the lineages, remaining in the cells and their progeny even in the absence of triazole stress (Selmecki, Forche et al. 2006, Selmecki, Dulmage et al. 2009). Additionally, some of the populations accumulated other aneuploidies, specifically impacting chromosomes 3-7, and cells which had acquired several of these additional chromosomes or chromosomal portions in addition to the overrepresentation of Chromosome 5L typically possessed the highest levels of triazole resistance (Selmecki, Dulmage et al. 2009). Moreover, rather than exhibiting any fitness defects, several of these triazole-adapted isolates exhibited increased growth in comparison to the control populations, even in the absence of triazole stress (Selmecki, Dulmage et al. 2009). Importantly, aneuploidy involving additional portions of chromosome 5L represent a confirmed mechanism of clinical triazole resistance in *C. albicans*, first discovered as the mechanism conferring fluconazole resistance within a patient isolate (Selmecki, Gerami-Nejad et al. 2008).

The capacity for aneuploidy to provide adaptation to triazole stress is a relatively new concept in filamentous fungi. Until 2023, species of mold such as *Aspergilli* were assumed to be incapable of utilizing this strategy to alter susceptibility to any form of antifungal stress (Handelman and Osherov 2022, Barda, Sadhasivam et al. 2023). Two separate publications in 2023 revealed aneuploidies occurring in two separate human pathogenic *Aspergillus* species: *A. flavus* and *A. fumigatus* (Barda, Sadhasivam et al. 2023, Khateb, Gago et al. 2023). While neither publication provided any explanation or proposal for how these species were able to produce aneuploidy associated with reduced azole susceptibility, both works destroyed the illusion that aneuploidy-mediated adaptations were completely unobtainable to filamentous fungi. However, further evidence is needed to reveal the yet unknown mechanism through which *Aspergillus* can generate aneuploidies in association with triazole adaptation and to validate the relationship between aneuploidy accumulation and triazole susceptibility changes.

In this study, we demonstrate that the functions of SldA kinase and the protein SldB for the SAC are likely conserved in *A. fumigatus*, as both mutants display hypersensitivity to benzimidazoles. The slight difference in susceptibility level between Δ*sldA* and Δ*sldB* implied a greater importance of the SldA kinase function for mitotic regulation than for SldB, which has been noted previously in other species regarding phenotypes relating to SAC deficiency (Brimacombe, Burke et al. 2019). We also show that loss of either SAC component results in reduced triazole MIC, which is consistent with previous data on loss of the kinase ortholog Bub1 in *C. albicans* (Brimacombe, Burke et al. 2019). However, further analysis of growth within the MIC assay wells revealed that this reduced triazole susceptibility is due to a heteroresistance phenotype in both strains. Aneuploidy remains a likely explanation

for this observation, as CIN has been shown previously in other fungal pathogens to produce heterogeneous populations containing both genomically normal progeny and sub-populations of aneuploid progeny exhibiting heteroresistance to fluconazole (Semighini, Averette et al. 2011, Altamirano, Fang et al. 2017, Chang, Khanal Lamichhane et al. 2018, Kukurudz, Chapel et al. 2022, Sun, Li et al. 2023). Following exposure to triazole antifungals, aneuploidies which are most beneficial in this context are selected for and are represented in the later adapted populations (Kukurudz, Chapel et al. 2022).

In other species, crosstalk exists between the SAC and DNA damage (DDR) responses. For example, in budding yeast, lack of the function of Hsp90 results both in aneuploidy and failure of certain forms of DNA damage repair, (which can cause further genomic instabilities by allowing for increased accumulation of mutations) (Fu, Pastushok et al. 2008). In *C. elegans*, deletion of BUB1, SAN1 (the ortholog of BUBR1/MAD3), or Cdc20 all result in hypersensitivity to ionizing radiation (Bertolini, Wang et al. 2017). (Ionizing radiation is known to induce DNA damage in the form of double-strand breaks (DSB) and single-strand breaks (SSB) and also produces oxidative stress by generating reactive oxygen species (ROS)) (Borrego-Soto, Ortiz-Lopez et al. 2015). Deletion of BUB1 or Sgo1 in *C. albicans* renders the strains hypersensitive to DNA damage from UV exposure (which induces both DNA damage and thymine dimers which block transcription) (McKay, Stubbert et al. 2004). (Interestingly, the phenotype is more severe for loss of the kinase ortholog than for loss of a downstream target) (Brimacombe, Burke et al. 2019). However, *sldA* deletion in *A. nidulans* did not show hypersensitivity to the genotoxic agents DEO (a DNA alkylating agent) or camptothecin (which induces DSB), but resulted in hypersusceptibility to both sucrose and NaCl (De Souza, Hashmi et al. 2013).

In the current study, we found that the reduced susceptibility phenotypes of Δ*sldA* and Δ*sldB* were generalizable to compounds which inhibit the biosynthesis of ergosterol, but did not produce a difference in susceptibility by MIC assay to any additional form of stress. This result deviated slightly from studies in other species where aneuploidies appear to provide spontaneous combinations of CNV which reduce susceptibility to other forms of stress (Sionov, Chang et al. 2009, Sun, Li et al. 2023). This result may indicate that in *A. fumigatus*, combinations of whole or segmental chromosomal abnormalities which have potential to reduce susceptibility to other forms of stress based on the location of certain genes tend to inextricably carry fitness defects which are poorly tolerated, have only weak impacts on susceptibility which are unable to shift the MIC, and/or the viable combinations which are capable to impact susceptibility to other forms of stress occur more rarely than those which impact susceptibility to EBIs. Moreover, our results deviate from the previously reported hypersensitivity to DNA damage phenotypes for loss of the kinase orthologs in *C. elegans* and *C. albicans*, and from the hypersusceptibility to osmotic stressors reported for loss of SldA in *A. nidulans* (Efimov and Morris 1998, De Souza, Hashmi et al. 2013, Bertolini, Wang et al. 2017). Our Δ*sldA* and Δ*sldB* mutants did not exhibit altered sensitivity to chemical inducers of DNA damage or oxidative stress compared to the *wild type* strain.

The precise interactions with SAC components can vary (Bolanos-Garcia and Blundell 2011). Kinase orthologs of other species may play certain roles in DNA damage responses that are not conserved in *Aspergillus* species, or redundancies might exist in *A. fumigatus* which negate the impacts from loss of the kinase.

Another surprising result from the current study was the lack of hyperadaptability to triazole for our Δ*sldA* mutant. This deviates from reports of hyperadaptability to triazole in a *C. albicans* Bub1 deficient strain (Brimacombe, Burke et al. 2019). However, later results revealed that *wild type A. fumigatus* acquired and selected for partly transient triazole adaptations, aneuploid conidia, and a specific chromosome two duplication in a similar manner as Δ*sldA*. These results together indicate that *wild type A. fumigatus* likely possesses a mechanism for inducing CIN and aneuploidy in the context of triazole exposure. If this mechanism relies on SAC deficiency to a similar degree as occurs when *sldA* is deleted, this could account for the nearly identical patterns between *wild type* and Δ*sldA* regarding MIC increase and acquisition of aneuploid conidia, development of adaptations which were at least partially transient, and the independent selection for an identical specific chromosome duplication in two out of three lineages of each strain during *in vitro* adaptation to voriconazole. Unfortunately, the number of publications which associate aneuploidies with triazole adaptation in this species remains limited and no mechanism has been yet proposed whereby *A. fumigatus* could obtain aneuploidy in this context. However, prior studies in other eukaryotes show abnormal function or expression of SldA kinase orthologs is sufficient to cause dysregulation of SAC components leading to SAC failures and aneuploidy (Cahill, Lengauer et al. 1998, Basu, Bousbaa et al. 1999, Hanks, Coleman et al. 2004, Pinto, Vieira et al. 2008, Ando, Kakeji et al. 2010, Bolanos-Garcia and Blundell 2011, Ricke, Jeganathan et al. 2011). Overexpression of BUB1 leads to imbalanced phosphorylations that result in abnormally high activation of Aurora B, promoting destabilization of KT-MT attachments which triggers chromosome missegregation and aneuploidy (Pinsky, Kung et al. 2006, Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012, Fujibayashi, Isa et al. 2020). Meanwhile, abnormally low expression of BUB1 orthologs in human and budding yeast also results in CIN and aneuploidy, as this causes a failure to localize SAC components to the kinetochore, including a failure to localize BUBR1 (in human cells), Sgo1, PP2A, and Aurora B, which results in premature loss of chromosome cohesion, premature sister chromatid separation, and even chromosome fragmentation when tension is applied to the spindle (Dai, Wang et al. 2004, Hanks, Coleman et al. 2004, Meraldi and Sorger 2005, Matsuura, Matsumoto et al. 2006, Fernius and Hardwick 2007, Jeganathan, Malureanu et al. 2007, Niikura, Dixit et al. 2007, Baker, Jin et al. 2009, Suijkerbuijk, van Osch et al. 2010, Ricke, Jeganathan et al. 2012, Suijkerbuijk, van Dam et al. 2012, Carvalhal, Bader et al. 2022). Therefore, both overexpression and underexpression of the kinase ortholog can promote SAC failure leading to aneuploidy. Based on our results and these previously established patterns in other species, we hypothesize that *wild type A. fumigatus* may naturally modulate SAC pathway fidelity during triazole exposure. This would provide a means to allow for directed chromosome missegregation and enhance the generation of aneuploidy-based

fitness benefits. We hypothesize that rather than permanent loss of function of an SAC effector such as the SldA kinase, (which is the situation in our laboratory Δ*sldA* mutant), *wild type A. fumigatus* would likely benefit more by modulating SAC fidelity through reversible and inducible changes to the activity or the expression of key SAC components. To explore this possibility, we reviewed the literature for evidence which could support or dismiss this hypothesis. The limited data available concerning changes in the level of transcription of key SAC components in the context of triazole stress appears to support this hypothesis.

Previous studies in other species have revealed that overexpression or underexpression of the SldA kinase ortholog Bub1, overexpression of the Aurora B kinase (which both regulates and is regulated by Bub1) in human cancers, or a downstream target Cdc20 is sufficient to cause SAC failures (**Table C.1**, **C.2**, **C.3**) (Dai, Wang et al. 2004, Ibrahim, Diekmann et al. 2008, Pinto, Vieira et al. 2008, Baker, Jin et al. 2009, Ando, Kakeji et al. 2010, Ricke, Jeganathan et al. 2011, Kitagawa and Lee 2015, Shrestha, Rossi et al. 2021). To explore whether dysregulation of SAC component expression is a characteristic response to triazole stress in *A. fumigatus* and other species of pathogenic fungi, we analyzed datasets from RNA sequencing available on the popular fungal genomics database FungiDB. Two datasets were found which provide information comparing the transcriptional profile of *A. fumigatus* at baseline to that following exposure to subinhibitory triazole (itraconazole) (Basenko, Pulman et al. 2018, Sarkans, Gostev et al. 2018, Furukawa, van Rhijn et al. 2020). Both datasets reveal that expression of *sldA* is generally reduced in response to triazole stress (**Supplemental Figure C.21**). Interestingly, this pattern was even seen in four triazole resistant strains; Δ*nctA* (AFUB_029870), Δ*nctB* (AFUB_045980), HapB deletion (AFUB_030360), and one strain with a deletion in NADH-ubiquinone oxidoreductase 29.9 kDa subunit, (AFUB_026380), showing that even triazole resistant strains may initially respond to triazole exposures by reducing *sldA* expression (Basenko, Pulman et al. 2018, Sarkans, Gostev et al. 2018, Furukawa, van Rhijn et al. 2020). To determine whether other fungal pathogens share this pattern, we searched for datasets comparing expression of *wild type* to conditions relating to triazole exposure or adaptation. We discovered that in *Cryptococcus neoformans*, expression of the kinase ortholog (gene CNAG_03184 in strain grubii H99) also reduced following triazole exposure, (while anecdotally, the difference appears milder for a triazole-hypersusceptible Cfo1 deletion mutant) (Kim, Cho et al. 2012, Basenko, Pulman et al. 2018). Moreover, in *C. auris* transcription of the ortholog of the kinase (gene B9J08_004346 in the strain B8441) is reduced in lineages experimentally evolved to fluconazole compared to the susceptible parent isolate (Basenko, Pulman et al. 2018, Bing, Hu et al. 2020). Furthermore, during the *in vivo* development of triazole resistance in *C. albicans* within the course of a patient infection, expression of the Bub1 kinase (gene C4_03120C_A in strain SC5314) appears to have dropped when comparing the later isogenic isolates which had adapted in the course patient antifungal therapy to the original susceptible isolate (Dhamgaye, Bernard et al. 2012, Basenko, Pulman et al. 2018). When we analyzed data for changes in expression of the SldB/BUB3, Cdc20, Mad2, and Aurora B orthologs in each of these datasets, we found a similar pattern of mildly to moderately

reduced expression was observed for several genes, though the pattern was not clearly present for each gene in each species (**Supplemental Figure C.22**, **C.23**, **C.24**, and **C.25**) (Dhamgaye, Bernard et al. 2012, Kim, Cho et al. 2012, Basenko, Pulman et al. 2018, Sarkans, Gostev et al. 2018, Bing, Hu et al. 2020, Furukawa, van Rhijn et al. 2020). These results indicate that changes in transcription of key SAC components, including the SldA kinase ortholog, may be a generalizable response to triazole exposure among multiple species of fungal pathogens. Depending on the magnitude of transcriptional change and the absence corresponding changes to components which interact in the SAC pathway, these mild to moderate transcriptional changes in SAC component expression during triazole stress could be sufficient to imbalance the pathway. Future work should evaluate the potential for directed SAC dysregulation, via changes to transcription, regulation, or otherwise, to partly account for the ability of fungi to acquire and select for adaptive aneuploidies in response to triazole exposure.

Flow cytometry revealed that our Δ*sldA* and Δ*sldB* mutants both appear to produce sub-populations of conidia packaged with aberrant levels of genomic content, typically skewing toward gain of nuclear DNA. Though these mutants were maintained on GMM in the absence of any added stressor, the mutants may have selected for mean genome size increases during culture simply due to a natural preference towards progeny with better fitness, and/or against progeny with poorer fitness. As increases in genome size may be better tolerated on average than losses, the strains naturally gravitated towards production of small sub-populations of conidia tending to have increased genome size. Previous studies in other species show that average gain of material across a population of progeny is at least more favored in the context of triazole stress (Altamirano, Fang et al. 2017, Chang, Khanal Lamichhane et al. 2018, Kukurudz, Chapel et al. 2022). However, due to technical barriers, we did not attempt to isolate aneuploid spores by FACS for genomic DNA extraction and sequencing. Instead, WGS analysis of genome sequencing data was performed on DNA collected from homogeneous population samples, with the smaller representation of aneuploid nuclei/conidia dwarfed by the predominantly *wild type* progeny. Moreover, because the CNV produced within conidia likely include a random assortment of combinations, both viable and inviable, and both losses and gains of genomic content (though generally skewed toward gain within the aneuploid spores), across all eight chromosomes, the CNV analysis performed based on WGS data did not show amplification of any specific chromosomal aneuploidy within our Δ*sldA* mutant at baseline. We assume this is explainable due to the low-level of CNV variances across the genome which were present only in a small sub-population of conidia. Previous studies in *S. cerevisiae* and *Schizosaccharomyces pombe* show that loss of the kinase ortholog produced minuscule subpopulations of aneuploid offspring (Bernard, Hardwick et al. 1998, Basu, Bousbaa et al. 1999). Our CFU analysis and observation of growth within MIC wells (**Figure C.9**) indicated that Δ*sldA* spontaneously produces only a very small number of triazole heteroresistant progeny. Thus, the contribution of this small population with a CNV to the total CN of any one region of a chromosome was vastly diluted by the greater

abundance of genomically normal progeny, and any variations were insufficient to reach the threshold of detectability in standard CNV analysis, appearing simply as slight noise (**Figure C.14A**). Meanwhile, repetitive exposure of Δ*sldA* and *wild type* to triazole triggered the increased generation of aneuploidies and ultimately drove the selection for only CNV which provided the best fitness in the context of triazole stress. As the majority of progeny now possessed those specific (likely beneficial) CNV, our adapted lineages exhibited CNV detectable in WGS analysis. Interestingly, four of six lineages selected for a duplication impacting chromosome two (**Figure C.14B**,**D**,**F**, and **G**). The only article available currently which detected aneuploidy associated with triazole resistant *A. fumigatus* reported a similar chromosome two aneuploidy within clinical isolates from one patient with a pan-azole resistant *A. fumigatus* infection (Khateb, Gago et al. 2023). While it is yet unclear which genes in this region of the genome could impact triazole susceptibility, future work should explore which genes or gene combinations are impacted by this aneuploidy which could contribute towards triazole non-susceptibility in this species.

The fact that voriconazole adaptation began to disappear within five of six lineages in the absence of voriconazole (often with simultaneous changes in levels of aneuploid conidia production according to flow cytometry) is not surprising if these lineages obtained aneuploidy-mediated adaptations, as in other species such fitness benefit-conferring CNVs are often transient; retained only as long as triazole stress remains (Sionov, Chang et al. 2009, Sionov, Lee et al. 2010, Ford, Funt et al. 2015, Chang, Khanal Lamichhane et al. 2018, Todd and Selmecki 2020, Todd and Selmecki 2023). In prior studies with *C. albicans*, sustained repeated exposure to levels of triazole close to the MC50 of *wild type* overwhelmingly drove the acquisition of adaptive aneuploidy (segmental aneuploidy providing CNV), while repeated exposure to levels of fluconazole much higher than the MC50 tended to produce more mutation-based resistance mechanisms (Todd, Soisangwan et al. 2023). Combined with our results, this data suggests that certain triazole doses may drive the development of aneuploidy-based adaptations while others may promote selection of mutation-based adaptations. If true, this could raise important questions about the mode of resistance development in patient populations receiving different doses of triazole and at different intervals. This could explain why the only report of triazole resistant *A. fumigatus* patient isolates found to possess aneuploidies potentially associated with resistant phenotypes were obtained from patients with chronic pulmonary aspergillosis (CPA) or cerebral aspergilloma, while samples obtained from patients with invasive pulmonary aspergillosis (IPA) instead possessed primarily mutation-based mechanisms (Khateb, Gago et al. 2023).

The results of the present study, in combination with previous knowledge of the impact of deficiencies in SldA kinase ortholog-mediated phosphoregulation on chromosome stability across eukaryotes and on triazole susceptibility and generation of known resistance-conferring aneuploidies in *C. albicans*, suggest that aneuploidy generation may be achieved through bypassed or suppressed SAC checkpoint and could represent a natural strategy for acquiring aneuploidy-mediated triazole adaptations in fungal pathogens including *A.*

*fumigatus*. Future work will be required to explore this potential source of genomic variation providing novel genome combinations under antifungal stress conditions, as well as to confirm the contributions that specific aneuploidies and associated gene expression changes provide towards the development of triazole adaptation in *A. fumigatus*.

**List of components of the mitotic spindle, kinetochore, and/or SAC pathway for which alteration results in error-prone chromosome segregation (CIN):**

APC/C Subunit APC11 Mutation: (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). APC/C Subunit APC11 Overexpression: (Skrzypek and Hirschman 2011, Tutaj, Pogoda et al. 2019). APC/C Subunit CDC16 Mutation: (Hartwell and Smith 1985, Skrzypek and Hirschman 2011). Aurora B/Ipl1:Aurora B/Ipl1 Overexpression: (Meraldi, Honda et al. 2004). Aurora B/Ipl1 Underexpression: (Varshney and Sanyal 2019). Aurora B/Ipl1 Mutation: (Francisco, Wang et al. 1994, Biggins, Severin et al. 1999, Kim, Kang et al. 1999, Cheeseman, Anderson et al. 2002, Pinsky, Kung et al. 2006, Sandall, Severin et al. 2006). Bik1: (Skrzypek and Hirschman 2011, Prajapati, Rizvi et al. 2017). Bim1/EB1: (Skrzypek and Hirschman 2011, Loll-Krippleber, Feri et al. 2015, Prajapati, Rizvi et al. 2017). Bir1/Survivin Deletion: (Sandall, Severin et al. 2006, Skrzypek and Hirschman 2011). Borealin/Nbl1p: (Nakajima, Tyers et al. 2009). BUB1 Overexpression: (Pinsky, Kung et al. 2006, Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012, Fujibayashi, Isa et al. 2020). BUB1 Underexpression: (Meraldi and Sorger 2005, Fernius and Hardwick 2007, Jeganathan, Malureanu et al. 2007, Niikura, Dixit et al. 2007, Baker, Jin et al. 2009, Ricke, Jeganathan et al. 2012, Funk, Zasadil et al. 2016, Carvalhal, Bader et al. 2022). BUBR1 Underexpression: (Dai, Wang et al. 2004, Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Suijkerbuijk, van Osch et al. 2010, Suijkerbuijk, van Dam et al. 2012, Giam and Rancati 2015). BUB1 Mutation: (Basu, Bousbaa et al. 1999, Jeganathan, Malureanu et al. 2007, Yuen, Warren et al. 2007, Carvalhal, Bader et al. 2022). BUBR1 (BUB1B) Mutation: (Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Suijkerbuijk, van Osch et al. 2010, Suijkerbuijk, van Dam et al. 2012). BUB1 Deletion: (Bernard, Hardwick et al. 1998, Brimacombe, Burke et al. 2019). BUB3 (SldB) Mutation: (Babu, Jeganathan et al. 2003, Yuen, Warren et al. 2007, Giam and Rancati 2015). Cdc20: (Hartwell and Smith 1985, Li, Fang et al. 2009, Li, Fang et al. 2010, Thompson, Bakhoum et al. 2010, Giam and Rancati 2015, Loll-Krippleber, Feri et al. 2015, Hu, Jin et al. 2022). Cdc28/CDK1: (Yu, Jiang et al. 1996, Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). CENP-A Overexpression: (Tomonaga, Matsushita et al. 2003, Heun, Erhardt et al. 2006, Shrestha, Rossi et al. 2021). CENP-A Underexpression: (Blower and Karpen 2001, Heun, Erhardt et al. 2006, Brimacombe, Burke et al. 2019). CENP-E/CENP-meta/Kinesin 7: (Chan, Schaar et al. 1998, Chan, Jablonski et al. 1999, Putkey, Cramer et al. 2002, Thompson, Bakhoum et al. 2010, Giam and Rancati 2015, Resende, Monteiro et al. 2018, Yu, Zhong et al. 2019, She, Xu et al. 2022). CENP-F/HCP-1/2 Depletion: (Cheeseman, MacLeod et al. 2005, Thompson, Bakhoum et al. 2010). CENP-H/MCM16: (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011). CENP-I/CTF3: (Kouprina, Pashina et al. 1988, Daniel,

Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011, Tirupataiah, Jamir et al. 2014, Tutaj, Pogoda et al. 2019). CENP-L/IML3: (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011). CENP-N/CHL4: (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Andersen, Nelson et al. 2008, Skrzypek and Hirschman 2011). CEP57: (Matsuura, Matsumoto et al. 2006, Snape, Hanks et al. 2011, Seyedmousavi, Guillot et al. 2015). Cin8 (BimC/Kinesin-5/Eg5) alone: (Kovacovicova, Awadova et al. 2016, Mittal, Ghule et al. 2020, She, Zhong et al. 2020). Cin8/BimC/Eg5 and Kip1 simultaneously: (Saunders, Koshland et al. 1995, Mittal, Ghule et al. 2020). CLASP/CLS-2 Mutation: (Pereira, Pereira et al. 2006). CLASP/CLS-2 Depletion: (Cheeseman, MacLeod et al. 2005, Thompson, Bakhoum et al. 2010). Cut12/Skf1: (Troxell, Sweezy et al. 2001). Cyclin B Mutation: (Gehmlich, Haren et al. 2004). Dam1-DASH-DDD Complex Mutation: (Cheeseman, Anderson et al. 2002, Meraldi, Honda et al. 2004, Kitagawa and Lee 2015, Jin, Bokros et al. 2017). Dsn1: (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011). Dynein: (Gehmlich, Haren et al. 2004, Prevo, Cheerambathur et al. 2023). Dynactin (p27) Deletion: (Gama, Pereira et al. 2017). Histone H2A Mutation: (Pinto and Winston 2000, Brimacombe, Burke et al. 2019). Histone H3 (Underexpression): (Roschke and Kirsch 2010, Ragusa and Vagnarelli 2023). Histone H4 (HHF1,2): (Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011, Gordon, Zhu et al. 2023). HSF1 Overexpression: (Dai 2018). Hsp90: (Chen, Bradford et al. 2012). INCENP/Sli15 Mutation: (Sandall, Severin et al. 2006). Kar3/Cik1: (Chan, Jablonski et al. 1999, Mayer, Pot et al. 2004, Tytell and Sorger 2006, Skrzypek and Hirschman 2011, Jin, Liu et al. 2012, Duffy, Fam et al. 2016, Suzuki, Gupta et al. 2018, Morard, Macias et al. 2019, Mittal, Ghule et al. 2020). Kip1: (Skrzypek and Hirschman 2011, Mittal, Ghule et al. 2020). Kip3: (Skrzypek and Hirschman 2011, Duffy, Fam et al. 2016, Mittal, Ghule et al. 2020). Knl1/Spc105 Mutation: (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011, Omer Javed, Li et al. 2018, Angrisani and Fachinetti 2023). Knl1/Spc105 Depletion: (Pitayu-Nugroho, Aubry et al. 2023). Mad1 Deletion: (Li and Murray 1991, Kitagawa and Rose 1999, Giam and Rancati 2015). Mad2: (Kitagawa and Rose 1999, Dobles, Liberal et al. 2000, Bai, Ramanan et al. 2002, Skrzypek and Hirschman 2011, Giam and Rancati 2015, Vossen, Alhosawi et al. 2019). Microtubule/Spindle Chemical Stress (Benzimidazoles): (Chen, Bradford et al. 2012, Akbari Dana, Hashemi et al. 2019). Mif2: (Meeks-Wagner, Wood et al. 1986, Brown, Goetsch et al. 1993, Meluh and Koshland 1995, Skrzypek and Hirschman 2011). Misalignment of the spindle: (Tame, Raaijmakers et al. 2014). Mis12 Mutation: (Takahashi, Yamada et al. 1994, Venkei, Przewloka et al. 2011). Mps1: (Giam and Rancati 2015). Ndc80: (Kline-Smith, Sandall et al. 2005, Zhu, Sherlock et al. 2016, Chen, Tresenrider et al. 2017). Nsl1: (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011, Venkei, Przewloka et al. 2011). Nuf2: (Skrzypek and Hirschman 2011, Cheng, Vaisica et al. 2012, Chen, Tresenrider et al. 2017). Num1/nuMA/LIN-5: (Lorson, Horvitz et al. 2000, Gehmlich, Haren et al. 2004, Haren, Gnadt et al. 2009, Silk, Holland et al. 2009, van Toorn, Gooch et al. 2023). Pds5/Spo76/BimD: (Wang, Read et al. 2002). Plk1/Polo Mutation: (Barbosa, Martins et al. 2020). Rae1 Mutation: (Babu, Jeganathan et al. 2003). RZZ Inhibition: (Gama, Pereira et al. 2017). RZZ Component "Rough Deal"/ROD:

(Karess and Glover 1989, Barbosa, Sunkel et al. 2022). RZZ Component "Zeste-White 10"/ZW10: (Williams, Karr et al. 1992, Barbosa, Sunkel et al. 2022). RZZ Component ZWILCH: (Williams, Li et al. 2003). Separase/Cut1/Esp1 Mutation: (Funabiki, Kumada et al. 1996, Lianga, Dore et al. 2018). Separase/Cut1/Esp1 Overexpression: (Zhang, Ge et al. 2008). Securin/Cut2/Pds1 Mutation: (Funabiki, Kumada et al. 1996, Yamamoto, Guacci et al. 1996). Sgt1: (Kitagawa, Skowyra et al. 1999, Skrzypek and Hirschman 2011). Shugoshin Sgo1 Deletion: (Brimacombe, Burke et al. 2019). Skp1: (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). Smc1/SMC1A (cohesin complex member): (Yi, Wang et al. 2017). Spc24: (Wigge and Kilmartin 2001, Le Masson, Saveanu et al. 2002, Skrzypek and Hirschman 2011, Chen, Tresenrider et al. 2017). Spc25: (Skrzypek and Hirschman 2011, Chen, Tresenrider et al. 2017). Spindly/SPDL1 Overexpression: (Klimaszewska-Wisniewska, Buchholz et al. 2022). Spindly/SPDL-1 Depletion: (Gassmann, Essex et al. 2008, Barbosa, Martins et al. 2020). TUB4, SPC97, or SPC98 ($\gamma$-tubulin small complex ($\gamma$-TuSC): (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013).

## Materials and Methods

### Strains and Growth Conditions

To identify kinases which impact antifungal susceptibility, we used our kinase gene disruption mutant library generated in the CEA10 background in a previous study (Souza and Martin-Vicente et al., 2021). The *wild type* strain CEA10/A1163 was used as the background strain for all *Aspergillus fumigatus* genetic manipulations in this study. All strains were cultured on *Aspergillus* glucose minimal medium (GMM) agar at 37°C unless otherwise noted, with or without drug added into the medium as necessary (Shimizu et al., 2001). Submerged culture and broth microdilution assays were performed using either Roswell Park Memorial Institute (RPMI-1640) medium or sterile liquid GMM as indicated.

### Genetic Manipulations of *A. fumigatus*

Genetic manipulations described in this study were accomplished using a modified CRISPR-Cas9 technique which has been described previously (Al Abdallah et al., 2017). For generation of gene deletion mutants, the entire open reading frame (ORF) was replaced by a homologous repair template containing the hygromycin resistance cassette. The genetic sequence encoding the putative SldA and SldB in *A. fumigatus* were identified through BLAST search using verified gene sequences from the model organism *A. nidulans* (Efimov et al., 1998, Souza et al., 2013, Basenko et al., 2018). Optimal protospacer adjacent motif (PAM) sites were identified upstream and downstream of the gene of interest using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) made available online by the University of Georgia (Peng et al., 2015). As complete gene deletion requires induction of a DSB both upstream and downstream of the gene coding sequence within the DNA, we selected two optimal PAM sites and designed the repair template to contain

the gene encoding the hygromycin resistance cassette amplified from the plasmid pJMR2 (Rybak et al., 2019) as well as the 40bp microhomology upstream of the 5′ DSB and to 40bp downstream of the 3′ PAM cut site. The homology repair templates were generated by PCR using high fidelity TAQ polymerase mastermix and were screened for correct amplification. *In vitro* assembly of the ribonucleoprotein complexes (RNP), protoplast generation, and transformations were performed as previously described (Al Abdallah et al., 2017). For fungal transformations, we utilized GMM supplemented with 1.2 M sorbitol (Sorbitol Minimum Medium (SMM)) to allow the protoplasts to adequately recover prior to selection. Hygromycin resistant colonies were screened and genotyped by PCR to confirm correct integration into the genome (Martin-Vicente et al., 2020). For complementation of the deletion mutants, the entire ORF was re-inserted into the genome at its native locus, adding the phleomycin resistance cassette. Complementation required two repair template fragments to be made via PCR amplification and product purification. For the first fragment, we amplified the complete ORF for the gene of interest from *wild type* CEA10 using primers adding 40bp of homology to the 5′ DSB and 40bp homology to the phleomycin resistance sequence. We produced the second fragment by amplifying the phleomycin resistance repair cassette from the plasmid pAGRP (Fortwendel et al., 2012) using primers adding 40bp homology to 3′ end of the gene of interest ORF and 40bp homology to the 3′ PAM cut site in the genome. Consequently, the repair template fragments possessed overlapping regions which were merged *in vivo* during the transformation process by the endogenous repair machinery. The resulting gene transformation recapitulated the original sequence of the gene in its 5′ context while adding a phleomycin resistance selection cassette downstream of the gene of interest but upstream of the hygromycin cassette, which remained within the complemented mutant. Positive colonies resistant to both hygromycin and phleomycin were confirmed by multiple genotyping PCR reactions to verify correct integration of the homologous repair template (Martin-Vicente et al., 2020).

**Analysis of Growth and Development Phenotypes**

Analysis of radial growth rate was performed for each strain following a protocol described elsewhere (Fortwendel et al., 2005). Briefly, 5µl of a $10^6$ cfu/ml concentrated water stock was inoculated into the center of 100mm GMM agar plate and incubated at 37°C. The colony diameter for each strain was measured at 24H intervals and images of plates were captured at 96H of incubation for visual representation of colony formation on solid agarose medium. Determination of biomass accumulation was performed by inoculating pre-weighed culture tubes containing 5ml of GMM supplemented with 0.5% yeast extract (GMM+YE) (Martin-Vicente et al., 2020) to a final concentration of $10^5$ cfu/ml and incubating at 37°C for 24 or 48 hours with shaking at 250rpm. After indicated timepoints, the mycelium was dried and lyophilized for a minimum of 24 hours before the final dry weight was recorded. Germination assays were performed following a previously outlined protocol with modification (Fortwendel et al., 2004). Briefly, for each strain, coverslip cultures

containing GMM liquid medium were inoculated to a final concentration of $10^5$ cfu/ml and incubated at 37°C. Coverslips for each strain were removed from the cultures at the times indicated and the number of conidia with a visible germ tube were counted. For these analyses, each experiment was performed at minimum in triplicate and data is reported as mean ± standard deviation. Analysis for statistically significant differences between the mutant and *wild type* CEA10 groups were performed via one-way ANOVA with Tukey's or Dunnett's post-hoc test as noted in the figure (GraphPad Prism V9.5.1.).

**Spot Dilution Assays for Susceptibility to Stress-Inducing Compounds**

Spot Dilution Assays to determine susceptibility of strains were performed as previously described, with modifications (Souza and Martin-Vicente et al., 2021). Briefly, fresh conidial suspensions were prepared for each control and test strain. Assays were accomplished using 100mm size round petri plates containing 20ml of GMM with or without the appropriate final concentration of specified compound added homogeneously into the medium. For each strain, 10 µl of 10-fold serial dilutions of conidia ranging from $5x10^6$ to $5x10^3$ conidia/ml were deposited onto GMM agar plates supplemented with concentrations of compound indicated. GMM agar plates without drug were included in each experiment for growth control. Plates were incubated at 37°C for timepoints indicated within each figure, at which point plate growth was analyzed and images were captured.

**Broth Microdilution Assays for Susceptibility to Stress-Inducing Compounds**

The susceptibility profiles of stress-inducing compounds referenced in this study were determined for each strain by broth microdilution assays performed as previously described, with modification (Souza and Martin-Vicente et al., 2021). Briefly, 10 two-fold dilutions of the appropriate compound were prepared sterile liquid GMM in place of RPMI medium within 96-well plates. Fresh conidial stock suspensions were prepared and each well was inoculated with a total of $2x10^4$ conidia from the appropriate test or control strain. A no conidia and no drug control were included for each row within the first two wells. Plates were incubated at 35°C for 24 hours for echinocandin MEC (Souza and Martin-Vicente et al., 2021), or at 37°C for 48 hours for other compounds. After the appropriate incubation, the MIC or MEC was determined as applicable dependent on the compound used in the experiment. MIC was considered the concentration of compound which inhibited 100% of growth. For determining the susceptibility of *Aspergillus nidulans wild type* and mutant strains to voriconazole by broth microdilution assay, 0.5% yeast extract was added to the liquid medium (GMM+YE).

**Broth Microdilution Assay using Hyphal Fragments as Inoculum**

To test for the voriconazole resistance phenotype within mature hyphae rather than asexual spores, broth microdilution assays performed as previously described, with modification

(Souza and Martin-Vicente et al., 2021). Briefly, 10 two-fold dilutions of voriconazole were prepared with the lowest concentration of drug starting at 0.25 µg/ml in sterile liquid GMM in place of RPMI medium within 96-well plates. To generate pre-sporulating hyphae for use as inoculum, fresh conidial suspensions of each strain included in this experiment were spread onto GMM plates (without any drug added, $1x10^6$ total spores per plate, spread onto plate evenly using a disposable plate spreader). To ensure sufficient yield of young hyphal colonies, three agarose media plates were inoculated for each strain. Plates were incubated at 37°C until colonies began to appear, but before the spore-production stage of growth, (18-24 hours). Plates were removed from incubation simultaneously and hyphal sections were cored from the agar with the use of disposable glass pipettes. These agarose plugs containing hyphal fragments were then transferred to individual wells of the 96-well plates. Plates were incubated at 37°C until the cored sections of hyphae matured into visible growth within the wells of the 96-well plate which could be easily distinguished from the initial inoculum (5 Days post inoculation). Plates were then analyzed and the MIC was considered the column/concentration which inhibited 100% of growth. Experiments were repeated in triplicate for each strain, with a minimum of three samples per assay.

**Quantitation of Viability by CFU**

CFU assays were performed as has been previously described, with modifications (Lamoth et al., 2012). Briefly, fresh conidial suspensions of each control and test strain included in the experiment were evenly distributed onto GMM agar plates, (200 conidia total for each No Drug plate or 1,000 conidia for each + voriconazole plate), with either no drug added or containing a concentration of voriconazole indicated within the figure added homogeneously to the medium. Plates were allowed to incubate at 37°C for 48 hours. At this timepoint, the number of visible colonies was enumerated for each plate. Total CFU counts were obtained from a minimum of 5 plates for each strain and drug concentration included in the experiment.

**Fluorescent Staining and Microscopy**

Propidium iodide (PI) and calcofluor white (CFW) co-staining was performed as previously described (Souza and Martin-Vicente et al., 2021). Briefly, sterilized coverslips were submerged in 5ml of liquid GMM medium and inoculated with $1x10^3$ conidia of the appropriate strain. Coverslip cultures were incubated at 37°C. At the specified timepoints, coverslips with adherent hyphae were removed from incubation and washed with 50mM solution of morpholinepropanesulfonic acid buffer (MOPS) which was adjusted to pH of 6.7. Washed coverslips were then submerged in a fixative solution of 8% formalin, 25mM EGTA, 5mM $M_gSO_4$, 5% DMSO, and 0.2% Triton, for one hour at room temperature (RT). Coverslips with fixed adherent hyphae were then washed twice for 10 minutes with a 50mM solution of PIPES, then treated for one hour at 37°C with RNAse A at a final concentration of 100µg/ml prepared in PIPES buffer. Coverslips were then washed twice with MOPS

buffer. Following the second wash, coverslips were stained with a solution of 12.5µg/ml of propidium iodide and 1µg/ml of calcofluor white in a light-proof container for 5 minutes at room temperature. Coverslips were then washed twice with MOPS before being mounted onto glass microscope slides. Coverslips were analyzed immediately after being mounted to capture images of sufficient number and quality for analysis. Images of stained adherent hyphae were obtained using a Nikon NI-U upright fluorescence microscope equipped with both TRITC and DAPI filters and with use of the Nikon Elements software package.

**Extraction of genomic DNA for PCR screening and sequencing**

Extraction of genomic DNA from hyphal culture for PCR screening to confirm correct integration of homologous repair or for submission for sequencing was performed as described previously (Al Abdallah, Souza et al. 2018). Briefly, fresh conidial stocks were used to inoculate approximately $1x10^7$ conidia into liquid GMM, (250ml volumes). Cultures were incubated for 22H at 37°C and biomass was collected by filtration. Dried hyphal mats were crushed under liquid nitrogen and genomic DNA extracted using the Qiagen Plant Mini Kit (Qiagen). Samples of genomic DNA were assessed for purity by both Nanodrop and Qubit and submitted to SeqCenter (Pittsburgh, PA) for Illumina Whole Genome Sequencing and preliminary variant analysis.

Extraction of genomic DNA from conidia was performed following a previously established protocol (Zhao, Latge et al. 2019). Briefly, DNA was extracted from conidia using the MasterPure™ Yeast DNA Purification Kit with modification. Conidia were collected in sterile deionized water and centrifuged at 14,000 RPM for 5 minutes to pellet. The supernatant was discarded and 300ml of yeast cell lysis solution was added to each sample as well as .4ml of silica/glass beads. Lysis was performed by bead-beating for 8 minutes, then RNase was added and samples were RNased for a minimum of 30 minutes at 65°C. For the remainder of the process, purification of genomic DNA was conducted according to MasterPure™ Yeast DNA Purification Kit manufacturer recommendations. Samples of genomic DNA were assessed for purity by both Nanodrop and Qubit and submitted to SeqCenter (Pittsburgh, PA) for Illumina Whole Genome Sequencing and preliminary variant analysis.

**Assessment for Differential Expression of Triazole Resistance-Associated Genes by RTq-PCR**

Real Time-Quantitative PCR (RT-qPCR) assays to measure gene expression were performed as previously described, with slight modifications (Souza and Martin-Vicente et al., 2021). Cultures were prepared for total RNA extraction by inoculating $2x10^7$ total conidia into liquid GMM supplemented with 0.5% yeast extract (GMM+YE). For the + voriconazole groups, voriconazole was added into the liquid medium to a final concentration of .50µg/ml. Cultures were then incubated at 37°C and 250 RPM. At the times indicated, cultures for

each strain were removed from the shaking incubator and placed into ice until processing. Each sample of hyphal growth was processed for extraction of total RNA using the trizol extraction method and the ProtoScript II First Strand cDNA synthesis kit (New England BioLabs). Normalized dilutions of cDNA were added to iQ SYBR Green Supermix (2X) (BIORAD) combined with gene-specific forward and reverse primers for RT-qPCR reactions. Relative fold expression of the genes encoding the *A. fumigatus* 14$\alpha$-lanosterol demethylase enzymes Cyp51A or Cyp51B, the triazole-resistance associated efflux pumps AbcC/Cdr1B and AtrF, and the gene encoding SldA was examined using the $2-\Delta\Delta Ct$ method, as previously described (Livak et al., 2001).

**Murine Model of Invasive Aspergillosis (IA)**

The animal model of invasive pulmonary aspergillosis was employed as previously described (Souza and Martin-Vicente et al., 2021). Female CF-1 mice weighing approximately 25g were immunosuppressed by subcutaneous injection of 40mg/kg triamcinolone acetonide (TA) (Kenalog, Bristol-Meyers Squibb, Princeton, NJ, USA) given one day prior to infection (Day -1). Mice were also immunosuppressed by intraperitoneal injection of 150mg/kg cyclophosphamide (CP) administered every three days for the duration of the experiment, beginning on day -3. On day 0, mice were transiently anesthetized by application of inhaled isoflurane within an induction chamber with the primary and secondary flow rate set to 0.5 liters/minute and 2.5% isoflurane. Sedated mice were then inoculated by intranasal instillation of a total of $1x10^6$ conidia, suspended within 20µl of sterile saline solution. The health of each mouse was monitored at least twice daily for the duration of the experiment. Survival was recorded for 15 days post-inoculation. Mice which reached the criteria requiring humane euthanasia, including signs of distress and end stages of disease, were humanely euthanized by anoxia with $CO_2$ followed by secondary method of cervical dislocation. Statistical analysis for differences in survival between the no-conidia control, *wild type* CEA10, and mutant strain infected groups was calculated using the Mantel-Cox Log-rank test (Souza and Martin-Vicente et al., 2021).

**Flow Cytometric Analysis and Calculation of DNA Index**

Flow cytometric quantitation of DNA content was performed as previously described (Goto et al., 2011, Dos Reis et al., 2018). In brief, samples of conidia from each test and control strain were washed once with sterile 1X phosphate buffered saline (PBS), resuspended in PBS, and filtered through sterile gauze to remove clumped conidia. Washed conidia were then resuspended in a 70% concentration (v/v) solution of EtOH and fixed overnight at 4°C with agitation. Following ethanol fixation, the samples of conidia were washed twice with sterile PBS, then resuspended in 50mM sodium citrate buffer, (pH 7.5) and sonicated for three, 1 second pulses separated by a 2 second pause between each pulse. Following sonication, RNase A (Invitrogen, Waltham, MA) was added to each sample to a final concentration of 0.50mg/ml and samples were incubated for at least one hour at

50°C. After this incubation, proteinase K (Sigma-Aldrich, St. Louis, MO), was added to each sample to a final concentration of 1mg/ml and samples were incubated for at least two hours at 50°C. Conidia were then washed twice with sterile PBS and again resuspended in 50mM sodium citrate buffer. Samples were sonicated again as before. Samples were then divided between two tubes; One to be stained overnight with SYBR green DNA stain (Millipore Sigma), and one to act as an unstained control for baseline fluorescence for flow cytometry analyses. For the tubes of conidia to undergo staining, 10,000X SYBR Green was diluted in Tris-EDTA buffer (TE-buffer, pH 8.0) and added to each sample of resuspended, sonicated conidia to a final concentration of 2% (v/v). After addition of the stain, sample tubes were incubated overnight (16 hours) at 4°C with agitation in a light-proof container. Following staining, both stained and unstained samples of conidia were washed twice in sterile PBS. Washed conidia were resuspended in PBS with 0.25% (v/v) Triton X added to concentrations of approximately $1x10^7$ conidia/ml and transferred into tubes compatible for use with the flow cytometer. For each SYBR stained conidial sample, an equivalent sample of unstained conidia were included as a control for baseline fluorescence. Samples were analyzed by flow cytometry using a NovoCyte 3000 unit (Miao et al., 2021). The cytometer was set to a low flow rate of 1,000 cells/second and a minimum of 70,000 events were captured using FSC and SSC gating strategies previously optimized for *A. fumigatus* conidia. Measures of fluorescence were obtained using a 488nm laser for excitation and the FITC channel (530 ± 30nm) for detection of emission.

DNA index was calculated following previously published examples in the literature (Danielsen et al., 2016). To quantitate the DNA index for each sample, the mean $G_1$ peak fluorescence was first found for the *wild type* CEA10 sample. Then, the mean $G_1$ peak fluorescence was determined for each experimental sample. Finally, the mean $G_1$ peak fluorescence of the test sample was divided by the mean $G_1$ peak fluorescence of the control CEA10 data, (obtained within the same analysis as the sample), resulting in the DNA Index score for the sample.

**Laboratory experimental adaptation to voriconazole**

Three lineages each of CEA10 and Δ*sldA* were subjected to nine cycles of repetitive growth and transfer on solid GMM agarose media containing the mold-active medical triazole voriconazole. At Day 0, $1x10^6$ total spores of fresh conidial stock from separate parental lineages was spread evenly using a disposable plate spreader onto three sets of *Aspergillus* minimal medium plates (GMM) containing voriconazole added to a final concentration of 0.50µg/ml. Each set contained three plates, for a total of nine CEA10 plates and nine Δ*sldA* plates. From here onward, each set of three were kept separate from all others and treated as a distinct lineage. Plates were allowed to grow at 37°C until sufficiently sporulated for harvesting (between 5 and 7 days). At this time, all plates were harvested on the same day. When harvesting, all three lineages of each parent strain were harvested separately, allowing for sufficient time between harvesting each lineage to prevent contamination, thus

maintaining lineage isolation. (During this process, each step of passaging is designated as one "Generation". Each generation of growth on voriconazole-containing medium is denoted in the corresponding figures by a "+" and a number, e.g., Generation +1.) These stocks were also tested via MIC assay to discern changes in susceptibility to voriconazole. To begin the second generation, we followed a population transfer strategy: Equivalent inoculum of conidia from Generation +1 were used to inoculate three plates each of GMM containing 1.0µg/ml voriconazole. Again, plates were incubated at 37°C for 5-7 days until sufficient sporulation occurred. Lineages were then harvested independently of each other as before. This care to prevent contamination was continued for the duration of the experiment. The concentration of voriconazole in the medium was only increased once more during the process to 1.5µg/ml at Generation +4. We continued the pattern of growth on GMM with 1.5µg/ml voriconazole added to the media and harvesting until a total of 9 voriconazole-adapted generations had been achieved and monitored changes in triazole susceptibility among the lineages throughout the experiment.

**Repeated transfer of voriconazole-adapted lineages over drug-free medium**

We began a series of step-wise transfers of conidial samples from Generation +9 of each lineage identically as described for the process of *in vitro* voriconazole adaptation, but without addition of voriconazole to the medium. At the first "Generation" of drug-free growth, we transferred an equal inoculum of conidia from Generation +9 of each lineage to GMM medium without triazole added and allowed the plates to grow until sporulation. As before, conidia were harvested from these plates separately to prevent contamination. Equal volumes were transferred from these stocks to the next Generation of drug-free plates. We continued this process until we had achieved five generations of growth in the absence of drug. Samples of conidia stocks collected at several generations throughout this process were assayed by MIC to monitor the dynamics of any changes in susceptibility to voriconazole over time in the absence of drug.

**Genome assembly**

To investigate whether genome size increased as a consequence of *sldA* deletion and/or voriconazole exposure, we first assembled the genomes of our 8 strains and compared cumulative assembly size. 152-bp paired end Illumina libraries were constructed and sequenced at SeqCenter (Pittsburgh, PA). For each of the 8 samples, we used Trim_Galore v0.6.6 to remove residual adapter sequences and to trim reads at low quality sites using the following parameters: "-q 30", "–stringency 1", "–gzip", "–length 100", and "–paired" (Martin 2011). For genome assembly, the adapter and quality trimmed reads were error corrected and assembled using SPAdes v3.15.3 with the "–careful" parameter and the default K-mer range (Bankevich, Nurk et al. 2012).

### Variant calling

Variant calling was provided by the sequencing center as an additional service. Files were received for each individual strain and variants were called based on comparison of sequences to the A1163 genome. Datasets were searched to determine any variants (single nucleotide substitution, deletion, or insertion), missing coverage evidence (deletion), and new junction evidence (rearrangements) within genes previously associated with triazole resistance development in *A. fumigatus* or genes associated with the SAC. Data which called predicted mutations in such genes were further analyzed for read alignment, percent of reads which indicated the variant was present, and total number of reads to ensure these were not falsely detected.

### Estimation of copy number variation

To further look into the role of genome expansion in our strains, we compared patterns of read depth. In this analysis the adapter and quality trimmed reads were mapped against the telomere to telomere genome assembly of *A. fumigatus* A1160 (Bowyer, Currin et al. 2022) (accession GCA_024220425.1) using the default settings in BWA-MEM v0.7.17 (Li and Durbin 2009, Li and Durbin 2010). Samtools v1.14 was used to convert sam to sorted bam format and index bam files (Li, Handsaker et al. 2009) and bamaddrg was used to add sample names to each sorted bam file (Danecek, Bonfield et al. 2021). Next, the Samtools "depth" command was used to create per nucleotide coverage values using the "-aa" parameter. Next, we used the bedtools v2 "makewindows" function to create a bed file of each 5kb window in the *A. fumigatus* A1160 reference genome (Quinlan 2014). Next, the bedtools "map" function was used to calculate the mean read depth across each 5kb window in the genome for each of the 8 samples. Finally, for each sample, the mean coverage values for each 5kb window were divided by the average coverage values across the entire genome to estimate copy number.

## FIGURE LEGENDS

**Figure 1 (C.1) Disruption of the *sldA* and *ssn3* genes results in reduced triazole susceptibility. A)** MIC assay comparing the *wild type A. fumigatus* progenitor strain (CEA10/A1163) susceptibility to that of the *sldA* gene disruption strain (*sldA*-1). **B)** MIC assay comparing CEA10 progenitor susceptibility to that of the *ssn3* gene disruption strain (*ssn3*-1). The MIC was defined as the concentration of triazole that inhibited 100% of growth. MICs were determined in RPMI after 48h at 37°C, according to the CLSI M38 document. Individual results from multiple experiments are shown within each graph. Bold central line denotes the Mean MIC. Error bars represent standard deviation (SD).

**Figure 2 A(C.2) and B(C.3) Loss of various components supporting chromosome sorting results in CIN. A)** Diagram of the mitotic spindle with approximate localization of components which support chromosome segregation. For A, a simplified spindle is shown. Many

components are not included, components are simplified and are not to scale. **B)** Diagram of a tensionless kinetochore with approximate locations of components which support chromosome segregation. Defects in all colored components shown in B result in CIN (see legend). For each component marked with a red octagon in the legend, evidence supports alteration also results in reduced triazole susceptibility/increased survival of progeny in triazole embedded medium in at least one species. For B, a simplified kinetochore is shown. Many components are not included, components are simplified and are not to scale. Associations between components are not all marked. (Note that examples of CIN could not be found for alterations impacting orthologs of CENP-T,W,S, or X in species searched. These are included in B to provide context for other associated kinetochore components but left uncolored).

**Figure 3 (C.4) The SAC pathway components SldA and SldB are not required for normal growth and development in *A. fumigatus*. A)** Colony morphologies of CEA10, Δ*sldA*, *sldA* complement (Δ*sldA* + *sldA*), Δ*sldB*, and *sldB* complement (Δ*sldB* + *sldB*) strains cultured for 96h on glucose minimal medium (GMM.) **B)** Quantitation of colony diameter on solid GMM over time. **C)** Quantitation of biomass accumulation in liquid GMM at 24- and 48-hour timepoints. **D)** Quantitation of germination assay in liquid GMM. A minimum of three independent tests per strain were performed for each experiment. For B-D, statistical analysis was performed using One Way ANOVA with Tukey's test for multiple comparisons. Error bars represent SD. * = Significant at P<.05; ** = Significant at P<.01; *** = Significant at P<.001.

**Figure 4 (C.5) Loss of *sldA* or *sldB* results in increased susceptibility to benomyl. A)** Spot-dilution assay plates comparing growth of the CEA10 progenitor strain to that of the *sldA* gene deletion strain and the *sldB* gene deletion strain on GMM in the absence (GMM No Drug) and presence of benomyl at increasing concentrations (0.80 µg/ml, 1.0 µg/ml, or 1.2 µg/ml). All images were acquired after 48h incubation at 37°C. **B)** MIC assay of the CEA10, Δ*sldA*, and Δ*sldB* strains by broth microdilution in GMM broth. MICs were determined after 48h at 37°C. Individual results from multiple experiments are shown within the graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Figure 5 (C.6) The SAC pathway components SldA and SldB are not required for normal nuclear number or positioning in *A. fumigatus*. A)** Representative images of 16h germlings stained with Propidium Iodide (PI) and Calcofluor White (CFW). White arrows indicate the location of septa. **B)** Quantification of nuclear number per interseptal hyphal compartment. Error bars represent SD. Individual results from multiple analyses of stained samples are shown within the graph. Statistical analysis by One Way ANOVA with Dunnett's post hoc test.

**Figure 6 (C.7) Loss of gene *sldA* or *sldB* reduces susceptibility to inhibitors of ergosterol biosynthesis. A)** A schematic of the ergosterol biosynthesis pathway with specified points of chemical inhibition noted. MIC results are shown for the ergosterol biosynthesis inhibitors

**B)** fluvastatin, **C)** terbinafine, **D)** voriconazole, **E)** itraconazole, **F)** posaconazole, and **G)** isavuconazole and for the ergosterol-binding polyene compound, **H)** Amphotericin B. Assays were performed as in Figure 4B. Individual results from multiple experiments are shown within the graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Figure 7 (C.8) Loss of gene *sldA* or *sldB* does not impact susceptibility to varieties of cell wall, DNA damage, oxidative stress, or host stress. A)** Mice (n = 10 / group) were chemotherapeutically immune suppressed with both cyclophosphamide and triamcinolone acetonide and intranasally inoculated with 1 x $10^6$ conidia of the indicated strain. Survival was followed for 15 days post-inoculation. Analysis for significance was assessed using the Mantel-Cox Log-rank test. **B-G)** MIC/MEC results for select stress inducing compounds Caspofungin **B)**, Methyl methanesulfonate [MMS] **C)**, Paraquat **D)**, 4-Nitroquinoline N-oxide **E)**, NaCl **F)** and Sucrose **G)**. The minimum inhibitory concentration or minimum effective concentration was determined as applicable. Individual results from multiple experiments are shown within each graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Figure 8 (C.9) The Δ*sldA* and Δ*sldB* triazole tolerance phenotype is heterogeneous within the conidial population of each mutant. A)** Micrographs representative of growth of the *wild type* and mutant strains within MIC plate wells at 2µg/ml voriconazole concentration after 48 hours incubation at 37°C. Images were captured at 10x objective. **B)** Analysis for spore viability by CFU count in the presence and absence of voriconazole. All analyses were performed using GMM medium and counts were performed after 48 hours of incubation at 37°C. Minimum of five replicates were assessed per strain/drug combination. Error bars represent SD. Statistical analysis by One Way ANOVA with Tukey post hoc test. * = Significant at P<.05 ** = Significant at P<.01 *** = Significant at P<.001 **** = Significant at P<.0001. Individual results from multiple experiments are shown within each graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Figure 9 (C.10) Conidia of the Δ*sldA* and Δ*sldB* mutants display higher mean DNA content by flow cytometric analysis. A)** Yeast cells from both diploid and haploid strains of *S. cerevisiae* were processed alongside *wild type* CEA10 conidia to detect mean DNA content of by flow cytometry. Histogram results from a representative analysis are shown. DNA content is shown on the x-axis as measured by fluorescence detected on the FITC channel, and normalized count on the y-axis. **B)** Flow cytometric assay comparing the genome content of *wild type* CEA10 conidia to that of the Δ*sldA* and Δ*sldB* deletion strains. The DNA Index was calculated based on histogram data and is shown for each strain. A minimum of 70,000 events were measured per sample. DNA content is displayed as the FITC signal on the x-axis, with normalized count on the y-axis. Dotted line denotes G1 peak fluorescence in CEA10.

**Figure 10 (C.11) Repeated voriconazole exposure results in transient increases in triazole MIC.** The MIC was determined for each strain by broth microdilution using GMM broth.

MICs were determined after 48h at 37°C. Mean results were calculated for each lineage from a minimum of two assays with lineages in triplicate and are shown within the graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Figure 11 (C.12) Repeated voriconazole exposure results in transient increases in conidial DNA content. A)** Comparison of DNA content of the CEA10 conidial population to that of the CEA10 voriconazole-adapted lineages at generation +9 of voriconazole exposure and following an additional five passages over drug-free media. **B)** Comparison of DNA content of CEA10 conidial population to that of the Δ*sldA* strain and its voriconazole-adapted lineages at generation +9 of voriconazole exposure and following an additional five passages over drug-free media. Normalized count is plotted on the y-axis and DNA content indicated by FITC signal on the x-axis. Black dotted line denotes $G_1$ peak fluorescence of CEA10 (A and B) before voriconazole adaptation. Red dotted line (B only) denotes $G_1$ peak fluorescence of Δ*sldA* before voriconazole adaptation. For each histogram, the corresponding DNA Index of the respective strain is shown.

**Figure 12 (C.13) Location of singular mutations found in the genes encoding Cyp51A or Hmg1. A)** Δ*sldA*.3 developed a mutation in the Cyp51A sequence which results in a substitution of Threonine (T) at position 50 for an Isoleucine (I). **B)** CEA10.3 developed a mutation in the Hmg1 sequence which results in a deletion of the Glycine at position 483.

**Figure 13 (C.14) Copy number profiles across Δ*sldA* and lineages exposed to voriconazole.** Copy number across the entire genome compared to the wild type A1160 reference of Δ*sldA* **A)**, CEA10.1 **B)**, CEA10.2 **C)**, CEA10.3 **D)**, Δ*sldA*.1 **E)**, Δ*sldA*.2 **F)**, and Δ*sldA*.3 **G)**. The X-axis corresponds to positions along the eight *A. fumigatus* A1160 reference chromosomes. For each plot, the relative copy number (left Y-axis and colored line) and raw copy number for the sample of interest (right Y axis and gray line) are depicted. Copy number was estimated for each non-overlapping 5 Kb region in the genome. Deviations from 1 in the colored lines represent copy number differences between the two samples being compared.

**Supplemental Figure 1 (C.15) Predicted functional domains and sequence homology of the *A. fumigatus SldA* kinase. A)** Schematic of domains in the *A. fumigatus* SldA protein important for SAC functions. The predicted locations of functional domains are shown based on sequence homology to characterized kinase orthologs of other species. **B)** Graphic summary of NCBI BLASTp "hits" to the *A. fumigatus SldA* protein sequence for *A. fumigatus*, *A. nidulans*, *S. cerevisiae*, *C. neoformans*, *C. auris*, *C. dubliniensis*, and *C. albicans*. Generated using the NCBI BLASTp tool available online at https://blast.ncbi.nlm.nih.gov/Blast.cgi using the SldA protein sequence of strain A1163 obtained from FungiDB as query. **C)** Phylogenetic tree based on sequence similarity between SldA orthologs of *A. fumigatus*, *A. nidulans*, *S. cerevisiae*, *C. neoformans*, *C. auris*, *C. dubliniensis*, and *C. albicans*. Generated using FungiDB Clustal Omega multiple sequence alignment tool Distance Tree of Results.

**Supplemental Figure 2 (C.16) Schematic for construction of gene deletion mutants. A)** Schematic of *sldA* gene deletion by CRISPR-Cas9-mediated excision and subsequent replacement with a PCR-amplified homologous repair template containing the gene encoding for resistance to hygromycin. **B)** Schematic of *sldA* gene complementation by CRISPR-mediated re-insertion of the entire ORF into the genome at the native locus joined with a homologous repair template containing the gene encoding resistance to phleomycin. **C)** Schematic of *sldB* gene deletion by CRISPR-Cas9-mediated excision and subsequent replacement with a PCR-amplified homologous repair template containing the gene encoding for resistance to hygromycin. **D)** Schematic of *sldB* gene complementation by CRISPR-mediated re-insertion of the entire ORF into the genome at the native locus joined with a homologous repair template containing the gene encoding resistance to phleomycin.

**Supplemental Figure 3 (C.17) The role of *sldA* in triazole susceptibility is conserved in *A. nidulans*.** The MIC to voriconazole was determined by broth microdilution assay in GMM supplemented with 0.5% yeast extract (GMM+YE) after 48h at 37°C. Individual results from multiple experiments are shown within the graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Supplemental Figure 4 (C.18) Loss of *sldA* does not result in differential expression relative to *wild type* of genes commonly associated with triazole resistance.** Analysis for differential expression of resistance-associated genes by RTq-PCR. Strains were assessed for differences in expression at baseline and upon exposure to sub-inhibitory level of voriconazole (0.50 µg/ml). Expression was analyzed after 4h of incubation in the presence of drug at 37°C. Error bars represent SD. Statistical analysis by One Way ANOVA with Tukey's post hoc test. * = Significant at P<.05 ** = Significant at P<.01 *** = Significant at P<.001 **** = Significant at P<.0001.

**Supplemental Figure 5 (C.19) The Δ*sldA* and Δ*sldB* mutant hyphae exhibit reduced triazole susceptibility. A)** Schematic for analysis for voriconazole susceptibility using hyphae. At Day 0, $1x10^6$ conidia from CEA10 and Δ*sldA* were spread evenly onto three sets of GMM agar plates. Plates were incubated at 37°C until colonies appeared ( 18-24 Hours). Cores of agar containing hyphal plugs were removed from the agar and transferred to individual wells of a 96-well plate which had been prepared for performance of a voriconazole MIC assay. Plates were allowed to incubate at 37°C until the cored sections of hyphae matured into visible growth within the wells of the 96-well plate which could be easily distinguished from the initial inoculum (5 Days post inoculation). Plates were then analyzed and the voriconazole concentration within the wells at which growth ceased was scored as the MIC. **B)** MIC results from hyphal cores. Each strain was tested in triplicate for a minimum of three plates per strain. Individual results from multiple experiments are shown within each graph. Bold central line denotes the Mean MIC. Error bars represent SD.

**Supplemental Figure 6 (C.20) Schematic of *in vitro* experimental adaptation to voriconazole.** At Day 0, $1x10^6$ total conidia from CEA10 and Δ*sldA* were spread evenly onto GMM

plates containing voriconazole at a final concentration of 0.50 µg/ml. Plates were incubated at 37°C until sufficiently sporulated for conidial harvesting ( 5 to 7 days). Conidial stocks harvested from this first passage comprised Voriconazole Adapted Generation +1 and were tested via MIC assay to discern changes in susceptibility to voriconazole. The second generation of voriconazole adaptation was generated by transferring a set volume of conidia from Generation +1 to GMM plates containing a final concentration of 1.0 µg/ml voriconazole. The plates were again allowed to incubate at 37°C until all plates sufficiently sporulated for harvesting. The concentration of voriconazole in the medium was only increased once more during the process to 1.5 µg/ml at generation +4. The process was repeated until a total of nine voriconazole-adapted generations had been achieved. After nine passages over drug-embedded agar, conidial suspensions from each strain were then passaged over drug-free medium for a total of five generations of growth in the absence of triazole.

**Supplemental Figure 7 A(C.21), B(C.22), C(C.23), D(C.24), and E(C.25) Transcriptomics data reveals SAC component ortholog ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts. A)** Plots showing general reduction of expression of the SldA kinase or its orthologs in species in triazole exposure/adaptation contexts. **B)** Plots showing general reduction of expression of SldB or its orthologs in species in triazole exposure/adaptation contexts. **C)** Plots showing general reduction of expression of Cdc20 or its orthologs in species in triazole exposure/adaptation contexts. **D)** Plots showing general reduction of expression of Mad2 or its orthologs in species in triazole exposure/adaptation contexts. **E)** Plots showing general reduction of expression of Aurora B kinase or its orthologs in species in triazole exposure/adaptation contexts. The dataset by Furukawa et al., 2020 reports expression of *wild type A. fumigatus* at baseline and in the presence of itraconazole (grown in 50ml of Vogel's minimal medium containing 1.0% glucose for 18H at 37°C, 180RPM, then transferred to 50 ml of RPMI-1640 medium containing 2.0% glucose and 165mM MOPS buffer (pH 7.0) with or without itraconazole (0.5mg/L) and cultured for an additional 4H). This dataset also reports expression pattern for two triazole resistant mutants, Δ*nctA* (AFUB_029870) and Δ*nctB* (AFUB_045980), under the same conditions. Methods for this experiment are available within the publication at doi: 10.1038/s41467-019-14191-1. The dataset by Bowyer et al., 2020 reports expression of *wild type A. fumigatus* (strain A1160) at baseline and in the presence of itraconazole (grown in 50 ml RPMI for 18H at 37°C, 200RPM, then transferred to 50ml RPMI with or without itraconazole (1mg/L) and cultured for an additional 4H). This dataset also reports expression pattern for two triazole resistant strains made in the A1160 background; one possessing a deletion in HapB (AFUB_030360), and one with a deletion in NADH-ubiquinone oxidoreductase 29.9 kDa subunit (AFUB_026380) designated strain 29.9, under the same conditions. Methods for this experiment are available at BioSamples under submission identifier GENERA200202, (https://www.ebi.ac.uk/biosamples/samples/SAMEG315535). The dataset by Kim et al., 2012 reports expression in *wild type Cryptococcus neoformans* strain grubii H99 at baseline and in the presence of fluconazole, (grown overnight in YPD medium (1% yeast extract, 2% bacto-peptone and 2% glucose) at 30°C with shaking, then transferred to 50 ml

of fresh YPD medium with or without fluconazole (10 g/ml) and cultured for an additional 3H). This dataset also reports expression pattern under the same conditions for a Cfo1 deletion mutant which displays a hypersusceptibility phenotype to triazoles made in the grubii H99 background. Methods for this experiment are available within the publication at doi: 10.1016/j.fgb.2012.08.006. The dataset by Dhamgaye et al., 2012 reports baseline comparison of expression in a fluconazole susceptible *Candida albicans* clinical isolate, Gu4, to a fluconazole resistant *Candida albicans* clinical isolate, Gu5, collected from the same patient following fluconazole therapy. Strains were grown in YPD rich medium (2% glucose, 1% bactopeptone, 1% yeast extract) at 30°C for 48H. Methods for this experiment are available within the publication at doi: 10.1186/1471-2164-13-396. The dataset by Bing et al., 2020 reports baseline comparison of expression in a fluconazole susceptible *Candida auris* strain, BJCA001, to fluconazole resistant *Candida auris* lineages generated using the BJCA001 background through laboratory *in vitro* evolution to fluconazole (passaged in RPMI 1640 with the concentration of fluconazole increasing sequentially from 32mg/L to 128mg/L through nine total passages). Results for "resistant strain" expression level display a mean transcription level from combined results of eighteen distinct colonies derived from three *in vitro* triazole-adapted lineages. Methods for this experiment are available within the publication at doi: 10.1128/AAC.01466-20.

**Table 1 A(C.1), B(C.2), and C(C.3)** Alterations to many components which support chromosome segregation results in CIN.

**Table 2 (C.4)** Comparison of DNA Index and Voriconazole MIC for Each Lineage at Generation +9 and -5.

**Table 3 (C.5)** WGS with variant analysis revealed few mutations occurred in voriconazole-adapted lineages in genes known to influence triazole susceptibility

**Figures**

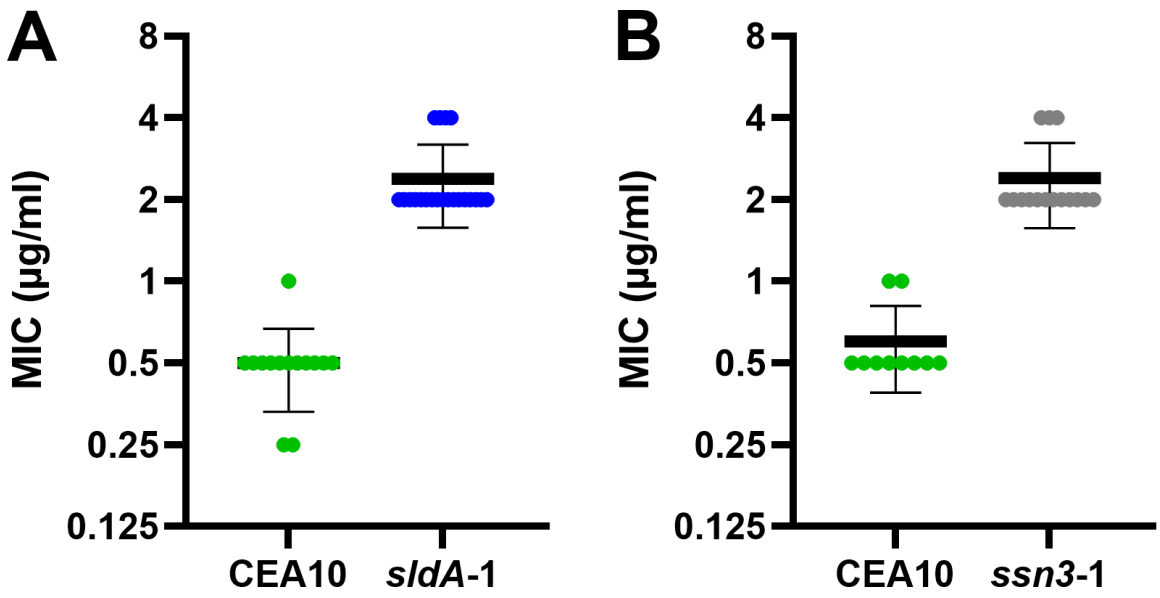


**Figure C.1: Figure 1: Disruption of the *sldA* and *ssn3* genes results in reduced triazole susceptibility**

.

.

.

.

.

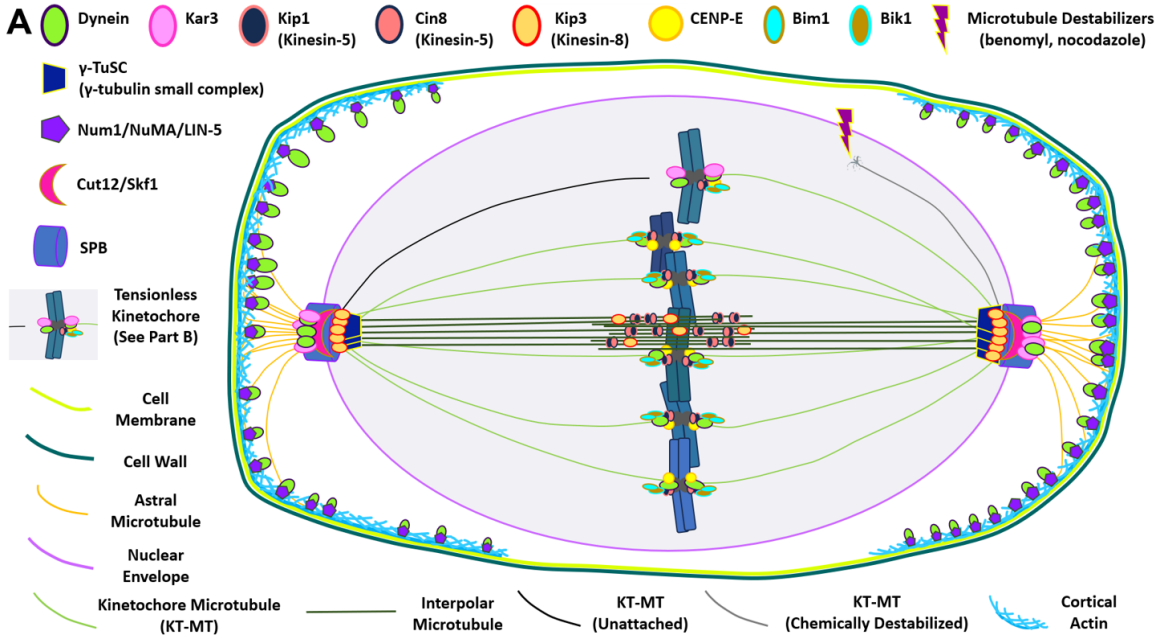


**Figure C.2: Figure 2A: Loss of various components supporting chromosome sorting results in CIN**

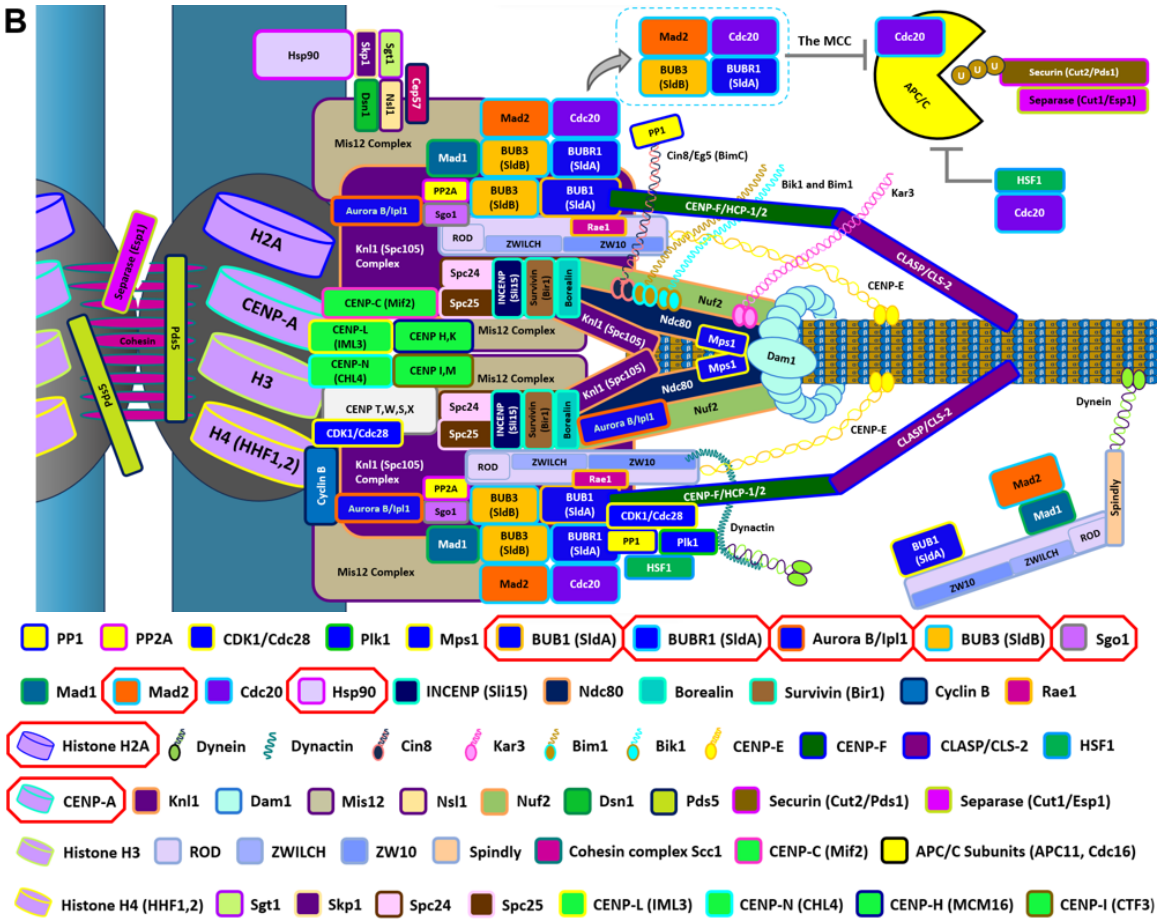


**Figure C.3: Figure 2B: Loss of various components supporting chromosome sorting results in CIN**

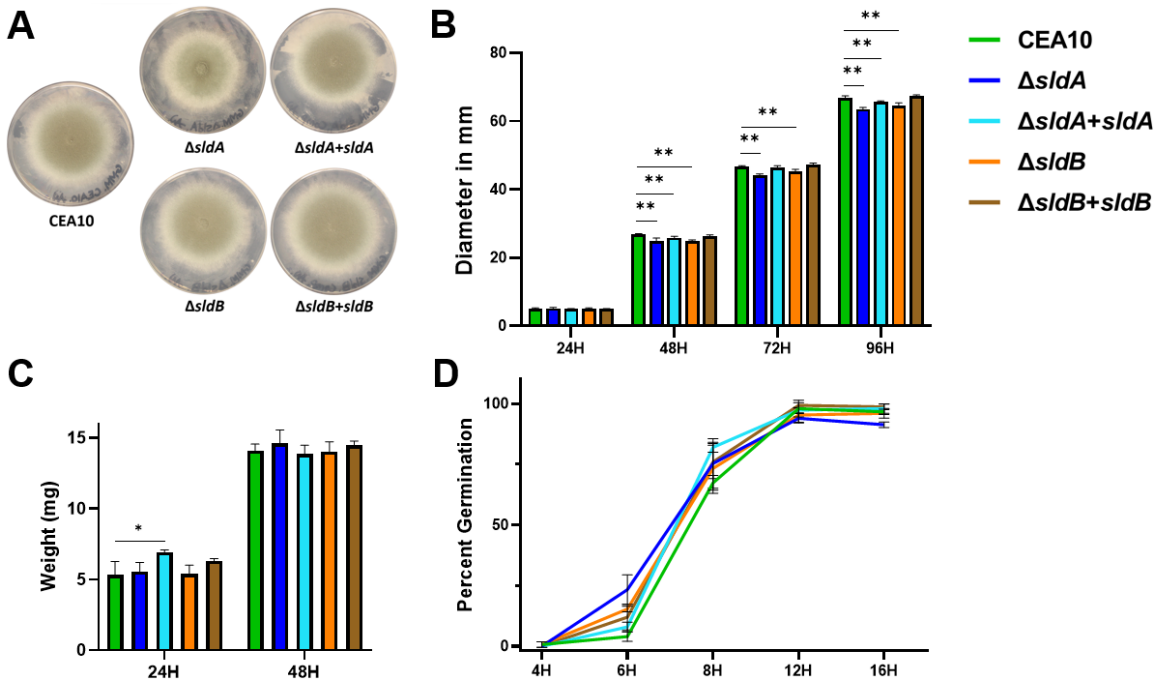


**Figure C.4: Figure 3: The SAC pathway components SldA and SldB are not required for normal growth and development in *A. fumigatus***

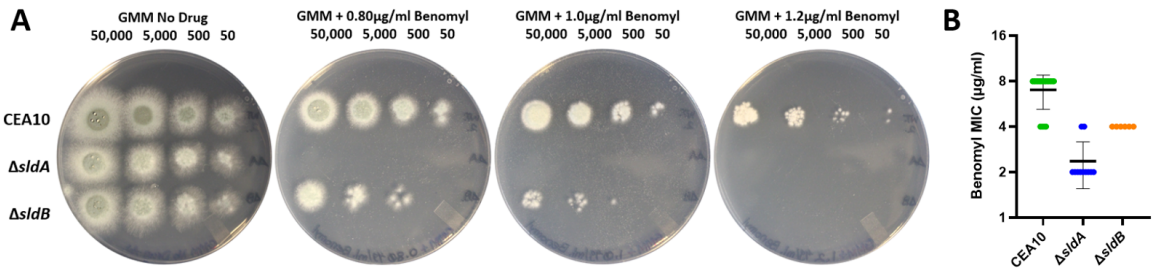


**Figure C.5: Figure 4: Loss of *sldA* or *sldB* results in increased susceptibility to benomyl**

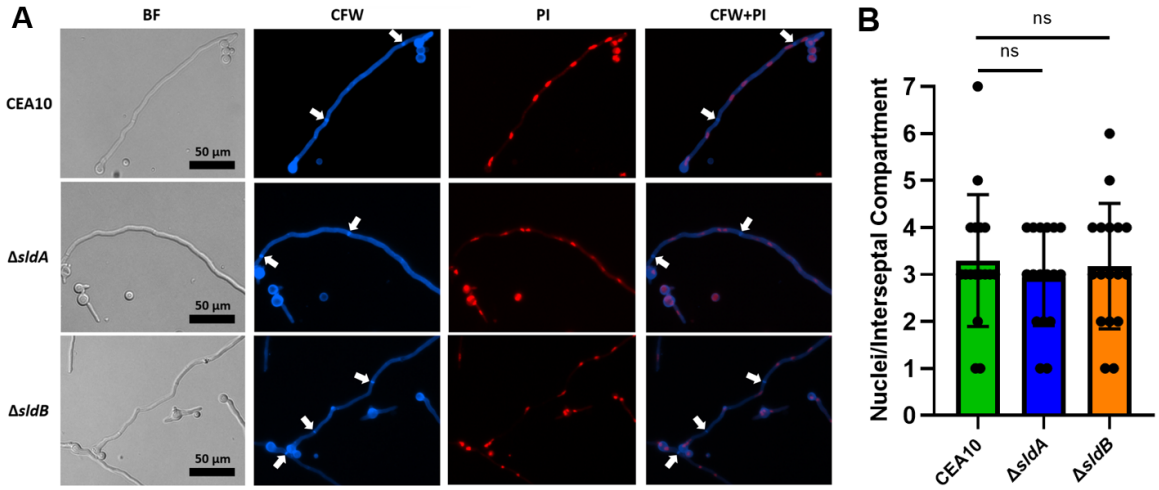


**Figure C.6: Figure 5: The SAC pathway components SldA and SldB are not required for normal nuclear number or positioning in *A. fumigatus***

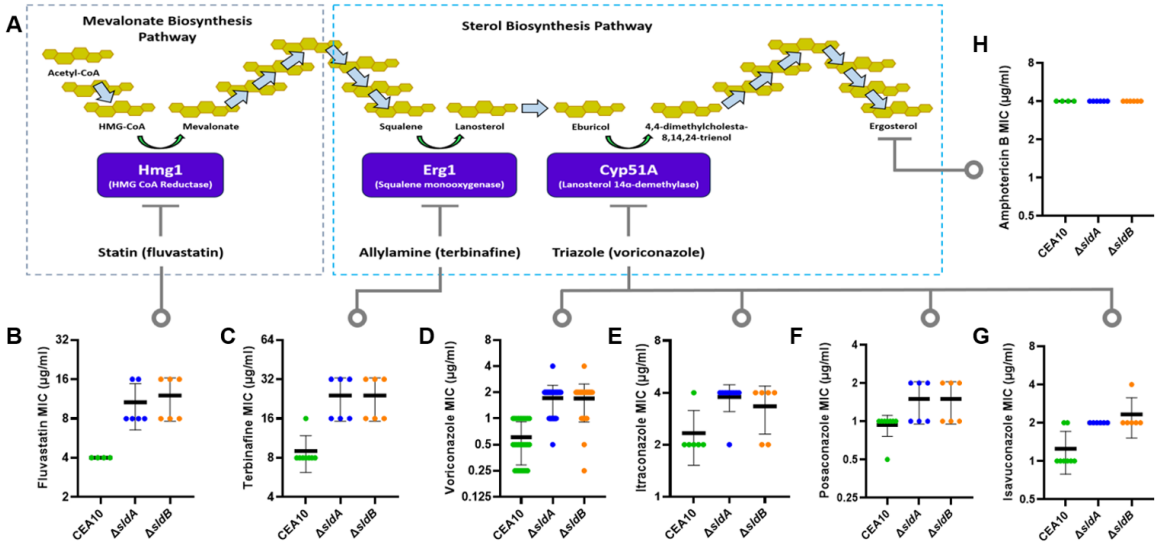


**Figure C.7: Figure 6: Loss of gene *sldA* or *sldB* reduces susceptibility to inhibitors of ergosterol biosynthesis**

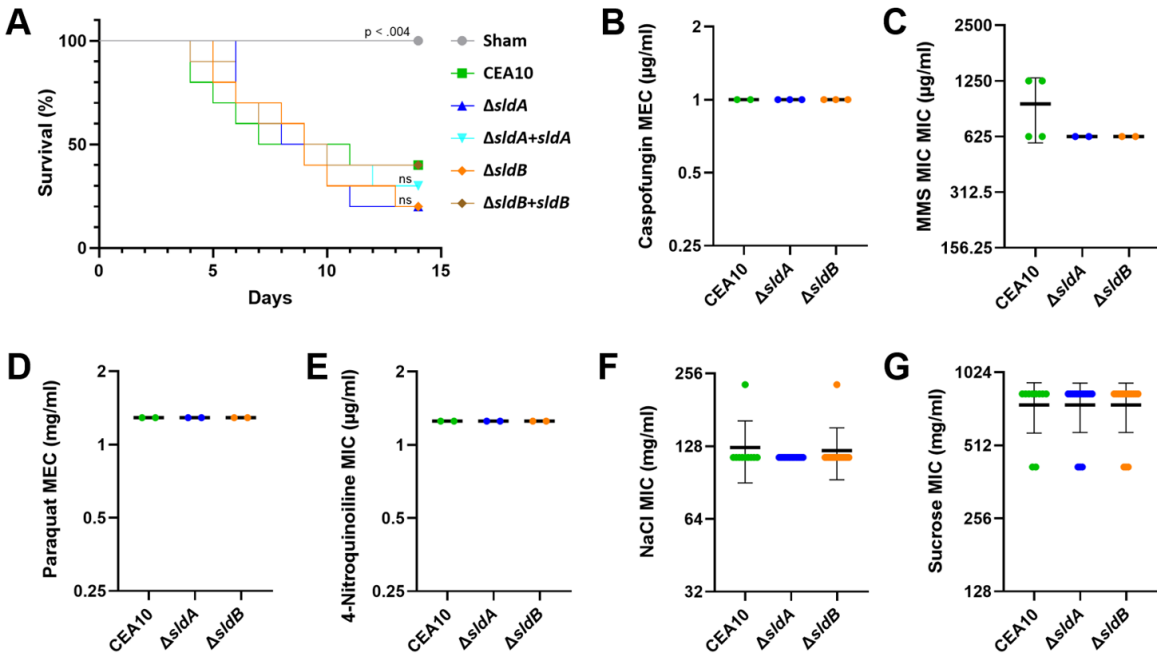


**Figure C.8: Figure 7: Loss of gene *sldA* or *sldB* does not impact susceptibility to varieties of cell wall, DNA damage, oxidative stress, or host stress**

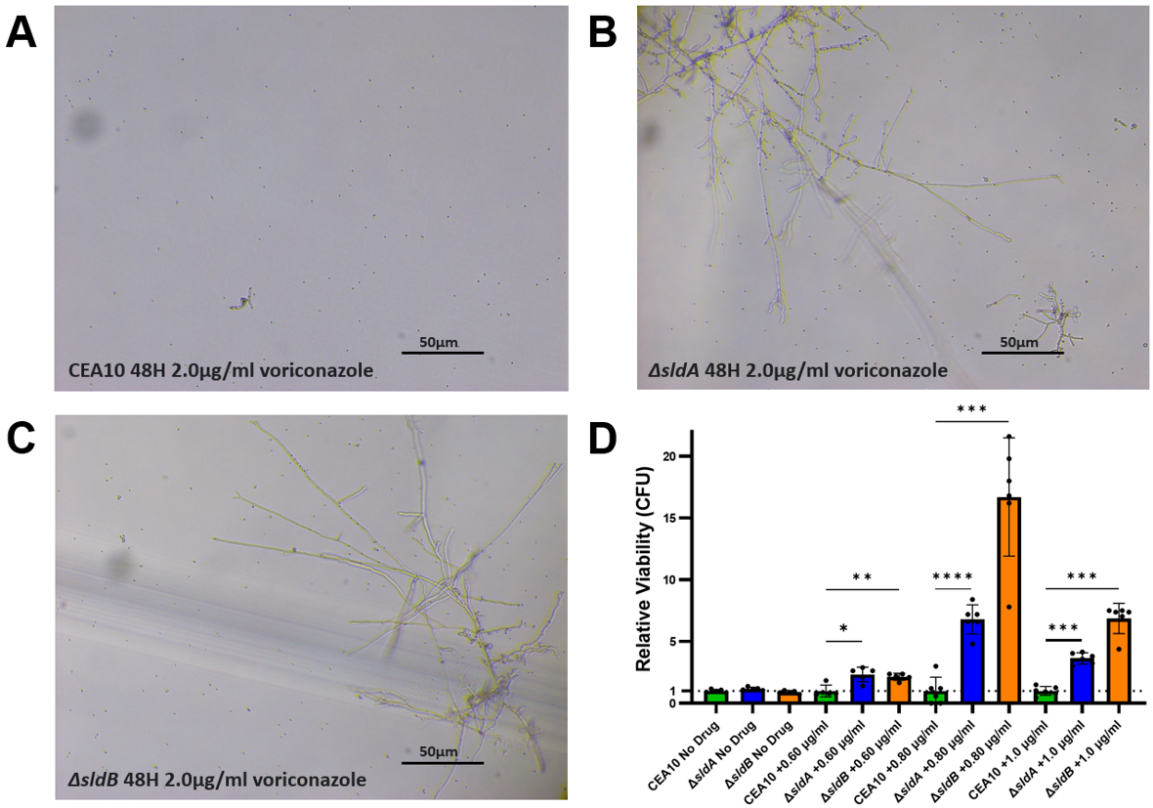


**Figure C.9: Figure 8: The Δ*sldA* and Δ*sldB* triazole tolerance phenotype is heterogeneous within the conidial population of each mutant**

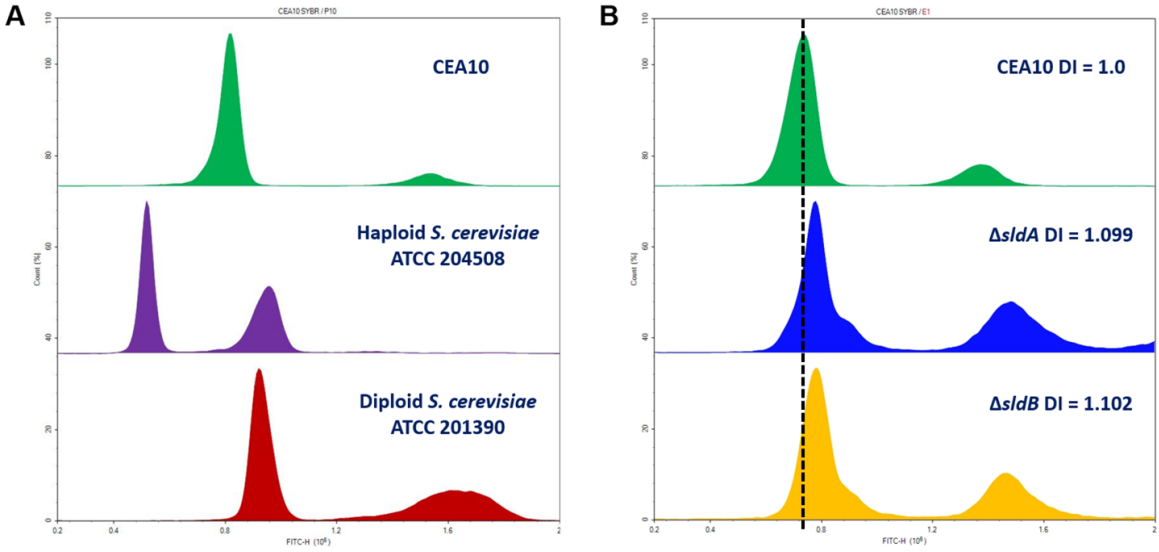


**Figure C.10: Figure 9: Conidia of the Δ*sldA* and Δ*sldB* mutants display higher mean DNA content by flow cytometric analysis**

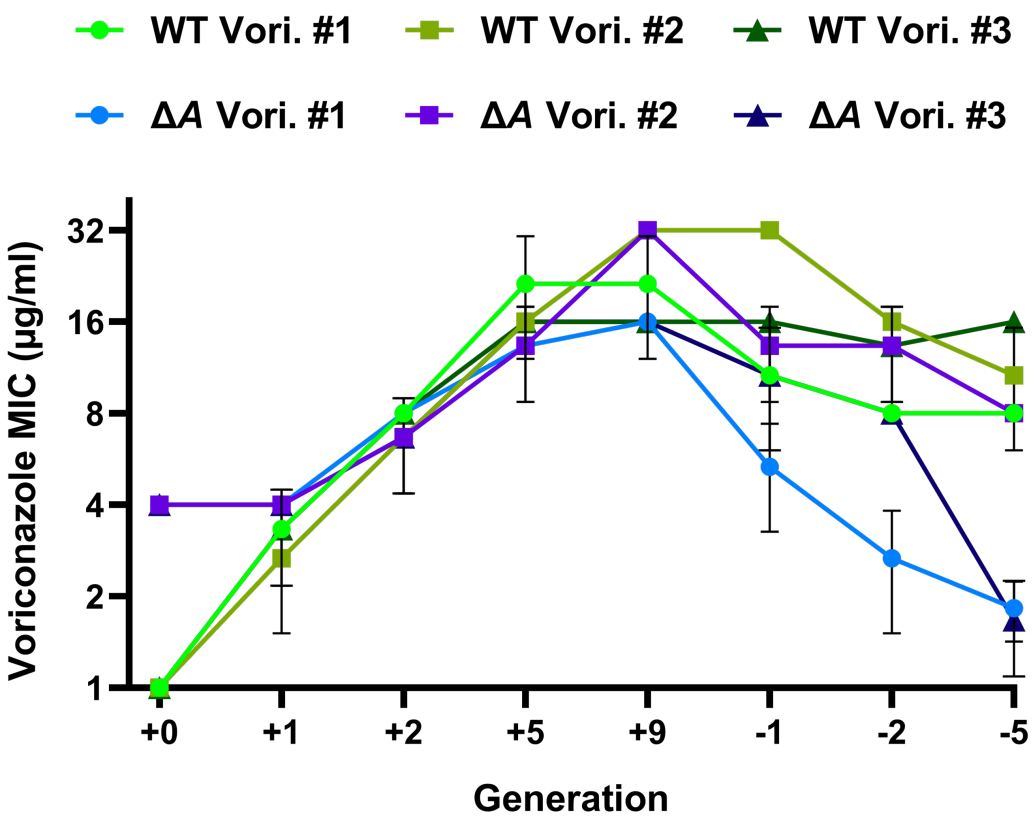


**Figure C.11: Figure 10: Repeated voriconazole exposure results in transient increases in triazole MIC**

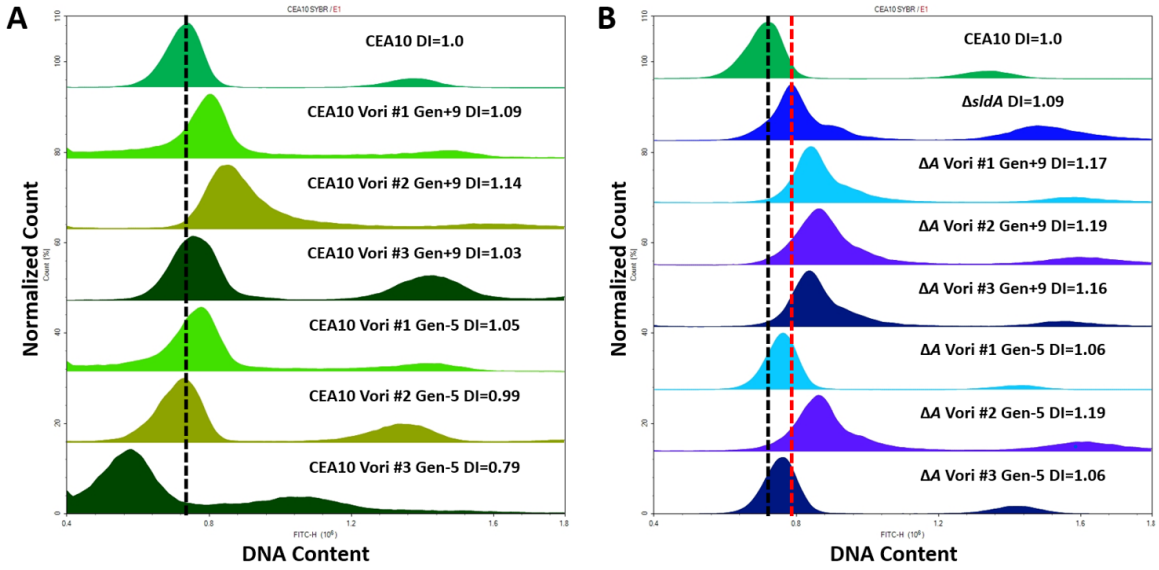


**Figure C.12: Figure 11: Repeated voriconazole exposure results in transient increases in conidial DNA content**

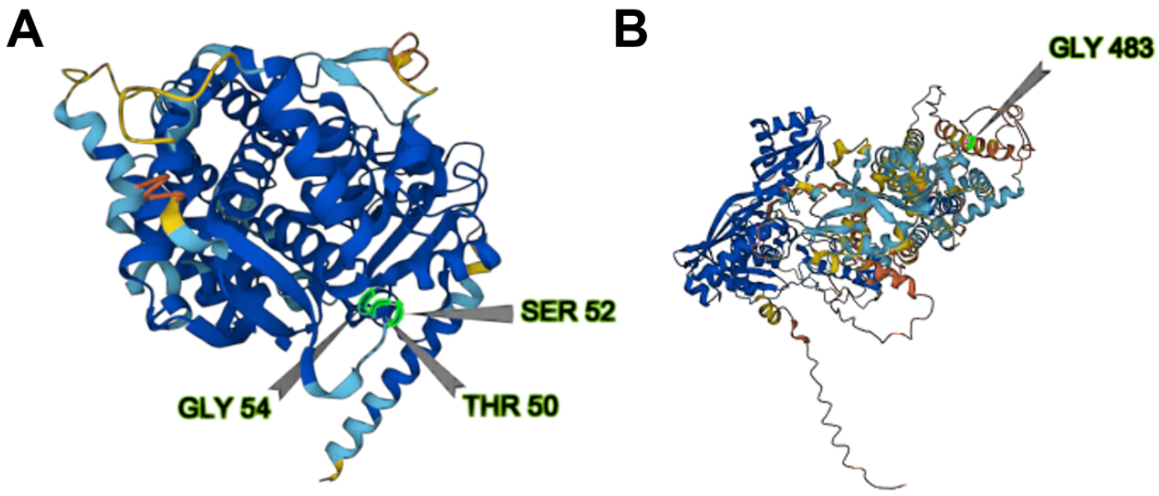


**Figure C.13: Figure 12: Location of singular mutations found in the genes encoding Cyp51A or Hmg1**

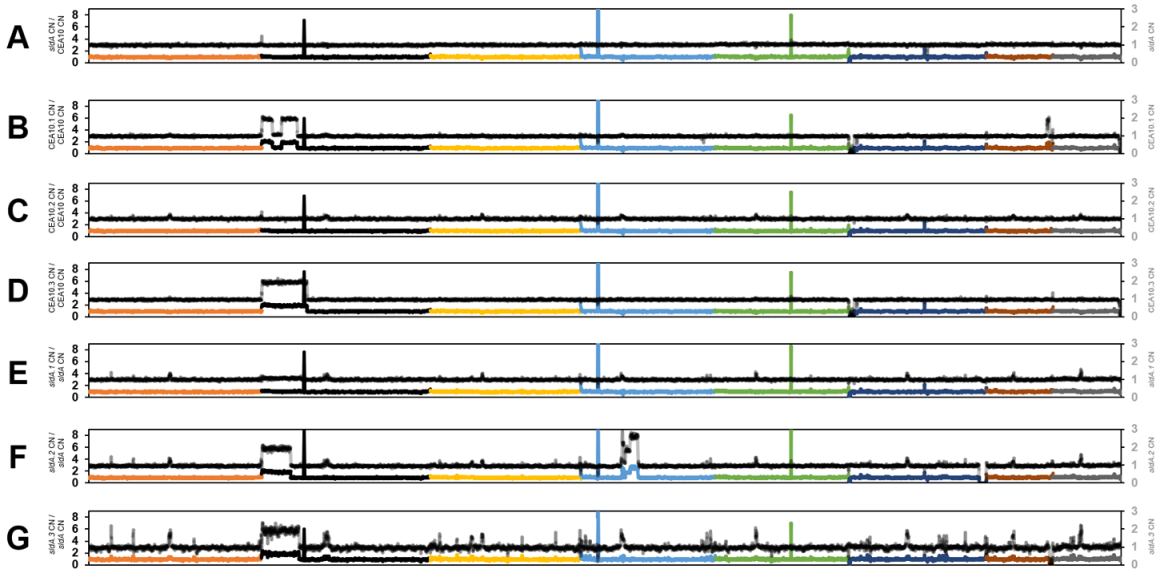


**Figure C.14: Figure 13: Copy number profiles across Δ*sldA* and lineages exposed to voriconazole**

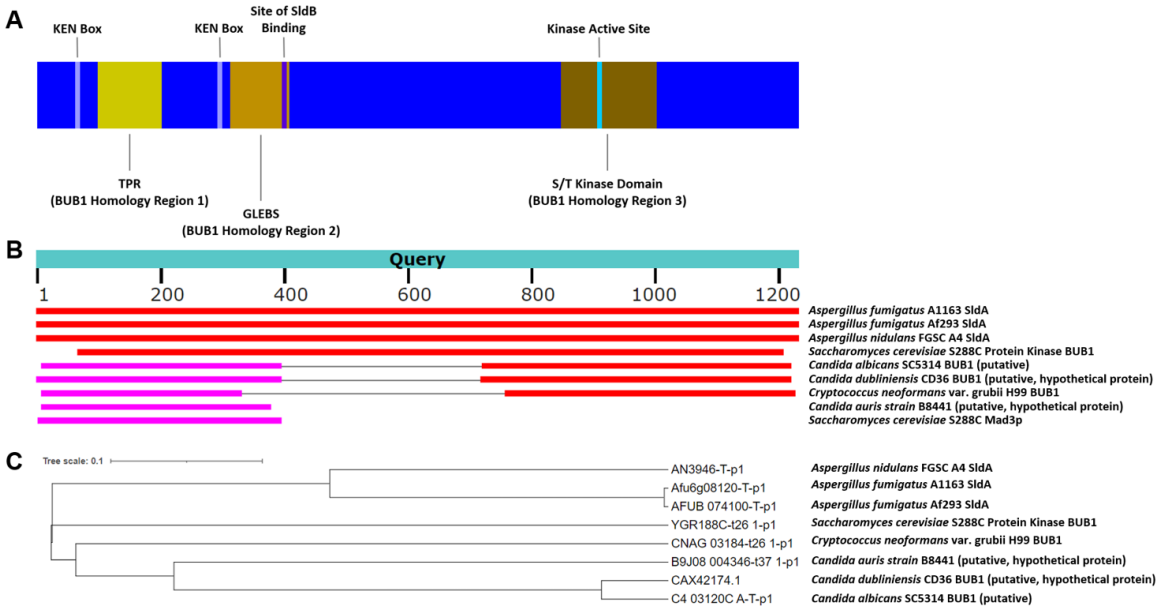


**Figure C.15: Supplemental Figure 1: Predicted functional domains and sequence homology of the *A. fumigatus SldA* kinase**

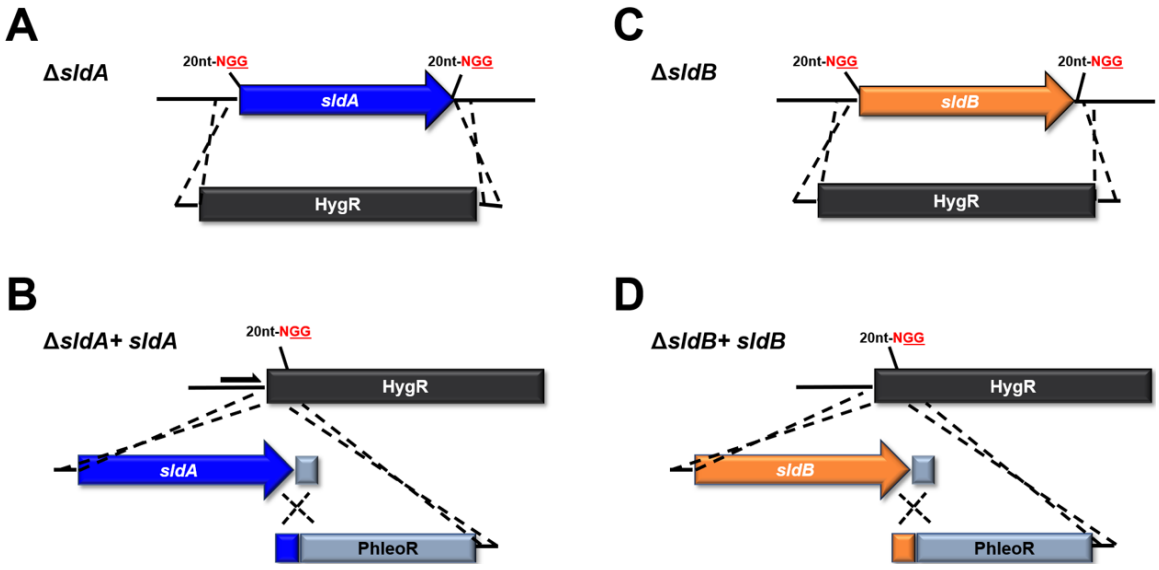


**Figure C.16: Supplemental Figure 2: Schematic for construction of gene deletion and gene complimented mutants**

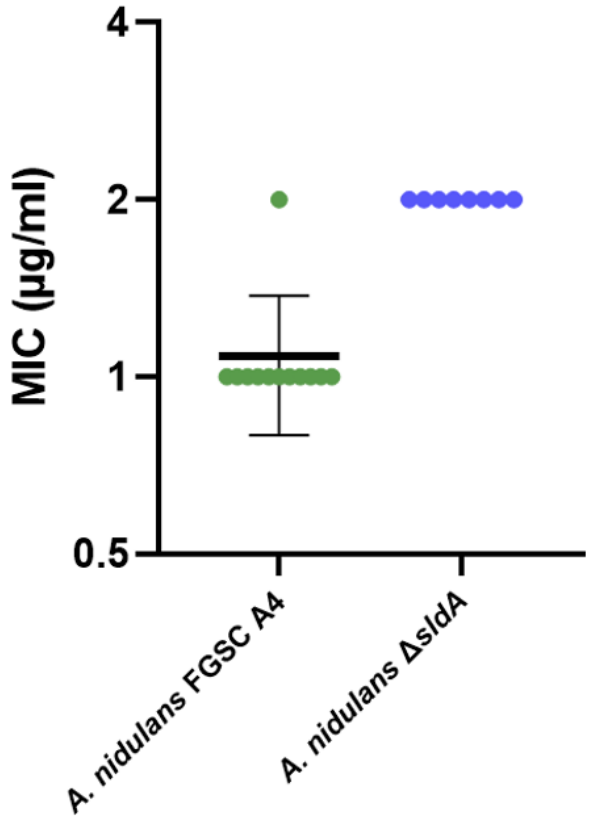


**Figure C.17: Supplemental Figure 3: The role of *sldA* in triazole susceptibility is conserved in *A. nidulans***

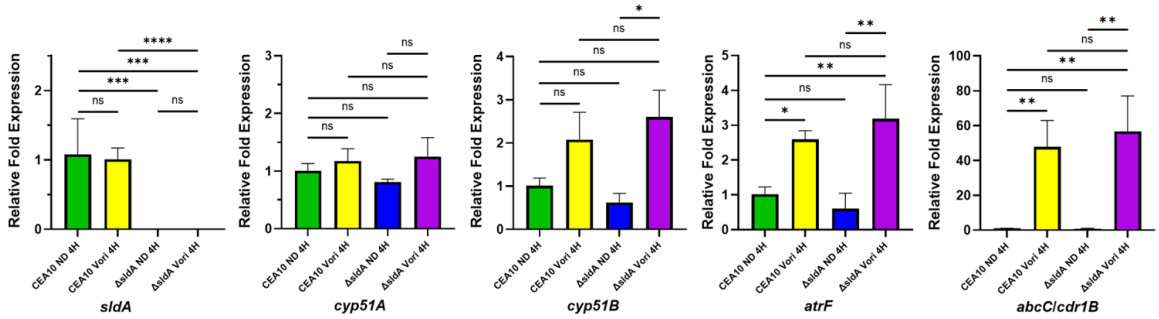


**Figure C.18: Supplemental Figure 4: Loss of *sldA* does not result in differential expression relative to *wild type* of genes commonly associated with triazole resistance**

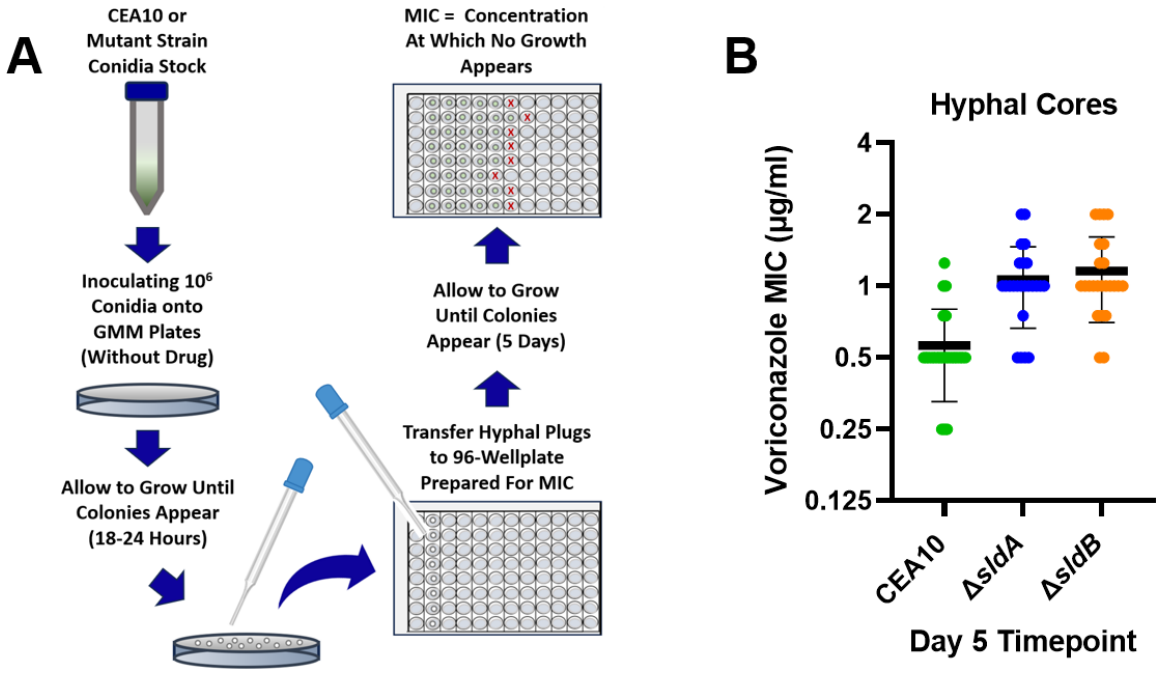


**Figure C.19: Supplemental Figure 5: The Δ*sldA* and Δ*sldB* mutant hyphae exhibit reduced triazole susceptibility**

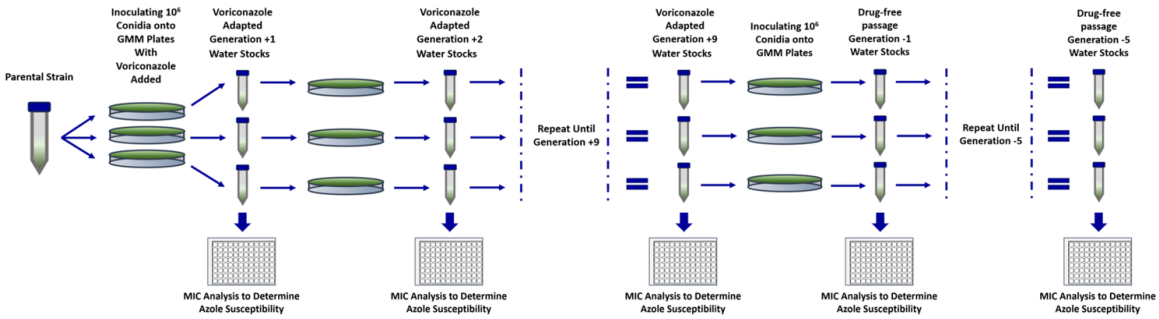


**Figure C.20: Supplemental Figure 6: Schematic of *in vitro* experimental adaptation to voriconazole**

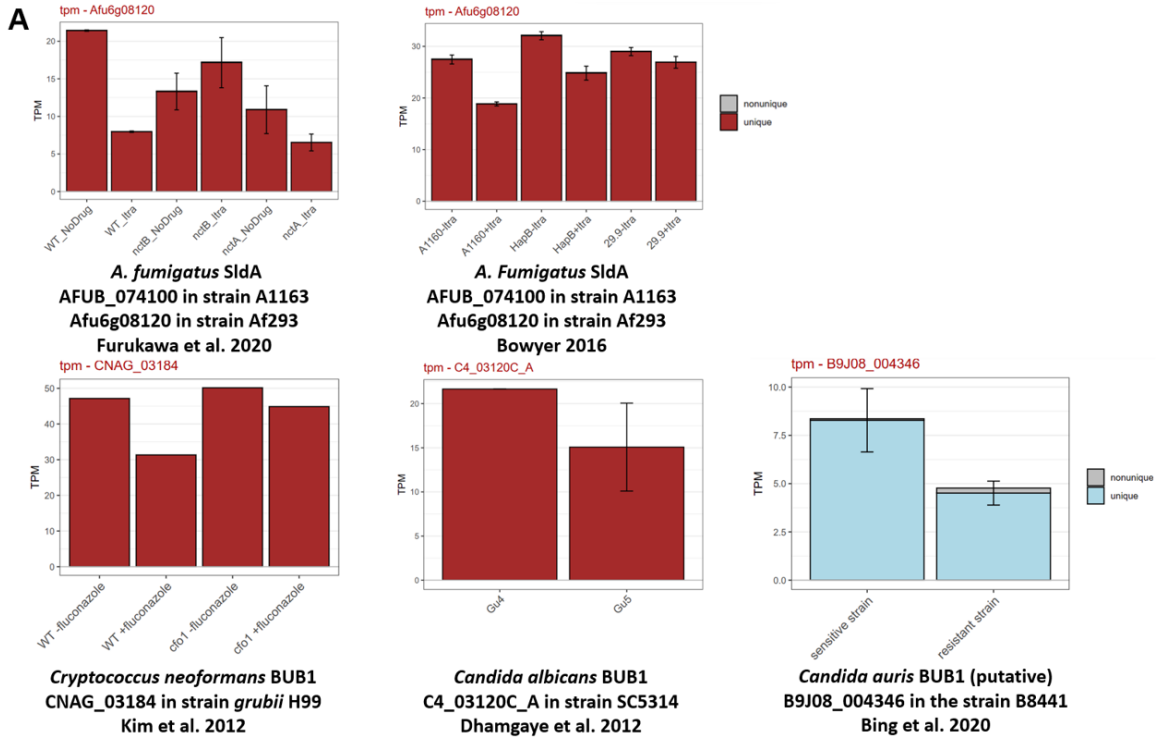


**Figure C.21: Supplemental Figure 7A: Transcriptomics data reveals SldA/BUB1 kinase ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts**

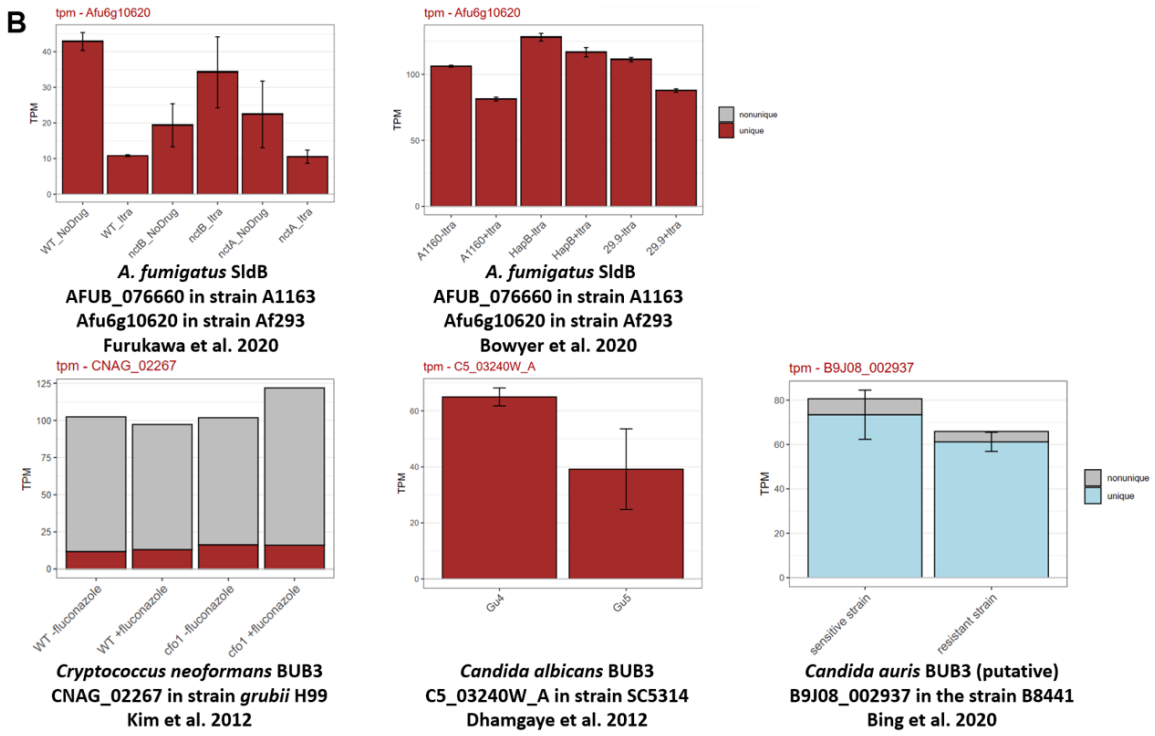


**Figure C.22: Supplemental Figure 7B: Transcriptomics data reveals SldB/BUB3 ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts**

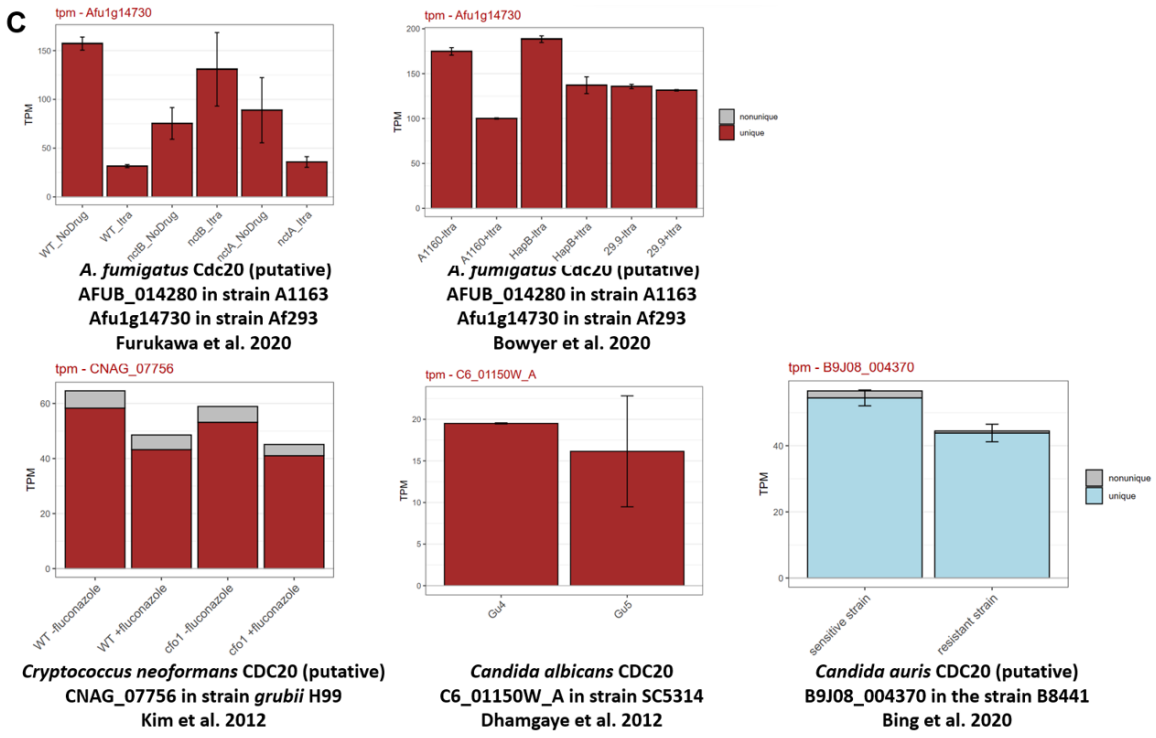


**Figure C.23: Supplemental Figure 7C: Transcriptomics data reveals Cdc20 ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts**

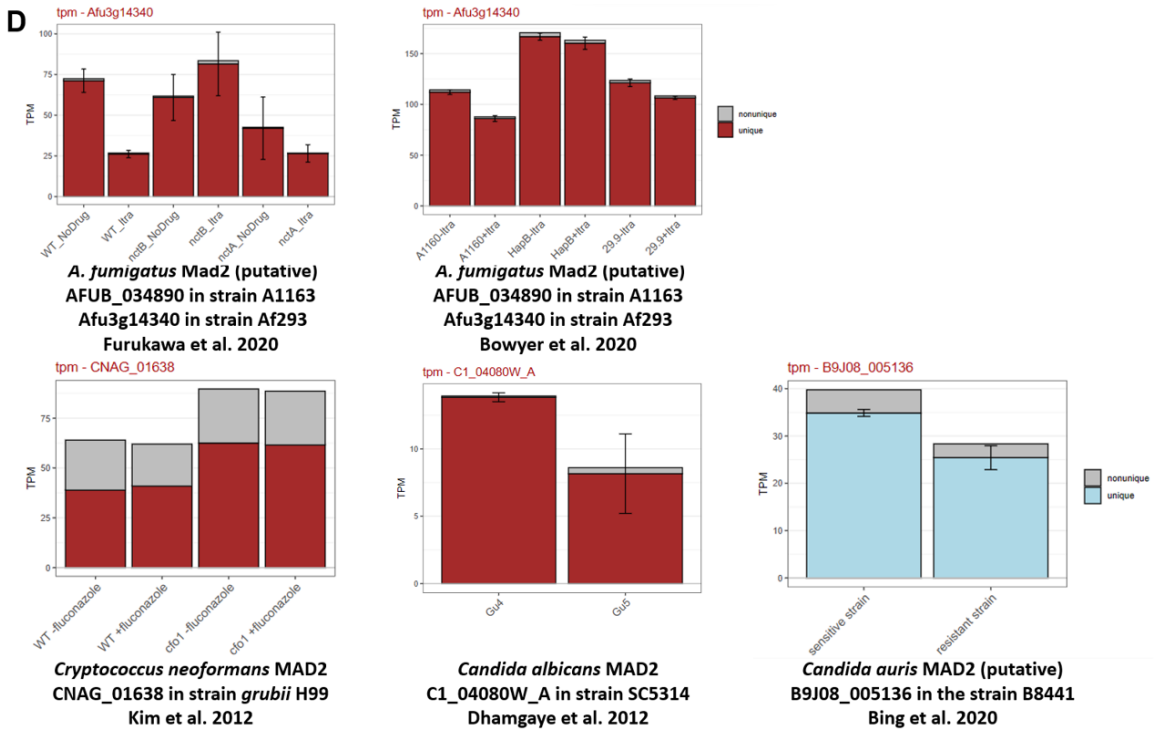


**Figure C.24: Supplemental Figure 7D: Transcriptomics data reveals Mad2 ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts**

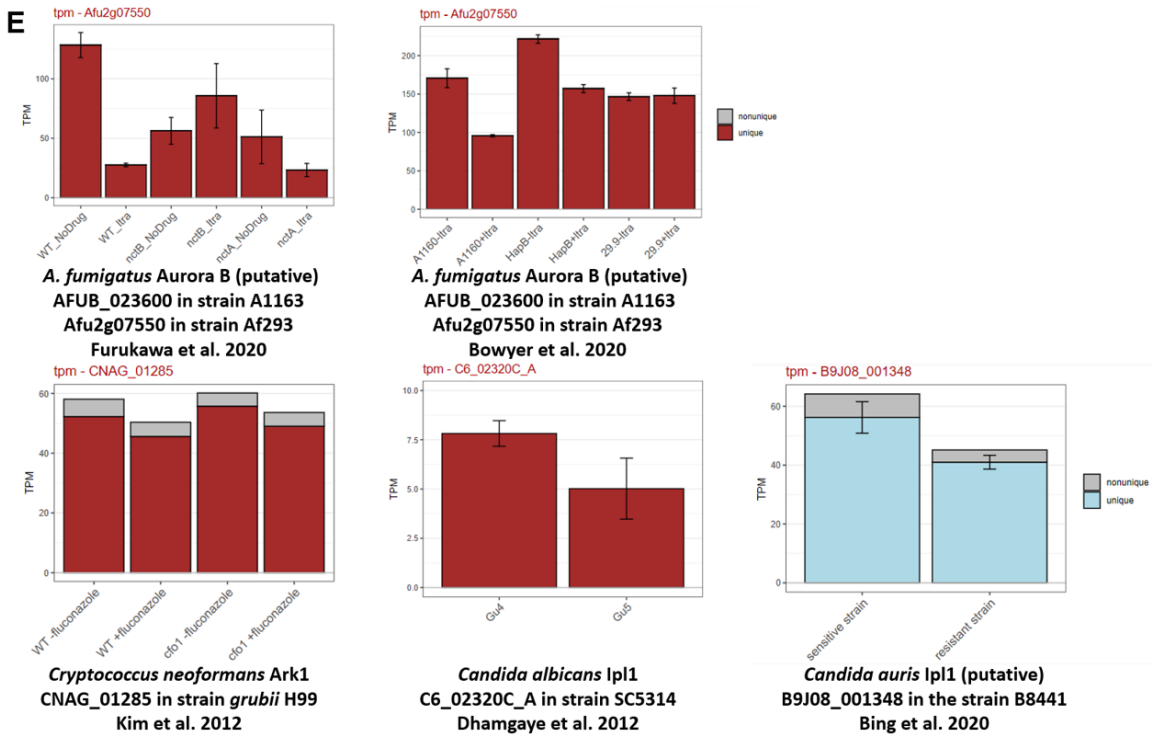


**Figure C.25: Supplemental Figure 7E: Transcriptomics data reveals Aurora B kinase ortholog expression among fungal pathogens often appears mildly to moderately reduced in triazole contexts**

**Table C.1:** Table 1 Part A: Alterations to many components which support chromosome segregation results in CIN

| |
|---|
| **APC/C Subunit APC11 Mutation:** (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). |
| **APC/C Subunit APC11 Overexpression:** (Skrzypek and Hirschman 2011, Tutaj, Pogoda et al. 2019). |
| **APC/C Subunit CDC16 Mutation:** (Hartwell and Smith 1985, Skrzypek and Hirschman 2011). |
| **Aurora B/Ipl1:Aurora B/Ipl1 Overexpression:** (Meraldi, Honda et al. 2004). |
| **Aurora B/Ipl1 Underexpression:** (Varshney and Sanyal 2019). |
| **Aurora B/Ipl1 Mutation:** (Francisco, Wang et al. 1994, Biggins, Severin et al. 1999, Kim, Kang et al. 1999, Cheeseman, Anderson et al. 2002, Pinsky, Kung et al. 2006, Sandall, Severin et al. 2006). |
| **Bik1:** (Skrzypek and Hirschman 2011, Prajapati, Rizvi et al. 2017). |
| **Bim1/EB1:** (Skrzypek and Hirschman 2011, Loll-Krippleber, Feri et al. 2015, Prajapati, Rizvi et al. 2017). |
| **Bir1/Survivin Deletion:** (Sandall, Severin et al. 2006, Skrzypek and Hirschman 2011). |
| **Borealin/Nbl1p:** (Nakajima, Tyers et al. 2009). |
| **BUB1 Overexpression:** (Pinsky, Kung et al. 2006, Ricke, Jeganathan et al. 2011, Ricke, Jeganathan et al. 2012, Fujibayashi, Isa et al. 2020). |
| **BUB1 Underexpression:** (Meraldi and Sorger 2005, Fernius and Hardwick 2007, Jeganathan, Malureanu et al. 2007, Niikura, Dixit et al. 2007, Baker, Jin et al. 2009, Ricke, Jeganathan et al. 2012, Funk, Zasadil et al. 2016, Carvalhal, Bader et al. 2022). |
| **BUBR1 Underexpression:** (Dai, Wang et al. 2004, Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Suijkerbuijk, van Osch et al. 2010, Suijkerbuijk, van Dam et al. 2012, Giam and Rancati 2015). |
| **BUB1 Mutation:** (Basu, Bousbaa et al. 1999, Jeganathan, Malureanu et al. 2007, Yuen, Warren et al. 2007, Carvalhal, Bader et al. 2022). |
| **BUBR1 (BUB1B) Mutation:** (Hanks, Coleman et al. 2004, Matsuura, Matsumoto et al. 2006, Suijkerbuijk, van Osch et al. 2010, Suijkerbuijk, van Dam et al. 2012). |
| **BUB1 Deletion:** (Bernard, Hardwick et al. 1998, Brimacombe, Burke et al. 2019). |
| **BUB3 (SldB) Mutation:** (Babu, Jeganathan et al. 2003, Yuen, Warren et al. 2007, Giam and Rancati 2015). |
| **Cdc20:** (Hartwell and Smith 1985, Li, Fang et al. 2009, Li, Fang et al. 2010, Thompson, Bakhoum et al. 2010, Giam and Rancati 2015, Loll-Krippleber, Feri et al. 2015, Hu, Jin et al. 2022). |
| **Cdc28/CDK1:** (Yu, Jiang et al. 1996, Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). |
| **CENP-A Overexpression:** (Tomonaga, Matsushita et al. 2003, Heun, Erhardt et al. 2006, Shrestha, Rossi et al. 2021). |
| **CENP-A Underexpression:** (Blower and Karpen 2001, Heun, Erhardt et al. 2006, Brimacombe, Burke et al. 2019). |
| **CENP-E/CENP-meta/Kinesin 7:** (Chan, Schaar et al. 1998, Chan, Jablonski et al. 1999, Putkey, Cramer et al. 2002, Thompson, Bakhoum et al. 2010, Giam and Rancati 2015, Resende, Monteiro et al. 2018, Yu, Zhong et al. 2019, She, Xu et al. 2022). |
| **CENP-F/HCP-1/2 Depletion:** (Cheeseman, MacLeod et al. 2005, Thompson, Bakhoum et al. 2010). |
| **CENP-H/MCM16:** (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011). |
| **CENP-I/CTF3:** (Kouprina, Pashina et al. 1988, Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011, Tirupataiah, Jamir et al. 2014, Tutaj, Pogoda et al. 2019). |

**Table C.2:** Table 1 Part B: Alterations to many components which support chromosome segregation results in CIN

| |
|---|
| **CENP-L/IML3:** (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011). |
| **CENP-N/CHL4:** (Daniel, Keyes et al. 2006, Yuen, Warren et al. 2007, Andersen, Nelson et al. 2008, Skrzypek and Hirschman 2011). |
| **CEP57:** (Matsuura, Matsumoto et al. 2006, Snape, Hanks et al. 2011, Seyedmousavi, Guillot et al. 2015). |
| **Cin8 (BimC/Kinesin-5/Eg5) alone:** (Kovacovicova, Awadova et al. 2016, Mittal, Ghule et al. 2020, She, Zhong et al. 2020). |
| **Cin8/BimC/Eg5 and Kip1 simultaneously:** (Saunders, Koshland et al. 1995, Mittal, Ghule et al. 2020). |
| **CLASP/CLS-2 Mutation:** (Pereira, Pereira et al. 2006). |
| **CLASP/CLS-2 Depletion:** (Cheeseman, MacLeod et al. 2005, Thompson, Bakhoum et al. 2010). |
| **Cut12/Skf1:** (Troxell, Sweezy et al. 2001). |
| **Cyclin B Mutation:** (Gehmlich, Haren et al. 2004). |
| **Dam1-DASH-DDD Complex Mutation:** (Cheeseman, Anderson et al. 2002, Meraldi, Honda et al. 2004, Kitagawa and Lee 2015, Jin, Bokros et al. 2017). |
| **Dsn1:** (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011). |
| **Dynein:** (Gehmlich, Haren et al. 2004, Prevo, Cheerambathur et al. 2023). |
| **Dynactin (p27) Deletion:** (Gama, Pereira et al. 2017). |
| **Histone H2A Mutation:** (Pinto and Winston 2000, Brimacombe, Burke et al. 2019). |
| **Histone H3 (Underexpression):** (Roschke and Kirsch 2010, Ragusa and Vagnarelli 2023). |
| **Histone H4 (HHF1,2):** (Yuen, Warren et al. 2007, Skrzypek and Hirschman 2011, Gordon, Zhu et al. 2023). |
| **HSF1 Overexpression:** (Dai 2018). |
| **Hsp90:** (Chen, Bradford et al. 2012). |
| **INCENP/Sli15 Mutation:** (Sandall, Severin et al. 2006). |
| **Kar3/Cik1:** (Chan, Jablonski et al. 1999, Mayer, Pot et al. 2004, Tytell and Sorger 2006, Skrzypek and Hirschman 2011, Jin, Liu et al. 2012, Duffy, Fam et al. 2016, Suzuki, Gupta et al. 2018, Morard, Macias et al. 2019, Mittal, Ghule et al. 2020). |
| **Kip1:** (Skrzypek and Hirschman 2011, Mittal, Ghule et al. 2020). |
| **Kip3:** (Skrzypek and Hirschman 2011, Duffy, Fam et al. 2016, Mittal, Ghule et al. 2020). |
| **Knl1/Spc105 Mutation:** (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011, Omer Javed, Li et al. 2018, Angrisani and Fachinetti 2023). |
| **Knl1/Spc105 Depletion:** (Pitayu-Nugroho, Aubry et al. 2023). |
| **Mad1 Deletion:** (Li and Murray 1991, Kitagawa and Rose 1999, Giam and Rancati 2015). |
| **Mad2:** (Kitagawa and Rose 1999, Dobles, Liberal et al. 2000, Bai, Ramanan et al. 2002, Skrzypek and Hirschman 2011, Giam and Rancati 2015, Vossen, Alhosawi et al. 2019). |
| **Microtubule/Spindle Chemical Stress (Benzimidazoles):** (Chen, Bradford et al. 2012, Akbari Dana, Hashemi et al. 2019). |
| **Mif2:** (Meeks-Wagner, Wood et al. 1986, Brown, Goetsch et al. 1993, Meluh and Koshland 1995, Skrzypek and Hirschman 2011). |

**Table C.3:** Table 1 Part C: Alterations to many components which support chromosome segregation results in CIN

| |
|---|
| **Misalignment of the spindle:** (Tame, Raaijmakers et al. 2014). |
| **Mis12 Mutation:** (Takahashi, Yamada et al. 1994, Venkei, Przewloka et al. 2011). |
| **Mps1:** (Giam and Rancati 2015). |
| **Ndc80:** (Kline-Smith, Sandall et al. 2005, Zhu, Sherlock et al. 2016, Chen, Tresenrider et al. 2017). |
| **Nsl1:** (Nekrasov, Smith et al. 2003, Skrzypek and Hirschman 2011, Venkei, Przewloka et al. 2011). |
| **Nuf2:** (Skrzypek and Hirschman 2011, Cheng, Vaisica et al. 2012, Chen, Tresenrider et al. 2017). |
| **Num1/nuMA/LIN-5:** (Lorson, Horvitz et al. 2000, Gehmlich, Haren et al. 2004, Haren, Gnadt et al. 2009, Silk, Holland et al. 2009, van Toorn, Gooch et al. 2023). |
| **Pds5/Spo76/BimD:** (Wang, Read et al. 2002). |
| **Plk1/Polo Mutation:** (Barbosa, Martins et al. 2020). |
| **Rae1 Mutation:** (Babu, Jeganathan et al. 2003). |
| **RZZ Inhibition:** (Gama, Pereira et al. 2017). |
| **RZZ Component "Rough Deal"/ROD:** (Karess and Glover 1989, Barbosa, Sunkel et al. 2022). |
| **RZZ Component "Zeste-White 10"/ZW10:** (Williams, Karr et al. 1992, Barbosa, Sunkel et al. 2022). |
| **RZZ Component ZWILCH:** (Williams, Li et al. 2003). |
| **Separase/Cut1/Esp1 Mutation:** (Funabiki, Kumada et al. 1996, Lianga, Dore et al. 2018). |
| **Separase/Cut1/Esp1 Overexpression:** (Zhang, Ge et al. 2008). |
| **Securin/Cut2/Pds1 Mutation:** (Funabiki, Kumada et al. 1996, Yamamoto, Guacci et al. 1996). |
| **Sgt1:** (Kitagawa, Skowyra et al. 1999, Skrzypek and Hirschman 2011). |
| **Shugoshin Sgo1 Deletion:** (Brimacombe, Burke et al. 2019). |
| **Skp1:** (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). |
| **Smc1/SMC1A (cohesin complex member):** (Yi, Wang et al. 2017). |
| **Spc24:** (Wigge and Kilmartin 2001, Le Masson, Saveanu et al. 2002, Skrzypek and Hirschman 2011, Chen, Tresenrider et al. 2017). |
| **Spc25:** (Skrzypek and Hirschman 2011, Chen, Tresenrider et al. 2017). |
| **Spindly/SPDL1 Overexpression:** (Klimaszewska-Wisniewska, Buchholz et al. 2022). |
| **Spindly/SPDL-1 Depletion**: (Gassmann, Essex et al. 2008, Barbosa, Martins et al. 2020). |
| **TUB4, SPC97, or SPC98 (γ-tubulin small complex (γ-TuSC):** (Skrzypek and Hirschman 2011, Choy, O'Toole et al. 2013). |

**Table C.4:** Table 2: Comparison of DNA Index and Voriconazole MIC for Each Lineage at Generation +9 and -5

| Strain | DNA Index | Mean MIC (In GMM) |
|---|---|---|
| **CEA10** | **1** | **1.06** |
| **CEA10 Lineage 1 Gen+9** | **1.09** | **21.33** |
| **CEA10 Lineage 2 Gen+9** | **1.14** | **32** |
| **CEA10 Lineage 3 Gen+9** | **1.03** | **16** |
| **CEA10 Lineage 1 Gen-5** | **1.05** | **10.67** |
| **CEA10 Lineage 2 Gen-5** | **.99** | **16** |
| **CEA10 Lineage 3 Gen-5** | **0.79** | **16** |
| | | |
| **Δ*sldA*** | **1.09** | **4** |
| **Δ*sldA* Lineage 1 Gen+9** | **1.17** | **16** |
| **Δ*sldA* Lineage 2 Gen+9** | **1.19** | **32** |
| **Δ*sldA* Lineage 3 Gen+9** | **1.16** | **16** |
| **Δ*sldA* Lineage 1 Gen-5** | **1.06** | **1.83** |
| **Δ*sldA* Lineage 2 Gen-5** | **1.19** | **8** |
| **Δ*sldA* Lineage 3 Gen-5** | **1.06** | **1.67** |

**Table C.5:** Table 3: WGS with variant analysis revealed few mutations occurred in voriconazole-adapted lineages in genes known to influence triazole susceptibility

| Gene Name | Gene ID | Variant (Not Found in CEA10 Parental Strain) | DNA Source |
|---|---|---|---|
| *sldA* | AFUB_074100 | Δ*sldA* Mutation = *sldA* gene deletion | Hyphae |
| | | Δ*sldA* Lineage 1 Gen+9 Mutation = *sldA* gene deletion | Hyphae |
| | | Δ*sldA* Lineage 2 Gen+9 Mutation = *sldA* gene deletion | Hyphae |
| | | Δ*sldA* Lineage 3 Gen+9 Mutation = *sldA* gene deletion | Hyphae |
| | | Δ*sldA* Mutation = *sldA* gene deletion | Conidia |
| | | Δ*sldA* Lineage 1 Gen+9 Mutation = *sldA* gene deletion | Conidia |
| | | Δ*sldA* Lineage 2 Gen+9 Mutation = *sldA* gene deletion | Conidia |
| | | Δ*sldA* Lineage 3 Gen+9 Mutation = *sldA* gene deletion | Conidia |
| *sldB* | AFUB_076660 | None | NA |
| *cyp51A* | AFUB_063960 | Δ*sldA* Lineage 3 Gen+9 Mutation = T50I (ACC→ATC) | Conidia |
| *cyp51B* | AFUB_089270 | None | NA |
| *hmg1* ortholog result 1 | AFUB_020770 | CEA10 Lineage 3 Gen+9 Mutation = G483 deletion | Hyphae |
| | | CEA10 Lineage 3 Gen+9 Mutation = G483 deletion | Conidia |
| *hmg1* ortholog result 2 | AFUB_010660 | None | NA |
| *atrF* ortholog result 1 | AFUB_093930 | None | NA |
| *atrF* ortholog result 2 | AFUB_013880 | None | NA |
| *atrF* ortholog result 3 | AFUB_016810 | None | NA |
| *abcC* / *cdr1B* ortholog result 1 | AFUB_013880 | None | NA |
| *abcC* / *cdr1B* ortholog result 2 | AFUB_016810 | None | NA |
| *abcC* / *cdr1B* ortholog result 3 | AFUB_030790 | None | NA |
| *abcC* / *cdr1B* ortholog result 4 | AFUB_041770 | None | NA |
| *mdr1* ortholog result 1 | AFUB_045530 | None | NA |
| *mdr1* ortholog result 2 | AFUB_012160 | None | NA |
| *mdr1* ortholog result 3 | AFUB_044470 | None | NA |
| *mdr1* ortholog result 4 | AFUB_044820 | None | NA |
| *mdr1* ortholog result 5 | AFUB_053630 | None | NA |
| *mdr1* ortholog result 6 | AFUB_067110 | None | NA |
| *mdr1* ortholog result 7 | AFUB_071280 | None | NA |
| *mdr1* ortholog result 8 | AFUB_087060 | None | NA |
| *mdr1* ortholog result 9 | AFUB_094820 | None | NA |
| *mdr1* ortholog result 10 | AFUB_095220 | None | NA |
| *hapB* | AFUB_030360 | None | NA |
| *hapC* | AFUB_004250 | None | NA |
| *hapE* | AFUB_092980 | None | NA |
| *hapX* | AFUB_052420 | None | NA |
| *srbA* | AFUB_018340 | None | NA |
| *cdc20* | AFUB_014280 | None | NA |
| APC/C ortholog | AFUB_043100 | None | NA |
| *mad2* | AFUB_034890 | None | NA |
| *mps1* | AFUB_041010 | None | NA |
| *erg6* | AFUB_099400 | None | NA |
| *insA* | AFUB_064770 | None | NA |
| *hrdA* | AFUB_082650 | None | NA |
| Aurora Kinase A ortholog | AFUB_072000 | None | NA |
| Aurora Kinase B ortholog | AFUB_023600 | None | NA |
| Histone H2A.1 ortholog | AFUB_043640 | None | NA |
| Histone H2A.2 ortholog | AFUB_043640 | None | NA |
| Histone H3 ortholog | AFUB_013270 | None | NA |
| PP2A Regulatory Subunit A | AFUB_005980 | None | NA |
| PP2A Regulatory Subunit B | AFUB_082000 | None | NA |
| Securin ortholog result 1 | AFUB_052380 | None | NA |
| Securin ortholog result 2 | AFUB_015180 | None | NA |
| Cohesin Complex Subunit ortholog result 1 | AFUB_040850 | None | NA |
| Cohesin Complex Subunit ortholog result 2 | AFUB_095400 | None | NA |
| Cohesin Complex Subunit ortholog result 3 | AFUB_022880 | None | NA |
| Separase | AFUB_057260 | None | NA |
| topoisomerase II | AFUB_001050 | None | NA |
| topoisomerase II associated protein | AFUB_058370 | None | NA |

**Acknowledgements**

This work was supported by (R21AI142509 and R21AI139388) to JRF and by (R01AI143197) to PDR and JRF. All authors declare no conflict of interest.

## References

Akbari Dana, M., S. J. Hashemi, R. Daei Ghazvini, S. Khodavesi, M. Modiri, L. Nazemi, S. Darabian and S. Rezaie (2019). "Effect of benomyl and diazinon on acquired azole resistance in Aspergillus flavus and expression of mdr1 and cyp51c genes." Curr Med Mycol 5(2): 27-32.

Al Abdallah, Q., W. Ge and J. R. Fortwendel (2017). "A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus: In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates." mSphere 2(6).

Al Abdallah, Q., A. C. O. Souza, A. Martin-Vicente, W. Ge and J. R. Fortwendel (2018). "Whole-genome sequencing reveals highly specific gene targeting by in vitro assembled Cas9-ribonucleoprotein complexes in Aspergillus fumigatus." Fungal Biol Biotechnol 5: 11.

Altamirano, S., D. Fang, C. Simmons, S. Sridhar, P. Wu, K. Sanyal and L. Kozubowski (2017). "Fluconazole-Induced Ploidy Change in Cryptococcus neoformans Results from the Uncoupling of Cell Growth and Nuclear Division." mSphere 2(3).

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool." J Mol Biol 215(3): 403-410.

Andersen, M. P., Z. W. Nelson, E. D. Hetrick and D. E. Gottschling (2008). "A genetic screen for increased loss of heterozygosity in Saccharomyces cerevisiae." Genetics 179(3): 1179-1195.

Ando, K., Y. Kakeji, H. Kitao, M. Iimori, Y. Zhao, R. Yoshida, E. Oki, K. Yoshinaga, T. Matumoto, M. Morita, Y. Sakaguchi and Y. Maehara (2010). "High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer." Cancer Sci 101(3): 639-645.

Angrisani, A. and D. Fachinetti (2023). "The KaryoCreate technology generates specific aneuploid karyotypes on demand." Cell Rep Methods 3(6): 100514.

Aruanno, M., E. Glampedakis and F. Lamoth (2019). "Echinocandins for the Treatment of Invasive Aspergillosis: from Laboratory to Bedside." Antimicrob Agents Chemother 63(8).

Babu, J. R., K. B. Jeganathan, D. J. Baker, X. Wu, N. Kang-Decker and J. M. van Deursen (2003). "Rae1 is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation." J Cell Biol 160(3): 341-353.

Badali, H., C. Canete-Gibas, D. McCarthy, H. Patterson, C. Sanders, M. P. David, J. Mele, H. Fan and N. P. Wiederhold (2022). "Species Distribution and Antifungal Susceptibilities of Aspergillus Section Fumigati Isolates in Clinical Samples from the United States." J Clin Microbiol 60(5): e0028022.

Bai, C., N. Ramanan, Y. M. Wang and Y. Wang (2002). "Spindle assembly checkpoint component CaMad2p is indispensable for Candida albicans survival and virulence in mice." Mol Microbiol 45(1): 31-44.

Baker, D. J., F. Jin, K. B. Jeganathan and J. M. van Deursen (2009). "Whole chromosome instability caused by Bub1 insufficiency drives tumorigenesis through tumor suppressor gene loss of heterozygosity." Cancer Cell 16(6): 475-486.

Bakhoum, S. F., S. L. Thompson, A. L. Manning and D. A. Compton (2009). "Genome stability is ensured by temporal control of kinetochore-microtubule dynamics." Nat Cell Biol 11(1): 27-35.

Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N. Vyahhi, G. Tesler, M. A. Alekseyev and P. A. Pevzner (2012). "SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing." J Comput Biol 19(5): 455-477.

Barbosa, J., T. Martins, T. Bange, L. Tao, C. Conde and C. Sunkel (2020). "Polo regulates Spindly to prevent premature stabilization of kinetochore-microtubule attachments." EMBO J 39(2): e100789.

Barbosa, J., C. E. Sunkel and C. Conde (2022). "The Role of Mitotic Kinases and the RZZ Complex in Kinetochore-Microtubule Attachments: Doing the Right Link." Front Cell Dev Biol 10: 787294.

Barda, O., S. Sadhasivam, D. Gong, A. Doron-Faigenboim, V. Zakin, M. T. Drott and E. Sionov (2023). "Aneuploidy Formation in the Filamentous Fungus Aspergillus flavus in Response to Azole Stress." Microbiol Spectr 11(4): e0433922.

Basenko, E. Y., J. A. Pulman, A. Shanmugasundram, O. S. Harb, K. Crouch, D. Starns, S. Warrenfeltz, C. Aurrecoechea, C. J. Stoeckert, Jr., J. C. Kissinger, D. S. Roos and C. Hertz-Fowler (2018). "FungiDB: An Integrated Bioinformatic Resource for Fungi and Oomycetes." J Fungi (Basel) 4(1).

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B. C. Williams, C. Lopes, C. E. Sunkel and M. L. Goldberg (1999). "Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila." J Cell Biol 146(1): 13-28.

Benedict, K., B. R. Jackson, T. Chiller and K. D. Beer (2019). "Estimation of Direct Healthcare Costs of Fungal Diseases in the United States." Clin Infect Dis 68(11): 1791-1797.

Bernard, P., K. Hardwick and J. P. Javerzat (1998). "Fission yeast bub1 is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis." J Cell Biol 143(7): 1775-1787.

Biggins, S., F. F. Severin, N. Bhalla, I. Sassoon, A. A. Hyman and A. W. Murray (1999). "The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast." Genes Dev 13(5): 532-544.

Bing, J., T. Hu, Q. Zheng, J. F. Munoz, C. A. Cuomo and G. Huang (2020). "Experimental Evolution Identifies Adaptive Aneuploidy as a Mechanism of Fluconazole Resistance in Candida auris." Antimicrob Agents Chemother 65(1).

Blower, M. D. and G. H. Karpen (2001). "The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions." Nat Cell Biol 3(8): 730-739.

Bolanos-Garcia, V. M. and T. L. Blundell (2011). "BUB1 and BUBR1: multifaceted kinases of the cell cycle." Trends Biochem Sci 36(3): 141-150.

Bowman, J. C., P. S. Hicks, M. B. Kurtz, H. Rosen, D. M. Schmatz, P. A. Liberator and C. M. Douglas (2002). "The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro." Antimicrob Agents Chemother 46(9): 3001-3012.

Bowyer, P., M. J. Bromley and D. W. Denning (2020). "Linking calcium signaling and mitochondrial function in fungal drug resistance." Proc Natl Acad Sci U S A 117(3): 1254-1256.

Bowyer, P., A. Currin, D. Delneri and M. G. Fraczek (2022). "Telomere-to-telomere genome sequence of the model mould pathogen Aspergillus fumigatus." Nat Commun 13(1): 5394.

Bradley, K., A. Le-Mahajan, B. Morris, T. Peritz, T. Chiller, K. Forsberg, N. S. Nunnally, S. R. Lockhart, J. A. W. Gold and J. M. Gould (2022). "Fatal Fungicide-Associated Triazole-Resistant Aspergillus fumigatus Infection, Pennsylvania, USA." Emerg Infect Dis 28(9): 1904-1905.

Brimacombe, C. A., J. E. Burke, J. Y. Parsa, S. Catania, T. R. O'Meara, J. N. Witchley, L. S. Burrack, H. D. Madhani and S. M. Noble (2019). "A natural histone H2A variant lacking the Bub1 phosphorylation site and regulated depletion of centromeric histone CENP-A foster evolvability in Candida albicans." PLoS Biol 17(6): e3000331.

Brown, M. T., L. Goetsch and L. H. Hartwell (1993). "MIF2 is required for mitotic spindle integrity during anaphase spindle elongation in Saccharomyces cerevisiae." J Cell Biol 123(2): 387-403.

Burgel, P. R., M. T. Baixench, M. Amsellem, E. Audureau, J. Chapron, R. Kanaan, I. Honore, J. Dupouy-Camet, D. Dusser, C. H. Klaassen, J. F. Meis, D. Hubert and A. Paugam (2012). "High prevalence of azole-resistant Aspergillus fumigatus in adults with cystic fibrosis exposed to itraconazole." Antimicrob Agents Chemother 56(2): 869-874.

Cadena, J., G. R. Thompson, 3rd and T. F. Patterson (2021). "Aspergillosis: Epidemiology, Diagnosis, and Treatment." Infect Dis Clin North Am 35(2): 415-434.

Cahill, D. P., C. Lengauer, J. Yu, G. J. Riggins, J. K. Willson, S. D. Markowitz, K. W. Kinzler and B. Vogelstein (1998). "Mutations of mitotic checkpoint genes in human cancers." Nature 392(6673): 300-303. Camahort, R., M. Shivaraju, M. Mattingly, B. Li, S. Nakanishi, D. Zhu, A. Shilatifard, J. L. Workman and J. L. Gerton (2009). "Cse4 is part of an octameric nucleosome in budding yeast." Mol Cell 35(6): 794-805.

Camps, S. M., B. E. Dutilh, M. C. Arendrup, A. J. Rijs, E. Snelders, M. A. Huynen, P. E. Verweij and W. J. Melchers (2012). "Discovery of a HapE mutation that causes azole resistance in Aspergillus fumigatus through whole genome sequencing and sexual crossing." PLoS One 7(11): e50034.

Carvalhal, S., I. Bader, M. A. Rooimans, A. B. Oostra, J. A. Balk, R. G. Feichtinger, C. Beichler, M. R. Speicher, J. M. van Hagen, Q. Waisfisz, M. van Haelst, M. Bruijn, A. Tavares, J. A. Mayr, R. M. F. Wolthuis, R. A. Oliveira and J. de Lange (2022). "Biallelic BUB1 mutations cause microcephaly, developmental delay, and variable effects on cohesion and chromosome segregation." Sci Adv 8(3): eabk0114.

Chan, G. K., S. A. Jablonski, V. Sudakin, J. C. Hittle and T. J. Yen (1999). "Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC." J Cell Biol 146(5): 941-954.

Chan, G. K., B. T. Schaar and T. J. Yen (1998). "Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1." J Cell Biol 143(1): 49-63.

Chang, Y. C., A. Khanal Lamichhane and K. J. Kwon-Chung (2018). "Cryptococcus neoformans, Unlike Candida albicans, Forms Aneuploid Clones Directly from Uninucleated Cells under Fluconazole Stress." mBio 9(6).

Cheeseman, I. M., S. Anderson, M. Jwa, E. M. Green, J. Kang, J. R. Yates, 3rd, C. S. Chan, D. G. Drubin and G. Barnes (2002). "Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p." Cell 111(2): 163-172.

Cheeseman, I. M., I. MacLeod, J. R. Yates, 3rd, K. Oegema and A. Desai (2005). "The CENP-F-like proteins HCP-1 and HCP-2 target CLASP to kinetochores to mediate chromosome segregation." Curr Biol 15(8): 771-777.

Chelius, C., W. Huso, S. Reese, A. Doan, S. Lincoln, K. Lawson, B. Tran, R. Purohit, T. Glaros, R. Srivastava, S. D. Harris and M. R. Marten (2020). "Dynamic Transcriptomic and Phosphoproteomic Analysis During Cell Wall Stress in Aspergillus nidulans." Mol Cell Proteomics 19(8): 1310-1329.

Chen, G., W. D. Bradford, C. W. Seidel and R. Li (2012). "Hsp90 stress potentiates rapid cellular adaptation through induction of aneuploidy." Nature 482(7384): 246-250.

Chen, J., A. Tresenrider, M. Chia, D. T. McSwiggen, G. Spedale, V. Jorgensen, H. Liao, F. J. van Werven and E. Unal (2017). "Kinetochore inactivation by expression of a repressive mRNA." Elife 6.

Cheng, E., J. A. Vaisica, J. Ou, A. Baryshnikova, Y. Lu, F. P. Roth and G. W. Brown (2012). "Genome rearrangements caused by depletion of essential DNA replication proteins in Saccharomyces cerevisiae." Genetics 192(1): 147-160.

Choy, J. S., E. O'Toole, B. M. Schuster, M. J. Crisp, T. S. Karpova, J. G. McNally, M. Winey, M. K. Gardner and M. A. Basrai (2013). "Genome-wide haploinsufficiency screen reveals a novel role for gamma-TuSC in spindle organization and genome stability." Mol Biol Cell 24(17): 2753-2763.

Ciciro, Y., D. Ragusa and A. Sala (2024). "Expression of the checkpoint kinase BUB1 is a predictor of response to cancer therapies." Sci Rep 14(1): 4461.

Cornely, O. A., J. Maertens, D. J. Winston, J. Perfect, A. J. Ullmann, T. J. Walsh, D. Helfgott, J. Holowiecki, D. Stockelberg, Y. T. Goh, M. Petrini, C. Hardalo, R. Suresh and D. Angulo-Gonzalez (2007). "Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia." N Engl J Med 356(4): 348-359.

Cowen, L. E. and W. J. Steinbach (2008). "Stress, Drugs, and Evolution: the Role of Cellular Signaling in Fungal Drug Resistance." Eukaryotic Cell 7(5): 747-764.

Dagenais, T. R. and N. P. Keller (2009). "Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis." Clin Microbiol Rev 22(3): 447-465.

Dai, C. (2018). "The heat-shock, or HSF1-mediated proteotoxic stress, response in cancer: from proteomic stability to oncogenesis." Philos Trans R Soc Lond B Biol Sci 373(1738).

Dai, W., Q. Wang, T. Liu, M. Swamy, Y. Fang, S. Xie, R. Mahmood, Y. M. Yang, M. Xu and C. V. Rao (2004). "Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency." Cancer Res 64(2): 440-445.

Danecek, P., J. K. Bonfield, J. Liddle, J. Marshall, V. Ohan, M. O. Pollard, A. Whitwh -am, T. Keane, S. A. McCarthy, R. M. Davies and H. Li (2021). "Twelve years of SAMtools and BCFtools." Gigascience 10(2).

Daniel, J. A., B. E. Keyes, Y. P. Ng, C. O. Freeman and D. J. Burke (2006). "Diverse functions of spindle assembly checkpoint genes in Saccharomyces cerevisiae." Genetics 172(1): 53-65.

Danielsen, H. E., M. Pradhan and M. Novelli (2016). "Revisiting tumour aneuploidy - the place of ploidy assessment in the molecular era." Nat Rev Clin Oncol 13(5): 291-304.

Davies, A. E. and K. B. Kaplan (2010). "Hsp90-Sgt1 and Skp1 target human Mis12 complexes to ensure efficient formation of kinetochore-microtubule binding sites." J Cell Biol 189(2): 261-274.

De Souza, C. P., S. B. Hashmi, A. H. Osmani, P. Andrews, C. S. Ringelberg, J. C. Dunlap and S. A. Osmani (2013). "Functional analysis of the Aspergillus nidulans kinome." PLoS One 8(3): e58008.

De Vroey, C. (1979). "[Saphrophytic life of opportunistic fungi]." Dermatologica 159(Suppl 1): 47-55.

Denning, D. W., S. Park, C. Lass-Florl, M. G. Fraczek, M. Kirwan, R. Gore, J. Smith, A. Bueid, C. B. Moore, P. Bowyer and D. S. Perlin (2011). "High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease." Clin Infect Dis 52(9): 1123-1129.

Dhamgaye, S., M. Bernard, G. Lelandais, O. Sismeiro, S. Lemoine, J. Y. Coppee, S. Le Crom, R. Prasad and F. Devaux (2012). "RNA sequencing revealed novel actors of the acquisition of drug resistance in Candida albicans." BMC Genomics 13: 396.

Dhital, B., S. Santasusagna, P. Kirthika, M. Xu, P. Li, M. Carceles-Cordon, R. K. Soni, Z. Li, R. C. Hendrickson, M. J. Schiewer, W. K. Kelly, C. N. Sternberg, J. Luo, A. Lujambio, C. Cordon-Cardo, M. Alvarez-Fernandez, M. Malumbres, H. Huang, A. Ertel, J. Domingo-Domenech and V. Rodriguez-Bravo (2023). "Harnessing transcriptionally driven chromosomal instability adaptation to target therapy-refractory lethal prostate cancer." Cell Rep Med 4(2): 100937.

Ditchfield, C., V. L. Johnson, A. Tighe, R. Ellston, C. Haworth, T. Johnson, A. Mortlock, N. Keen and S. S. Taylor (2003). "Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores." J Cell Biol 161(2): 267-280.

Dobles, M., V. Liberal, M. L. Scott, R. Benezra and P. K. Sorger (2000). "Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2." Cell 101(6): 635-645.

Dos Reis, T. F., L. P. Silva, P. A. de Castro, P. B. Almeida de Lima, R. A. do Carmo, M. M. Marini, J. F. da Silveira, B. H. Ferreira, F. Rodrigues, I. Malavazi and G. H. Goldman (2018). "The Influence of Genetic Stability on Aspergillus fumigatus Virulence and Azole Resistance." G3 (Bethesda) 8(1): 265-278.

Dudakova, A., B. Spiess, M. Tangwattanachuleeporn, C. Sasse, D. Buchheidt, M. Weig, U. Gross and O. Bader (2017). "Molecular Tools for the Detection and Deduction of

Azole Antifungal Drug Resistance Phenotypes in Aspergillus Species." Clin Microbiol Rev 30(4): 1065-1091.

Duffy, S., H. K. Fam, Y. K. Wang, E. B. Styles, J. H. Kim, J. S. Ang, T. Singh, V. Larionov, S. P. Shah, B. Andrews, C. F. Boerkoel and P. Hieter (2016). "Overexpression screens identify conserved dosage chromosome instability genes in yeast and human cancer." Proc Natl Acad Sci U S A 113(36): 9967-9976.

Edgerton, H., V. Paolillo and B. R. Oakley (2015). "Spatial regulation of the spindle assembly checkpoint and anaphase-promoting complex in Aspergillus nidulans." Mol Microbiol 95(3): 442-457.

Efimov, V. P. and N. R. Morris (1998). "A screen for dynein synthetic lethals in Aspergillus nidulans identifies spindle assembly checkpoint genes and other genes involved in mitosis." Genetics 149(1): 101-116.

Elowe, S. (2011). "Bub1 and BubR1: at the interface between chromosome attachment and the spindle checkpoint." Mol Cell Biol 31(15): 3085-3093.

Escobar, N., S. R. Ordonez, H. A. B. Wösten, P.-J. A. Haas, H. de Cock and H. P. Haagsman (2016). "Hide, Keep Quiet, and Keep Low: Properties That Make Aspergillus fumigatus a Successful Lung Pathogen." Frontiers in Microbiology 7.

Farr, K. A. and M. A. Hoyt (1998). "Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint." Mol Cell Biol 18(5): 2738-2747.

Fernius, J. and K. G. Hardwick (2007). "Bub1 kinase targets Sgo1 to ensure efficient chromosome biorientation in budding yeast mitosis." PLoS Genet 3(11): e213.

Fisher, M. C. and D. W. Denning (2023). "The WHO fungal priority pathogens list as a game-changer." Nat Rev Microbiol 21(4): 211-212.

Ford, C. B., J. M. Funt, D. Abbey, L. Issi, C. Guiducci, D. A. Martinez, T. Delorey, B. Y. Li, T. C. White, C. Cuomo, R. P. Rao, J. Berman, D. A. Thompson and A. Regev (2015). "The evolution of drug resistance in clinical isolates of Candida albicans." Elife 4: e00662.

Francisco, L., W. Wang and C. S. Chan (1994). "Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation." Mol Cell Biol 14(7): 4731-4740.

Fu, Y., L. Pastushok and W. Xiao (2008). "DNA damage-induced gene expression in Saccharomyces cerevisiae." FEMS Microbiol Rev 32(6): 908-926.

Fujibayashi, Y., R. Isa, D. Nishiyama, N. Sakamoto-Inada, N. Kawasumi, J. Yamaguchi, S. Kuwahara-Ota, Y. Matsumura-Kimoto, T. Tsukamoto, Y. Chinen, Y. Shimura, T.

Kobayashi, S. Horiike, M. Taniwaki, H. Handa and J. Kuroda (2020). "Aberrant BUB1 Overexpression Promotes Mitotic Segregation Errors and Chromosomal Instability in Multiple Myeloma." Cancers (Basel) 12(8).

Funabiki, H., K. Kumada and M. Yanagida (1996). "Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes." EMBO J 15(23): 6617-6628.

Funk, L. C., L. M. Zasadil and B. A. Weaver (2016). "Living in CIN: Mitotic Infidelity and Its Consequences for Tumor Promotion and Suppression." Dev Cell 39(6): 638-652.

Furukawa, T., N. van Rhijn, M. Fraczek, F. Gsaller, E. Davies, P. Carr, S. Gago, R. Fortune-Grant, S. Rahman, J. M. Gilsenan, E. Houlder, C. H. Kowalski, S. Raj, S. Paul, P. Cook, J. E. Parker, S. Kelly, R. A. Cramer, J. P. Latge, S. Moye-Rowley, E. Bignell, P. Bowyer and M. J. Bromley (2020). "The negative cofactor 2 complex is a key regulator of drug resistance in Aspergillus fumigatus." Nat Commun 11(1): 427.

Gama, J. B., C. Pereira, P. A. Simoes, R. Celestino, R. M. Reis, D. J. Barbosa, H. R. Pires, C. Carvalho, J. Amorim, A. X. Carvalho, D. K. Cheerambathur and R. Gassmann (2017). "Molecular mechanism of dynein recruitment to kinetochores by the Rod-Zw10-Zwilch complex and Spindly." J Cell Biol 216(4): 943-960.

Gassmann, R., A. Essex, J. S. Hu, P. S. Maddox, F. Motegi, A. Sugimoto, S. M. O'Rourke, B. Bowerman, I. McLeod, J. R. Yates, 3rd, K. Oegema, I. M. Cheeseman and A. Desai (2008). "A new mechanism controlling kinetochore-microtubule interactions revealed by comparison of two dynein-targeting components: SPDL-1 and the Rod/Zwilch/Zw10 complex." Genes Dev 22(17): 2385-2399.

Gehmlich, K., L. Haren and A. Merdes (2004). "Cyclin B degradation leads to NuMA release from dynein/dynactin and from spindle poles." EMBO Rep 5(1): 97-103.

Giam, M. and G. Rancati (2015). "Aneuploidy and chromosomal instability in cancer: a jackpot to chaos." Cell Div 10: 3.

Gordon, M. R., J. Zhu, G. Sun and R. Li (2023). "Suppression of chromosome instability by targeting a DNA helicase in budding yeast." Mol Biol Cell 34(1): ar3.

Goto, G. H., A. Mishra, R. Abdulle, C. A. Slaughter and K. Kitagawa (2011). "Bub1-mediated adaptation of the spindle checkpoint." PLoS Genet 7(1): e1001282.

Gsaller, F., P. Hortschansky, T. Furukawa, P. D. Carr, B. Rash, J. Capilla, C. Müller, F. Bracher, P. Bowyer, H. Haas, A. A. Brakhage and M. J. Bromley (2016). "Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex." PLOS Pathogens 12(7): e1005775.

Handelman, M. and N. Osherov (2022). "Experimental and in-host evolution of triazole resistance in human pathogenic fungi." Front Fungal Biol 3: 957577.

Hanks, S., K. Coleman, S. Reid, A. Plaja, H. Firth, D. Fitzpatrick, A. Kidd, K. Mehes, R. Nash, N. Robin, N. Shannon, J. Tolmie, J. Swansbury, A. Irrthum, J. Douglas and N. Rahman (2004). "Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B." Nat Genet 36(11): 1159-1161.

Haren, L., N. Gnadt, M. Wright and A. Merdes (2009). "NuMA is required for proper spindle assembly and chromosome alignment in prometaphase." BMC Res Notes 2: 64.

Harrison, B. D., J. Hashemi, M. Bibi, R. Pulver, D. Bavli, Y. Nahmias, M. Wellington, G. Sapiro and J. Berman (2014). "A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole." PLoS Biol 12(3): e1001815.

Hartwell, L. H. and D. Smith (1985). "Altered fidelity of mitotic chromosome transmission in cell cycle mutants of S. cerevisiae." Genetics 110(3): 381-395.

He, Y., W. Fu, L. Du, H. Yao, Z. Hua, J. Li and Z. Lin (2022). "Discovery of a novel Aurora B inhibitor GSK650394 with potent anticancer and anti-aspergillus fumigatus dual efficacies in vitro." J Enzyme Inhib Med Chem 37(1): 109-117.

Heasley, L. R., J. G. DeLuca and S. M. Markus (2019). "Effectors of the spindle assembly checkpoint are confined within the nucleus of Saccharomyces cerevisiae." Biol Open 8(6).

Hernando, E., I. Orlow, V. Liberal, G. Nohales, R. Benezra and C. Cordon-Cardo (2001). "Molecular analyses of the mitotic checkpoint components hsMAD2, hBUB1 and hBUB3 in human cancer." International Journal of Cancer 95(4): 223-227.

Heun, P., S. Erhardt, M. D. Blower, S. Weiss, A. D. Skora and G. H. Karpen (2006). "Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores." Dev Cell 10(3): 303-315.

Hill, J. A., R. Ammar, D. Torti, C. Nislow and L. E. Cowen (2013). "Genetic and genomic architecture of the evolution of resistance to antifungal drug combinations." PLoS Genet 9(4): e1003390.

Hodiamont, C. J., K. M. Dolman, I. J. Ten Berge, W. J. Melchers, P. E. Verweij and D. Pajkrt (2009). "Multiple-azole-resistant Aspergillus fumigatus osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery." Med Mycol 47(2): 217-220.

Hoffmann, G., A. Samel-Pommerencke, J. Weber, A. Cuomo, T. Bonaldi and A. E. Ehrenhofer-Murray (2018). "A role for CENP-A/Cse4 phosphorylation on serine 33 in deposition at the centromere." FEMS Yeast Res 18(1).

Horta, M. A. C., J. L. Steenwyk, M. E. Mead, L. H. B. Dos Santos, S. Zhao, J. G. Gibbons, M. Marcet-Houben, T. Gabaldon, A. Rokas and G. H. Goldman (2022). "Examination

of Genome-Wide Ortholog Variation in Clinical and Environmental Isolates of the Fungal Pathogen Aspergillus fumigatus." mBio 13(4): e0151922.

Hoyt, M. A., L. Totis and B. T. Roberts (1991). "S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function." Cell 66(3): 507-517.

Hu, X., X. Jin, X. Cao and B. Liu (2022). "The Anaphase-Promoting Complex/Cyclosome Is a Cellular Ageing Regulator." Int J Mol Sci 23(23).

Ibrahim, B., S. Diekmann, E. Schmitt and P. Dittrich (2008). "In-silico modeling of the mitotic spindle assembly checkpoint." PLoS One 3(2): e1555.

J. Ramón De Lucas, A. I. D., Alfonso Mendoza* and Fernando Laborda (1998) "Use of flow-cytometry to distinguish between haploid and diploid strains of Aspergillus fumigatus." Fungal Genetics Newsletter, 7-9.

Jeganathan, K., L. Malureanu, D. J. Baker, S. C. Abraham and J. M. van Deursen (2007). "Bub1 mediates cell death in response to chromosome missegregation and acts to suppress spontaneous tumorigenesis." J Cell Biol 179(2): 255-267.

Jia, L., B. Li and H. Yu (2016). "The Bub1-Plk1 kinase complex promotes spindle checkpoint signalling through Cdc20 phosphorylation." Nat Commun 7: 10818.

Jin, F., M. Bokros and Y. Wang (2017). "The phosphorylation of a kinetochore protein Dam1 by Aurora B/Ipl1 kinase promotes chromosome bipolar attachment in yeast." Sci Rep 7(1): 11880.

Jin, F., M. Bokros and Y. Wang (2017). "Premature Silencing of the Spindle Assembly Checkpoint Is Prevented by the Bub1-H2A-Sgo1-PP2A Axis in Saccharomyces cerevisiae." Genetics 205(3): 1169-1178.

Jin, F., H. Liu, P. Li, H. G. Yu and Y. Wang (2012). "Loss of function of the Cik1/Kar3 motor complex results in chromosomes with syntelic attachment that are sensed by the tension checkpoint." PLoS Genet 8(2): e1002492.

Karess, R. E. and D. M. Glover (1989). "rough deal: a gene required for proper mitotic segregation in Drosophila." J Cell Biol 109(6 Pt 1): 2951-2961.

Kawashima, S. A., Y. Yamagishi, T. Honda, K. Ishiguro and Y. Watanabe (2010). "Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin." Science 327(5962): 172-177.

Khateb, A., S. Gago, M. Bromley, M. Richardson and P. Bowyer (2023). "Aneuploidy Is Associated with Azole Resistance in Aspergillus fumigatus." Antimicrob Agents Chemother 67(4): e0125322.

Kim, J., Y. J. Cho, E. Do, J. Choi, G. Hu, B. Cadieux, J. Chun, Y. Lee, J. W. Kronstad and W. H. Jung (2012). "A defect in iron uptake enhances the susceptibility of Cryptococcus neoformans to azole antifungal drugs." Fungal Genet Biol 49(11): 955-966.

Kim, J. H., J. S. Kang and C. S. Chan (1999). "Sli15 associates with the ipl1 protein kinase to promote proper chromosome segregation in Saccharomyces cerevisiae." J Cell Biol 145(7): 1381-1394.

Kitagawa, K., D. Skowyra, S. J. Elledge, J. W. Harper and P. Hieter (1999). "SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex." Mol Cell 4(1): 21-33.

Kitagawa, M. and S. H. Lee (2015). "The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis." Front Cell Dev Biol 3: 14.

Kitagawa, R. and A. M. Rose (1999). "Components of the spindle-assembly checkpoint are essential in Caenorhabditis elegans." Nat Cell Biol 1(8): 514-521.

Klimaszewska-Wisniewska, A., K. Buchholz, J. Durslewicz, E. S. Villodre, M. Gagat and D. Grzanka (2022). "SPDL1 Is an Independent Predictor of Patient Outcome in Colorectal Cancer." Int J Mol Sci 23(3).

Kline-Smith, S. L., S. Sandall and A. Desai (2005). "Kinetochore-spindle microtubule interactions during mitosis." Curr Opin Cell Biol 17(1): 35-46.

Koehler, P., M. Bassetti, A. Chakrabarti, S. C. A. Chen, A. L. Colombo, M. Hoenigl, N. Klimko, C. Lass-Florl, R. O. Oladele, D. C. Vinh, L. P. Zhu, B. Boll, R. Bruggemann, J. P. Gangneux, J. R. Perfect, T. F. Patterson, T. Persigehl, J. F. Meis, L. Ostrosky-Zeichner, P. L. White, P. E. Verweij, O. A. Cornely; European Confederation of Medical Mycology; International Society for Human Animal Mycology; Asia Fungal Working Group; INFOCUS LATAM/ISHAM Working Group; ISHAM Pan Africa Mycology Working Group; European Society for Clinical Microbiology; Infectious Diseases Fungal Infection Study Group; ESCMID Study Group for Infections in Critically Ill Patients; Interregional Association of Clinical Microbiology and Antimicrobial Chemotherapy; Medical Mycology Society of Nigeria; Medical Mycology Society of China Medicine Education Association; Infectious Diseases Working Party of the German Society for Haematology and Medical Oncology; Association of Medical Microbiology; Infectious Disease Canada. "Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance." Lancet Infect Dis 21(6): e149-e162.

Kops, G. J., B. A. Weaver and D. W. Cleveland (2005). "On the road to cancer: aneuploidy and the mitotic checkpoint." Nat Rev Cancer 5(10): 773-785.

Kosmidis, C. and D. W. Denning (2015). "The clinical spectrum of pulmonary aspergillosis." Thorax 70(3): 270-277.

Kouprina, N., O. B. Pashina, N. T. Nikolaishwili, A. M. Tsouladze and V. L. Larionov (1988). "Genetic control of chromosome stability in the yeast Saccharomyces cerevisiae." Yeast 4(4): 257-269.

Kovacovicova, K., T. Awadova, P. Mikel and M. Anger (2016). "In Vitro Maturation of Mouse Oocytes Increases the Level of Kif11/Eg5 on Meiosis II Spindles." Biol Reprod 95(1): 18.

Kukurudz, R. J., M. Chapel, Q. Wonitowy, A. R. Adamu Bukari, B. Sidney, R. Sierhuis and A. C. Gerstein (2022). "Acquisition of cross-azole tolerance and aneuploidy in Candida albicans strains evolved to posaconazole." G3 (Bethesda) 12(9).

Lamoth, F., P. R. Juvvadi, J. R. Fortwendel and W. J. Steinbach (2012). "Heat shock protein 90 is required for conidiation and cell wall integrity in Aspergillus fumigatus." Eukaryot Cell 11(11): 1324-1332.

Laniado-Laborín, R. and M. N. Cabrales-Vargas (2009). "Amphotericin B: side effects and toxicity." Rev Iberoam Micol 26(4): 223-227.

Lara-Gonzalez, P., F. G. Westhorpe and S. S. Taylor (2012). "The spindle assembly checkpoint." Curr Biol 22(22): R966-980.

Latge, J. P. and G. Chamilos (2019). "Aspergillus fumigatus and Aspergillosis in 2019." Clin Microbiol Rev 33(1).

Lazzarini, C., M. C. Esposto, A. Prigitano, M. Cogliati, G. De Lorenzis and A. M. Tortorano (2016). "Azole Resistance in Aspergillus fumigatus Clinical Isolates from an Italian Culture Collection." Antimicrob Agents Chemother 60(1): 682-685.

Le Masson, I., C. Saveanu, A. Chevalier, A. Namane, R. Gobin, M. Fromont-Racine, A. Jacquier and C. Mann (2002). "Spc24 interacts with Mps2 and is required for chromosome segregation, but is not implicated in spindle pole body duplication." Mol Microbiol 43(6): 1431-1443.

Leland, S., P. Nagarajan, A. Polyzos, S. Thomas, G. Samaan, R. Donnell, F. Marchetti and S. Venkatachalam (2009). "Heterozygosity for a Bub1 mutation causes female-specific germ cell aneuploidy in mice." Proc Natl Acad Sci U S A 106(31): 12776-12781.

Li, H. and R. Durbin (2009). "Fast and accurate short read alignment with Burrows-Wheeler transform." Bioinformatics 25(14): 1754-1760.

Li, H. and R. Durbin (2010). "Fast and accurate long-read alignment with Burrows-Wheeler transform." Bioinformatics 26(5): 589-595.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin and S. Genome Project Data Processing (2009). "The Sequence Alignment/Map format and SAMtools." Bioinformatics 25(16): 2078-2079.

Li, M., X. Fang, D. J. Baker, L. Guo, X. Gao, Z. Wei, S. Han, J. M. van Deursen and P. Zhang (2010). "The ATM-p53 pathway suppresses aneuploidy-induced tumorigenesis." Proc Natl Acad Sci U S A 107(32): 14188-14193.

Li, M., X. Fang, Z. Wei, J. P. York and P. Zhang (2009). "Loss of spindle assembly checkpoint-mediated inhibition of Cdc20 promotes tumorigenesis in mice." J Cell Biol 185(6): 983-994.

Li, R. and A. W. Murray (1991). "Feedback control of mitosis in budding yeast." Cell 66(3): 519-531.

Lianga, N., C. Dore, E. K. Kennedy, E. Yeh, E. C. Williams, C. M. Fortinez, A. Wang, K. S. Bloom and A. D. Rudner (2018). "Cdk1 phosphorylation of Esp1/Separase functions with PP2A and Slk19 to regulate pericentric Cohesin and anaphase onset." PLoS Genet 14(3): e1007029.

Lionakis, M. S., R. E. Lewis and D. P. Kontoyiannis (2018). "Breakthrough Invasive Mold Infections in the Hematology Patient: Current Concepts and Future Directions." Clin Infect Dis 67(10): 1621-1630.

Liu, H., S. Rankin and H. Yu (2013). "Phosphorylation-enabled binding of SGO1-PP2A to cohesin protects sororin and centromeric cohesion during mitosis." Nat Cell Biol 15(1): 40-49.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods 25(4): 402-408.

Loll-Krippleber, R., A. Feri, M. Nguyen, C. Maufrais, J. Yansouni, C. d'Enfert and M. Legrand (2015). "A FACS-optimized screen identifies regulators of genome stability in Candida albicans." Eukaryot Cell 14(3): 311-322.

Long, N., L. Zeng, S. Qiao, L. Li and G. Zhong (2018). "Aspergillus fumigatus Afssn3-Afssn8 Pair Reverse Regulates Azole Resistance by Conferring Extracellular Polysaccharide, Sphingolipid Pathway Intermediates, and Efflux Pumps to Biofilm." Antimicrob Agents Chemother 62(3).

Lorson, M. A., H. R. Horvitz and S. van den Heuvel (2000). "LIN-5 is a novel component of the spindle apparatus required for chromosome segregation and cleavage plane specification in Caenorhabditis elegans." J Cell Biol 148(1): 73-86.

Maertens, J. A., Raad, II, K. A. Marr, T. F. Patterson, D. P. Kontoyiannis, O. A. Cornely, E. J. Bow, G. Rahav, D. Neofytos, M. Aoun, J. W. Baddley, M. Giladi, W. J. Heinz, R. Herbrecht, W. Hope, M. Karthaus, D. G. Lee, O. Lortholary, V. A. Morrison, I. Oren, D. Selleslag, S. Shoham, G. R. Thompson, 3rd, M. Lee, R. M. Maher, A. H. Schmitt-Hoffmann, B. Zeiher and A. J. Ullmann (2016). "Isavuconazole versus voriconazole for primary treatment of

invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial." Lancet 387(10020): 760-769.

Martin-Vicente, A., A. C. O. Souza, A. V. Nywening, W. Ge and J. R. Fortwendel (2020). "Overexpression of the Aspergillus fumigatus Small GTPase, RsrA, Promotes Polarity Establishment during Germination." J Fungi (Basel) 6(4).

Martin, M. (2011). "Cutadapt removes adapter sequences from high-throughput sequencing reads." EMBnet. journal 17(1): 10-12.

Matsuura, S., Y. Matsumoto, K. Morishima, H. Izumi, H. Matsumoto, E. Ito, K. Tsutsui, J. Kobayashi, H. Tauchi, Y. Kajiwara, S. Hama, K. Kurisu, H. Tahara, M. Oshimura, K. Komatsu, T. Ikeuchi and T. Kajii (2006). "Monoallelic BUB1B mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome." Am J Med Genet A 140(4): 358-367.

Mattos, E. C., G. Palmisano and G. H. Goldman (2020). "Phosphoproteomics of Aspergillus fumigatus Exposed to the Antifungal Drug Caspofungin." mSphere 5(3).

Mattos, E. C., L. P. Silva, C. Valero, P. A. de Castro, T. F. Dos Reis, L. F. C. Ribeiro, M. R. Marten, R. Silva-Rocha, C. Westmann, C. da Silva, C. A. Taft, N. Al-Furaiji, M. Bromley, U. H. Mortensen, J. P. Benz, N. A. Brown and G. H. Goldman (2020). "The Aspergillus fumigatus Phosphoproteome Reveals Roles of High-Osmolarity Glycerol Mitogen-Activated Protein Kinases in Promoting Cell Wall Damage and Caspofungin Tolerance." mBio 11(1).

Mayer, M. L., I. Pot, M. Chang, H. Xu, V. Aneliunas, T. Kwok, R. Newitt, R. Aebersold, C. Boone, G. W. Brown and P. Hieter (2004). "Identification of protein complexes required for efficient sister chromatid cohesion." Mol Biol Cell 15(4): 1736-1745.

Mayr, A. and C. Lass-Florl (2011). "Epidemiology and antifungal resistance in invasive Aspergillosis according to primary disease: review of the literature." Eur J Med Res 16(4): 153-157.

Mead, M. E., P. A. de Castro, J. L. Steenwyk, J. P. Gangneux, M. Hoenigl, J. Prattes, R. Rautemaa-Richardson, H. Guegan, C. B. Moore, C. Lass-Florl, F. Reizine, C. Valero, N. Van Rhijn, M. J. Bromley, A. Rokas, G. H. Goldman, S. Gago and E. C. S. Group (2023). "COVID-19-Associated Pulmonary Aspergillosis Isolates Are Genomically Diverse but Similar to Each Other in Their Responses to Infection-Relevant Stresses." Microbiol Spectr 11(2): e0512822.

Meeks-Wagner, D., J. S. Wood, B. Garvik and L. H. Hartwell (1986). "Isolation of two genes that affect mitotic chromosome transmission in S. cerevisiae." Cell 44(1): 53-63.

Meluh, P. B. and D. Koshland (1995). "Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C." Mol Biol Cell 6(7): 793-807.

Meraldi, P., R. Honda and E. A. Nigg (2004). "Aurora kinases link chromosome segregation and cell division to cancer susceptibility." Curr Opin Genet Dev 14(1): 29-36.

Meraldi, P. and P. K. Sorger (2005). "A dual role for Bub1 in the spindle checkpoint and chromosome congression." EMBO J 24(8): 1621-1633.

Miao, J., H. M. E. Willems and B. M. Peters (2021). "Exogenous Reproductive Hormones nor Candida albicans Colonization Alter the Near Neutral Mouse Vaginal pH." Infect Immun 89(2).

Mittal, P., K. Ghule, D. Trakroo, H. K. Prajapati and S. K. Ghosh (2020). "Meiosis-Specific Functions of Kinesin Motors in Cohesin Removal and Maintenance of Chromosome Integrity in Budding Yeast." Mol Cell Biol 40(8).

Morard, M., L. G. Macias, A. C. Adam, M. Lairon-Peris, R. Perez-Torrado, C. Toft and E. Barrio (2019). "Aneuploidy and Ethanol Tolerance in Saccharomyces cerevisiae." Front Genet 10: 82.

Myslinski, E., M. A. Gerard, A. Krol and P. Carbon (2007). "Transcription of the human cell cycle regulated BUB1B gene requires hStaf/ZNF143." Nucleic Acids Res 35(10): 3453-3464.

Nakajima, Y., R. G. Tyers, C. C. Wong, J. R. Yates, 3rd, D. G. Drubin and G. Barnes (2009). "Nbl1p: a Borealin/Dasra/CSC-1-like protein essential for Aurora/Ipl1 complex function and integrity in Saccharomyces cerevisiae." Mol Biol Cell 20(6): 1772-1784.

Nekrasov, V. S., M. A. Smith, S. Peak-Chew and J. V. Kilmartin (2003). "Interactions between centromere complexes in Saccharomyces cerevisiae." Mol Biol Cell 14(12): 4931-4946.

Niikura, Y., A. Dixit, R. Scott, G. Perkins and K. Kitagawa (2007). "BUB1 mediation of caspase-independent mitotic death determines cell fate." J Cell Biol 178(2): 283-296.

Novak, B. and J. J. Tyson (2021). "Mechanisms of signalling-memory governing progression through the eukaryotic cell cycle." Curr Opin Cell Biol 69: 7-16.

Omer Javed, A., Y. Li, J. Muffat, K. C. Su, M. A. Cohen, T. Lungjangwa, P. Aubourg, I. M. Cheeseman and R. Jaenisch (2018). "Microcephaly Modeling of Kinetochore Mutation Reveals a Brain-Specific Phenotype." Cell Rep 25(2): 368-382 e365.

Parums, D. V. (2022). "Editorial: The World Health Organization (WHO) Fungal Priority Pathogens List in Response to Emerging Fungal Pathogens During the COVID-19 Pandemic." Med Sci Monit 28: e939088.

Patterson, T. F., W. R. Kirkpatrick, M. White, J. W. Hiemenz, J. R. Wingard, B. Dupont, M. G. Rinaldi, D. A. Stevens and J. R. Graybill (2000). "Invasive aspergillosis. Disease

spectrum, treatment practices, and outcomes. I3 Aspergillus Study Group." Medicine (Baltimore) 79(4): 250-260.

Patterson, T. F., G. R. Thompson, 3rd, D. W. Denning, J. A. Fishman, S. Hadley, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, M. H. Nguyen, B. H. Segal, W. J. Steinbach, D. A. Stevens, T. J. Walsh, J. R. Wingard, J. A. Young and J. E. Bennett (2016). "Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America." Clin Infect Dis 63(4): e1-e60.

Peng, D. and R. Tarleton (2015). "EuPaGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens." Microb Genom 1(4): e000033.

Pennisi, R., P. Ascenzi and A. di Masi (2015). "Hsp90: A New Player in DNA Repair?" Biomolecules 5(4): 2589-2618.

Pereira, A. L., A. J. Pereira, A. R. Maia, K. Drabek, C. L. Sayas, P. J. Hergert, M. Lince-Faria, I. Matos, C. Duque, T. Stepanova, C. L. Rieder, W. C. Earnshaw, N. Galjart and H. Maiato (2006). "Mammalian CLASP1 and CLASP2 cooperate to ensure mitotic fidelity by regulating spindle and kinetochore function." Mol Biol Cell 17(10): 4526-4542.

Pinsky, B. A., C. Kung, K. M. Shokat and S. Biggins (2006). "The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores." Nat Cell Biol 8(1): 78-83.

Pinto, I. and F. Winston (2000). "Histone H2A is required for normal centromere function in Saccharomyces cerevisiae." EMBO J 19(7): 1598-1612.

Pinto, M., J. Vieira, F. R. Ribeiro, M. J. Soares, R. Henrique, J. Oliveira, C. Jeronimo and M. R. Teixeira (2008). "Overexpression of the mitotic checkpoint genes BUB1 and BUBR1 is associated with genomic complexity in clear cell kidney carcinomas." Cell Oncol 30(5): 389-395.

Pitayu-Nugroho, L., M. Aubry, K. Laband, H. Geoffroy, T. Ganeswaran, A. Primadhanty, J. C. Canman and J. Dumont (2023). "Kinetochore component function in C. elegans oocytes revealed by 4D tracking of holocentric chromosomes." Nat Commun 14(1): 4032.

Prajapati, H. K., S. M. Rizvi, I. Rathore and S. K. Ghosh (2017). "Microtubule-associated proteins, Bik1 and Bim1, are required for faithful partitioning of the endogenous 2 micron plasmids in budding yeast." Mol Microbiol 103(6): 1046-1064.

Prevo, B., D. K. Cheerambathur, W. C. Earnshaw and A. Desai (2023). "Kinetochore dynein is sufficient to biorient chromosomes and remodel the outer kinetochore." bioRxiv.

Primorac, I., J. R. Weir, E. Chiroli, F. Gross, I. Hoffmann, S. van Gerwen, A. Ciliberto and A. Musacchio (2013). "Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling." Elife 2: e01030.

Putkey, F. R., T. Cramer, M. K. Morphew, A. D. Silk, R. S. Johnson, J. R. McIntosh and D. W. Cleveland (2002). "Unstable kinetochore-microtubule capture and chromosomal instability following deletion of CENP-E." Dev Cell 3(3): 351-365.

Qiao, J., W. Liu and R. Li (2008). "Antifungal resistance mechanisms of Aspergillus." Nihon Ishinkin Gakkai Zasshi 49(3): 157-163.

Quinlan, A. R. (2014). "BEDTools: The Swiss-Army Tool for Genome Feature Analysis." Curr Protoc Bioinformatics 47: 11 12 11-34.

Ragusa, D. and P. Vagnarelli (2023). "Contribution of histone variants to aneuploidy: a cancer perspective." Front Genet 14: 1290903.

Resende, L. P., A. Monteiro, R. Bras, T. Lopes and C. E. Sunkel (2018). "Aneuploidy in intestinal stem cells promotes gut dysplasia in Drosophila." J Cell Biol 217(11): 3930-3946.

Resendiz Sharpe, A., K. Lagrou, J. F. Meis, A. Chowdhary, S. R. Lockhart, P. E. Verweij and I. E. A. R. S. w. group (2018). "Triazole resistance surveillance in Aspergillus fumigatus." Med Mycol 56(suppl_1): 83-92.

Ricke, R. M., K. B. Jeganathan, L. Malureanu, A. M. Harrison and J. M. van Deursen (2012). "Bub1 kinase activity drives error correction and mitotic checkpoint control but not tumor suppression." J Cell Biol 199(6): 931-949.

Ricke, R. M., K. B. Jeganathan and J. M. van Deursen (2011). "Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation." J Cell Biol 193(6): 1049-1064.

Rivero-Menendez, O., A. Alastruey-Izquierdo, E. Mellado and M. Cuenca-Estrella (2016). "Triazole Resistance in Aspergillus spp.: A Worldwide Problem?" J Fungi (Basel) 2(3).

Roberts, B. T., K. A. Farr and M. A. Hoyt (1994). "The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase." Mol Cell Biol 14(12): 8282-8291.

Roschke, A. V. and I. R. Kirsch (2010). "Targeting karyotypic complexity and chromosomal instability of cancer cells." Curr Drug Targets 11(10): 1341-1350.

Roy, B., S. J. Y. Han, A. N. Fontan, S. Jema and A. P. Joglekar (2022). "Aurora B phosphorylates Bub1 to promote spindle assembly checkpoint signaling." Curr Biol 32(1): 237-247 e236.

Rybak, J. M., J. R. Fortwendel and P. D. Rogers (2019). "Emerging threat of triazole-resistant Aspergillus fumigatus." J Antimicrob Chemother 74(4): 835-842.

Rybak, J. M., W. Ge, N. P. Wiederhold, J. E. Parker, S. L. Kelly, P. D. Rogers and J. R. Fortwendel (2019). "Mutations in hmg1, Challenging the Paradigm of Clinical Triazole Resistance in Aspergillus fumigatus." mBio 10(2).

Sakurikar, N., J. M. Eichhorn and T. C. Chambers (2012). "Cyclin-dependent kinase-1 (Cdk1)/cyclin B1 dictates cell fate after mitotic arrest via phosphoregulation of antiapoptotic Bcl-2 proteins." J Biol Chem 287(46): 39193-39204.

Sandall, S., F. Severin, I. X. McLeod, J. R. Yates, 3rd, K. Oegema, A. Hyman and A. Desai (2006). "A Bir1-Sli15 complex connects centromeres to microtubules and is required to sense kinetochore tension." Cell 127(6): 1179-1191.

Sarkans, U., M. Gostev, A. Athar, E. Behrangi, O. Melnichuk, A. Ali, J. Minguet, J. C. Rada, C. Snow, A. Tikhonov, A. Brazma and J. McEntyre (2018). "The BioStudies database-one stop shop for all data supporting a life sciences study." Nucleic Acids Res 46(D1): D1266-D1270.

Saunders, W. S., D. Koshland, D. Eshel, I. R. Gibbons and M. A. Hoyt (1995). "Saccharomyces cerevisiae kinesin- and dynein-related proteins required for anaphase chromosome segregation." J Cell Biol 128(4): 617-624.

Schliekelman, M., D. O. Cowley, R. O'Quinn, T. G. Oliver, L. Lu, E. D. Salmon and T. Van Dyke (2009). "Impaired Bub1 function in vivo compromises tension-dependent checkpoint function leading to aneuploidy and tumorigenesis." Cancer Res 69(1): 45-54.

Selmecki, A., A. Forche and J. Berman (2006). "Aneuploidy and isochromosome formation in drug-resistant Candida albicans." Science 313(5785): 367-370.

Selmecki, A., A. Forche and J. Berman (2010). "Genomic plasticity of the human fungal pathogen Candida albicans." Eukaryot Cell 9(7): 991-1008.

Selmecki, A., M. Gerami-Nejad, C. Paulson, A. Forche and J. Berman (2008). "An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1." Mol Microbiol 68(3): 624-641.

Selmecki, A. M., K. Dulmage, L. E. Cowen, J. B. Anderson and J. Berman (2009). "Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance." PLoS Genet 5(10): e1000705.

Semighini, C. P., A. F. Averette, J. R. Perfect and J. Heitman (2011). "Deletion of Cryptococcus neoformans AIF ortholog promotes chromosome aneuploidy and fluconazole-resistance in a metacaspase-independent manner." PLoS Pathog 7(11): e1002364.

Seyedmousavi, S., J. Guillot, P. Arne, G. S. de Hoog, J. W. Mouton, W. J. Melchers and P. E. Verweij (2015). "Aspergillus and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease." Med Mycol 53(8): 765-797.

She, Z. Y., M. F. Xu, S. Y. Jiang and Y. L. Wei (2022). "Kinesin-7 CENP-E is essential for chromosome alignment and spindle assembly of mouse spermatocytes." Biochim Biophys Acta Mol Cell Res 1869(9): 119306.

She, Z. Y., N. Zhong, K. W. Yu, Y. Xiao, Y. L. Wei, Y. Lin, Y. L. Li and M. H. Lu (2020). "Kinesin-5 Eg5 is essential for spindle assembly and chromosome alignment of mouse spermatocytes." Cell Div 15: 6.

Shimizu, K. and N. P. Keller (2001). "Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans." Genetics 157(2): 591-600.

Shrestha, R. L., A. Rossi, D. Wangsa, A. K. Hogan, K. S. Zaldana, E. Suva, Y. J. Chung, C. L. Sanders, S. Difilippantonio, T. S. Karpova, B. Karim, D. R. Foltz, D. Fachinetti, P. D. Aplan, T. Ried and M. A. Basrai (2021). "CENP-A overexpression promotes aneuploidy with karyotypic heterogeneity." J Cell Biol 220(4).

Silk, A. D., A. J. Holland and D. W. Cleveland (2009). "Requirements for NuMA in maintenance and establishment of mammalian spindle poles." J Cell Biol 184(5): 677-690.

Singh, P., M. E. Pesenti, S. Maffini, S. Carmignani, M. Hedtfeld, A. Petrovic, A. Srinivasamani, T. Bange and A. Musacchio (2021). "BUB1 and CENP-U, Primed by CDK1, Are the Main PLK1 Kinetochore Receptors in Mitosis." Mol Cell 81(1): 67-87 e69.

Sionov, E., Y. C. Chang, H. M. Garraffo and K. J. Kwon-Chung (2009). "Heteroresistance to fluconazole in Cryptococcus neoformans is intrinsic and associated with virulence." Antimicrob Agents Chemother 53(7): 2804-2815.

Sionov, E., H. Lee, Y. C. Chang and K. J. Kwon-Chung (2010). "Cryptococcus neoformans overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes." PLoS Pathog 6(4): e1000848.

Skrzypek, M. S., J. Binkley, G. Binkley, S. R. Miyasato, M. Simison and G. Sherlock (2017). "The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data." Nucleic Acids Res 45(D1): D592-D596.

Skrzypek, M. S. and J. Hirschman (2011). "Using the Saccharomyces Genome Database (SGD) for analysis of genomic information." Curr Protoc Bioinformatics Chapter 1: 1 20 21-21 20 23.

Snape, K., S. Hanks, E. Ruark, P. Barros-Nunez, A. Elliott, A. Murray, A. H. Lane, N. Shannon, P. Callier, D. Chitayat, J. Clayton-Smith, D. R. Fitzpatrick, D. Gisselsson, S. Jacquemont, K. Asakura-Hay, M. A. Micale, J. Tolmie, P. D. Turnpenny, M. Wright, J. Douglas and N. Rahman (2011). "Mutations in CEP57 cause mosaic variegated aneuploidy syndrome." Nat Genet 43(6): 527-529.

Souza, A. C. O., A. Martin-Vicente, A. V. Nywening, W. Ge, D. J. Lowes, B. M. Peters and J. R. Fortwendel (2021). "Loss of Septation Initiation Network (SIN) kinases blocks

tissue invasion and unlocks echinocandin cidal activity against Aspergillus fumigatus." PLoS Pathog 17(8): e1009806.

Stemmann, O., A. Neidig, T. Kocher, M. Wilm and J. Lechner (2002). "Hsp90 enables Ctf13p/Skp1p to nucleate the budding yeast kinetochore." Proc Natl Acad Sci U S A 99(13): 8585-8590.

Stoler, S., K. C. Keith, K. E. Curnick and M. Fitzgerald-Hayes (1995). "A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis." Genes Dev 9(5): 573-586.

Stopsack, K. H., C. A. Whittaker, T. A. Gerke, M. Loda, P. W. Kantoff, L. A. Mucci and A. Amon (2019). "Aneuploidy drives lethal progression in prostate cancer." Proc Natl Acad Sci U S A 116(23): 11390-11395.

Straight, A. F., A. S. Belmont, C. C. Robinett and A. W. Murray (1996). "GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion." Curr Biol 6(12): 1599-1608.

Suijkerbuijk, S. J., T. J. van Dam, G. E. Karagoz, E. von Castelmur, N. C. Hubner, A. M. Duarte, M. Vleugel, A. Perrakis, S. G. Rudiger, B. Snel and G. J. Kops (2012). "The vertebrate mitotic checkpoint protein BUBR1 is an unusual pseudokinase." Dev Cell 22(6): 1321-1329.

Suijkerbuijk, S. J., M. H. van Osch, F. L. Bos, S. Hanks, N. Rahman and G. J. Kops (2010). "Molecular causes for BUBR1 dysfunction in the human cancer predisposition syndrome mosaic variegated aneuploidy." Cancer Res 70(12): 4891-4900.

Sun, L. L., H. Li, T. H. Yan, Y. B. Cao, Y. Y. Jiang and F. Yang (2023). "Aneuploidy enables cross-tolerance to unrelated antifungal drugs in Candida parapsilosis." Front Microbiol 14: 1137083.

Suzuki, A., A. Gupta, S. K. Long, R. Evans, B. L. Badger, E. D. Salmon, S. Biggins and K. Bloom (2018). "A Kinesin-5, Cin8, Recruits Protein Phosphatase 1 to Kinetochores and Regulates Chromosome Segregation." Curr Biol 28(17): 2697-2704 e2693.

Takahashi, K., H. Yamada and M. Yanagida (1994). "Fission yeast minichromosome loss mutants mis cause lethal aneuploidy and replication abnormality." Mol Biol Cell 5(10): 1145-1158.

Tame, M. A., J. A. Raaijmakers, B. van den Broek, A. Lindqvist, K. Jalink and R. H. Medema (2014). "Astral microtubules control redistribution of dynein at the cell cortex to facilitate spindle positioning." Cell Cycle 13(7): 1162-1170.

Taylor, S. S., E. Ha and F. McKeon (1998). "The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase." J Cell Biol 142(1): 1-11.

Thompson, S. L., S. F. Bakhoum and D. A. Compton (2010). "Mechanisms of chromosomal instability." Curr Biol 20(6): R285-295.

Tirupataiah, S., I. Jamir, I. Srividya and K. Mishra (2014). "Yeast Nkp2 is required for accurate chromosome segregation and interacts with several components of the central kinetochore." Mol Biol Rep 41(2): 787-797.

Todd, R. T., A. L. Braverman and A. Selmecki (2018). "Flow Cytometry Analysis of Fungal Ploidy." Curr Protoc Microbiol 50(1): e58. Todd, R. T., A. Forche and A. Selmecki (2017). "Ploidy Variation in Fungi: Polyploidy, Aneuploidy, and Genome Evolution." Microbiol Spectr 5(4).

Todd, R. T. and A. Selmecki (2020). "Expandable and reversible copy number amplification drives rapid adaptation to antifungal drugs." Elife 9.

Todd, R. T. and A. Selmecki (2023). "Copy Number Variation and Allele Ratio Analysis in Candida albicans Using Whole Genome Sequencing Data." Methods Mol Biol 2658: 105-125.

Todd, R. T., N. Soisangwan, S. Peters, B. Kemp, T. Crooks, A. Gerstein and A. Selmecki (2023). "Antifungal Drug Concentration Impacts the Spectrum of Adaptive Mutations in Candida albicans." Mol Biol Evol 40(1).

Tomonaga, T., K. Matsushita, S. Yamaguchi, T. Oohashi, H. Shimada, T. Ochiai, K. Yoda and F. Nomura (2003). "Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer." Cancer Res 63(13): 3511-3516.

Troxell, C. L., M. A. Sweezy, R. R. West, K. D. Reed, B. D. Carson, A. L. Pidoux, W. Z. Cande and J. R. McIntosh (2001). "pkl1(+)and klp2(+): Two kinesins of the Kar3 subfamily in fission yeast perform different functions in both mitosis and meiosis." Mol Biol Cell 12(11): 3476-3488.

Tutaj, H., E. Pogoda, K. Tomala and R. Korona (2019). "Gene overexpression screen for chromosome instability in yeast primarily identifies cell cycle progression genes." Curr Genet 65(2): 483-492.

Tytell, J. D. and P. K. Sorger (2006). "Analysis of kinesin motor function at budding yeast kinetochores." J Cell Biol 172(6): 861-874.

Ullmann, A. J., J. H. Lipton, D. H. Vesole, P. Chandrasekar, A. Langston, S. R. Tarantolo, H. Greinix, W. Morais de Azevedo, V. Reddy, N. Boparai, L. Pedicone, H. Patino and S. Durrant (2007). "Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease." N Engl J Med 356(4): 335-347.

van Paassen, J., A. Russcher, A. W. In 't Veld-van Wingerden, P. E. Verweij and E. J. Kuijper (2016). "Emerging aspergillosis by azole-resistant Aspergillus fumigatus at an intensive care unit in the Netherlands, 2010 to 2013." Euro Surveill 21(30).

van Toorn, M., A. Gooch, S. Boerner and T. Kiyomitsu (2023). "NuMA deficiency causes micronuclei via checkpoint-insensitive k-fiber minus-end detachment from mitotic spindle poles." Curr Biol 33(3): 572-580 e572.

Varshney, N. and K. Sanyal (2019). "Aurora kinase Ipl1 facilitates bilobed distribution of clustered kinetochores to ensure error-free chromosome segregation in Candida albicans." Mol Microbiol 112(2): 569-587.

Venkei, Z., M. R. Przewloka and D. M. Glover (2011). "Drosophila Mis12 complex acts as a single functional unit essential for anaphase chromosome movement and a robust spindle assembly checkpoint." Genetics 187(1): 131-140.

Vossen, M. L., H. M. Alhosawi, K. J. Aney and L. S. Burrack (2019). "CaMad2 Promotes Multiple Aspects of Genome Stability Beyond Its Direct Function in Chromosome Segregation." Genes (Basel) 10(12).

Wang, S. W., R. L. Read and C. J. Norbury (2002). "Fission yeast Pds5 is required for accurate chromosome segregation and for survival after DNA damage or metaphase arrest." J Cell Sci 115(Pt 3): 587-598.

Watanabe, Y. and T. S. Kitajima (2005). "Shugoshin protects cohesin complexes at centromeres." Philos Trans R Soc Lond B Biol Sci 360(1455): 515-521, discussion 521.

Wigge, P. A. and J. V. Kilmartin (2001). "The Ndc80p complex from Saccharomyces cerevisiae contains conserved centromere components and has a function in chromosome segregation." J Cell Biol 152(2): 349-360.

Williams, B. C., T. L. Karr, J. M. Montgomery and M. L. Goldberg (1992). "The Drosophila l(1)zw10 gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset." J Cell Biol 118(4): 759-773.

Williams, B. C., Z. Li, S. Liu, E. V. Williams, G. Leung, T. J. Yen and M. L. Goldberg (2003). "Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions." Mol Biol Cell 14(4): 1379-1391.

Yamamoto, A., V. Guacci and D. Koshland (1996). "Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae." J Cell Biol 133(1): 85-97.

Yang, Y., D. Tsuchiya and S. Lacefield (2015). "Bub3 promotes Cdc20-dependent activation of the APC/C in S. cerevisiae." J Cell Biol 209(4): 519-527.

Yi, F., Z. Wang, J. Liu, Y. Zhang, Z. Wang, H. Xu, X. Li, N. Bai, L. Cao and X. Song (2017). "Structural Maintenance of Chromosomes protein 1: Role in Genome Stability and Tumorigenesis." Int J Biol Sci 13(8): 1092-1099.

Yu, K. W., N. Zhong, Y. Xiao and Z. Y. She (2019). "Mechanisms of kinesin-7 CENP-E in kinetochore-microtubule capture and chromosome alignment during cell division." Biol Cell 111(6): 143-160.

Yu, Y., Y. W. Jiang, R. J. Wellinger, K. Carlson, J. M. Roberts and D. J. Stillman (1996). "Mutations in the homologous ZDS1 and ZDS2 genes affect cell cycle progression." Mol Cell Biol 16(10): 5254-5263.

Yuen, K. W., C. D. Warren, O. Chen, T. Kwok, P. Hieter and F. A. Spencer (2007). "Systematic genome instability screens in yeast and their potential relevance to cancer." Proc Natl Acad Sci U S A 104(10): 3925-3930.

Zhang, N., G. Ge, R. Meyer, S. Sethi, D. Basu, S. Pradhan, Y. J. Zhao, X. N. Li, W. W. Cai, A. K. El-Naggar, V. Baladandayuthapani, F. S. Kittrell, P. H. Rao, D. Medina and D. Pati (2008). "Overexpression of Separase induces aneuploidy and mammary tumorigenesis." Proc Natl Acad Sci U S A 105(35): 13033-13038.

Zhao, S., J. P. Latge and J. G. Gibbons (2019). "Genome Sequences of Two Strains of the Food Spoilage Mold Aspergillus fischeri." Microbiol Resour Announc 8(50).

Zhu, Y. O., G. Sherlock and D. A. Petrov (2016). "Whole Genome Analysis of 132 Clinical Saccharomyces cerevisiae Strains Reveals Extensive Ploidy Variation." G3 (Bethesda) 6(8): 2421-2434.

Zilberberg, M. D., R. Harrington, J. R. Spalding and A. F. Shorr (2019). "Burden of hospitalizations over time with invasive aspergillosis in the United States, 2004-2013." BMC Public Health 19(1): 591.

# List of References

A. V. Nywening, A. C. O. Souza* A. Martin-Vicente* (*Co-first authors) et al. (2021). "Loss of Septation Initiation Network (SIN) kinases blocks tissue invasion and unlocks echinocandin cidal activity against Aspergillus fumigatus". In: *PLoS Pathog* 17.8, e1009806. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1009806. URL: https://www.ncbi.nlm.nih.gov/pubmed/34370772.

Abdallah, Q. Al et al. (2018). "Whole-genome sequencing reveals highly specific gene targeting by in vitro assembled Cas9-ribonucleoprotein complexes in Aspergillus fumigatus". In: *Fungal Biol Biotechnol* 5. Edition: 20180605, p. 11. ISSN: 2054-3085 (Electronic) 2054-3085 (Linking). DOI: 10.1186/s40694-018-0057-2. URL: https://www.ncbi.nlm.nih.gov/pubmed/29992034.

Abdolrasouli, A. et al. (June 2, 2015). "Genomic Context of Azole Resistance Mutations in Aspergillus fumigatus Determined Using Whole-Genome Sequencing". In: *mBio* 6.3, e00536. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mBio.00536-15.

Abrieu, A. et al. (July 13, 2001). "Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint". In: *Cell* 106.1, pp. 83–93. ISSN: 0092-8674 (Print) 0092-8674 (Linking). DOI: 10.1016/s0092-8674(01)00410-x.

Adams, T. H., J. K. Wieser, and J. H. Yu (Mar. 1998). "Asexual sporulation in Aspergillus nidulans". In: *Microbiol Mol Biol Rev* 62.1, pp. 35–54. ISSN: 1092-2172 (Print) 1098-5557 (Electronic) 1092-2172 (Linking). DOI: 10.1128/MMBR.62.1.35-54.1998.

Agarwal, R. (Mar. 2009). "Allergic bronchopulmonary aspergillosis". In: *Chest* 135.3, pp. 805–826. ISSN: 1931-3543 (Electronic) 0012-3692 (Linking). DOI: 10.1378/chest.08-2586.

Akbari Dana, M. et al. (June 2019). "Effect of benomyl and diazinon on acquired azole resistance in Aspergillus flavus and expression of mdr1 and cyp51c genes". In: *Curr Med Mycol* 5.2, pp. 27–32. ISSN: 2423-3439 (Print) 2423-3420 (Electronic) 2423-3420 (Linking). DOI: 10.18502/cmm.5.2.1158.

Al Abdallah, Q., W. Ge, and J. R. Fortwendel (Dec. 2017). "A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus: In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates". In: *mSphere* 2.6. ISSN: 2379-5042 (Print) 2379-5042. DOI: 10.1128/mSphere.00446-17.

Alcazar-Fuoli, L. and E. Mellado (2012). "Ergosterol biosynthesis in Aspergillus fumigatus: its relevance as an antifungal target and role in antifungal drug resistance". In: *Front Microbiol* 3, p. 439. ISSN: 1664-302X (Electronic) 1664-302X (Linking). DOI: 10.3389/fmicb.2012.00439.

Alshrefy, A. J. et al. (2022). "Incidence of Bacterial and Fungal Secondary Infections in COVID-19 Patients Admitted to the ICU". In: *Int J Gen Med* 15, pp. 7475–7485. ISSN: 1178-7074 (Print) 1178-7074 (Electronic) 1178-7074 (Linking). DOI: 10.2147/IJGM.S382687.

Altschul, S F et al. (1990). "Basic local alignment search tool". In: *J Mol Biol* 215.3, pp. 403–410. ISSN: 0022-2836 (Print) 0022-2836 (Linking). DOI: 10.1016/S0022-2836(05)80360-2. URL: https://www.ncbi.nlm.nih.gov/pubmed/2231712.

Amitani, R. et al. (Sept. 1995). "Purification and characterization of factors produced by Aspergillus fumigatus which affect human ciliated respiratory epithelium". In: *Infect Immun* 63.9, pp. 3266–71. ISSN: 0019-9567 (Print) 1098-5522 (Electronic) 0019-9567 (Linking). DOI: 10.1128/iai.63.9.3266-3271.1995.

Andersen, B. et al. (June 2011). "Associations between fungal species and water-damaged building materials". In: *Appl Environ Microbiol* 77.12, pp. 4180–8. ISSN: 1098-5336 (Electronic) 0099-2240 (Print) 0099-2240 (Linking). DOI: 10.1128/AEM.02513-10.

Anderson, C. A. et al. (Mar. 15, 2015). "Ploidy variation in multinucleate cells changes under stress". In: *Mol Biol Cell* 26.6, pp. 1129–40. ISSN: 1939-4586 (Electronic) 1059-1524 (Print) 1059-1524 (Linking). DOI: 10.1091/mbc.E14-09-1375.

Ando, K. et al. (Mar. 2010). "High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer". In: *Cancer Sci* 101.3, pp. 639–45. ISSN: 1349-7006 (Electronic) 1347-9032 (Linking). DOI: 10.1111/j.1349-7006.2009.01457.x.

Ansari, Z., D. Miller, and A. Galor (Sept. 1, 2013). "Current Thoughts in Fungal Keratitis: Diagnosis and Treatment". In: *Curr Fungal Infect Rep* 7.3, pp. 209–218. ISSN: 1936-3761 (Print) 1936-377X (Electronic) 1936-3761 (Linking). DOI: 10.1007/s12281-013-0150-110.1007/s12281-013-0150-1.

Aruanno, M, E Glampedakis, and F Lamoth (2019). "Echinocandins for the Treatment of Invasive Aspergillosis: from Laboratory to Bedside". In: *Antimicrob Agents Chemother* 63.8. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00399-19. URL: https://www.ncbi.nlm.nih.gov/pubmed/31138565.

Avkan-Oguz, V. et al. (Jan. 8, 2020). "Primary Cutaneous Aspergillosis in Immunocompetent Adults: Three Cases and a Review of the Literature". In: *Cureus* 12.1, e6600. ISSN: 2168-8184 (Print) 2168-8184 (Electronic) 2168-8184 (Linking). DOI: 10.7759/cureus.6600.

Bachewich, C., K. Masker, and S. Osmani (Jan. 2005). "The polo-like kinase PLKA is required for initiation and progression through mitosis in the filamentous fungus Aspergillus nidulans". In: *Mol Microbiol* 55.2, pp. 572–87. ISSN: 0950-382X (Print) 0950-382X (Linking). DOI: 10.1111/j.1365-2958.2004.04404.x.

Badali, H et al. (2022). "Species Distribution and Antifungal Susceptibilities of Aspergillus Section Fumigati Isolates in Clinical Samples from the United States". In: *J Clin Microbiol* 60.5, e0028022. ISSN: 1098-660X (Electronic) 0095-1137 (Print) 0095-1137 (Linking). DOI: 10.1128/jcm.00280-22. URL: https://www.ncbi.nlm.nih.gov/pubmed/35400175.

Bader, O. et al. (Aug. 2013). "cyp51A-Based mechanisms of Aspergillus fumigatus azole drug resistance present in clinical samples from Germany". In: *Antimicrob Agents Chemother* 57.8, pp. 3513–7. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00167-13.

Balkunde, Rachappa and Ram Dixit (2021). "Cytoskeleton | Functional interactions between actin filaments and microtubules in plant and animal cells". In: *Encyclopedia of Biological Chemistry: Third Edition*. Vol. 5. DOI: 10.1016/B978-0-12-819460-7.00303-0.

Ballard, E. et al. (Sept. 2019). "Recreation of in-host acquired single nucleotide polymorphisms by CRISPR-Cas9 reveals an uncharacterised gene playing a role in Aspergillus fumigatus azole resistance via a non-cyp51A mediated resistance mechanism". In: *Fungal Genet Biol* 130, pp. 98–106. ISSN: 1096-0937 (Electronic) 1087-1845 (Print) 1087-1845 (Linking). DOI: 10.1016/j.fgb.2019.05.005.

Barac, A. et al. (Apr. 13, 2017). "Complications of chronic necrotizing pulmonary aspergillosis: review of published case reports". In: *Rev Inst Med Trop Sao Paulo* 59, e19. ISSN: 1678-9946 (Electronic) 0036-4665 (Print) 0036-4665 (Linking). DOI: 10.1590/S1678-9946201759019.

Barda, O. et al. (Aug. 17, 2023). "Aneuploidy Formation in the Filamentous Fungus Aspergillus flavus in Response to Azole Stress". In: *Microbiol Spectr* 11.4, e0433922. ISSN: 2165-0497 (Electronic) 2165-0497 (Linking). DOI: 10.1128/spectrum.04339-22.

Basenko, E Y et al. (2018). "FungiDB: An Integrated Bioinformatic Resource for Fungi and Oomycetes". In: *J Fungi (Basel)* 4.1. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof4010039. URL: https://www.ncbi.nlm.nih.gov/pubmed/30152809.

Basu, J et al. (1999). "Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila". In: *J Cell Biol* 146.1, pp. 13–28. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). DOI: 10.1083/jcb.146.1.13. URL: https://www.ncbi.nlm.nih.gov/pubmed/10402457.

Bellete, B. et al. (Feb. 2010). "Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma". In: *Med Mycol* 48.1, pp. 197–200. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.3109/13693780902717018.

Benedict, K et al. (2019). "Estimation of Direct Healthcare Costs of Fungal Diseases in the United States". In: *Clin Infect Dis* 68.11, pp. 1791–1797. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/ciy776. URL: https://www.ncbi.nlm.nih.gov/pubmed/30204844.

Benet, T. et al. (Feb. 2013). "Estimation of the incubation period of invasive aspergillosis by survival models in acute myeloid leukemia patients". In: *Med Mycol* 51.2, pp. 214–8. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.3109/13693786.2012.687462.

Bharathi, M. J. et al. (Apr. 2007). "Microbial keratitis in South India: influence of risk factors, climate, and geographical variation". In: *Ophthalmic Epidemiol* 14.2, pp. 61–9. ISSN: 0928-6586 (Print) 0928-6586 (Linking). DOI: 10.1080/09286580601001347.

Bing, J. et al. (Dec. 16, 2020). "Experimental Evolution Identifies Adaptive Aneuploidy as a Mechanism of Fluconazole Resistance in Candida auris". In: *Antimicrob Agents Chemother* 65.1. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.01466-20.

Blanco, J. J. et al. (Apr. 2011). "[Chronic necrotizing pulmonary aspergillosis as a complication of silicosis]". In: *An Sist Sanit Navar* 34.1, pp. 109–14. ISSN: 2340-3527 (Electronic) 1137-6627 (Linking). DOI: 10.4321/s1137-66272011000100013.

Bolanos-Garcia, V M and T L Blundell (2011). "BUB1 and BUBR1: multifaceted kinases of the cell cycle". In: *Trends Biochem Sci* 36.3, pp. 141–150. ISSN: 0968-0004 (Print) 0968-0004 (Linking). DOI: 10.1016/j.tibs.2010.08.004. URL: https://www.ncbi.nlm.nih.gov/pubmed/20888775.

Bongomin, F. et al. (June 2, 2020). "Chronic Pulmonary Aspergillosis: Notes for a Clinician in a Resource-Limited Setting Where There Is No Mycologist". In: *J Fungi (Basel)* 6.2. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof6020075.

Bowman, J C et al. (2002). "The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro". In: *Antimicrob Agents Chemother* 46.9, pp. 3001–3012. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.46.9.3001-3012.2002. URL: https://www.ncbi.nlm.nih.gov/pubmed/12183260.

Bowyer, P, M J Bromley, and D W Denning (2020). "Linking calcium signaling and mitochondrial function in fungal drug resistance". In: *Proc Natl Acad Sci U S A* 117.3, pp. 1254–1256. ISSN: 1091-6490 (Electronic) 0027-8424 (Print) 0027-8424 (Linking). DOI: 10.1073/pnas.1920497117. URL: https://www.ncbi.nlm.nih.gov/pubmed/31900354.

Boyd, S. et al. (May 7, 2022). "Elevated Rates of Ventilator-Associated Pneumonia and COVID-19 Associated Pulmonary Aspergillosis in Critically Ill Patients with SARS-CoV2 Infection in the Second Wave: A Retrospective Chart Review". In: *Antibiotics (Basel)* 11.5. ISSN: 2079-6382 (Print) 2079-6382 (Electronic) 2079-6382 (Linking). DOI: 10.3390/antibiotics11050632.

Bradley, K et al. (2022). "Fatal Fungicide-Associated Triazole-Resistant Aspergillus fumigatus Infection, Pennsylvania, USA". In: *Emerg Infect Dis* 28.9, pp. 1904–1905. ISSN: 1080-6059 (Electronic) 1080-6040 (Print) 1080-6040 (Linking). DOI: 10.3201/eid2809.220517. URL: https://www.ncbi.nlm.nih.gov/pubmed/35997507.

Brady, D M and K G Hardwick (2000). "Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function". In: *Curr Biol* 10.11, pp. 675–678. ISSN: 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/s0960-9822(00)00515-7. URL: https://www.ncbi.nlm.nih.gov/pubmed/10837255.

Brimacombe, C A et al. (2019). "A natural histone H2A variant lacking the Bub1 phosphorylation site and regulated depletion of centromeric histone CENP-A foster evolvability in Candida albicans". In: *PLoS Biol* 17.6, e3000331. ISSN: 1545-7885 (Electronic) 1544-9173 (Print) 1544-9173 (Linking). DOI: 10.1371/journal.pbio.3000331. URL: https://www.ncbi.nlm.nih.gov/pubmed/31226107.

Brown, G. D. et al. (Dec. 19, 2012). "Hidden killers: human fungal infections". In: *Sci Transl Med* 4.165, 165rv13. ISSN: 1946-6242 (Electronic) 1946-6234 (Linking). DOI: 10.1126/scitranslmed.3004404.

Brown, L. et al. (Mar. 2021). "The global incidence and diagnosis of fungal keratitis". In: *Lancet Infect Dis* 21.3, e49–e57. ISSN: 1474-4457 (Electronic) 1473-3099 (Linking). DOI: 10.1016/S1473-3099(20)30448-5.

Brown, M. E. et al. (June 2021). "A novel 3D culture model of fungal keratitis to explore host-pathogen interactions within the stromal environment". In: *Exp Eye Res* 207, p. 108581. ISSN: 1096-0007 (Electronic) 0014-4835 (Print) 0014-4835 (Linking). DOI: 10.1016/j.exer.2021.108581.

Buil, J. B. et al. (Aug. 2019). "The fading boundaries between patient and environmental routes of triazole resistance selection in Aspergillus fumigatus". In: *PLoS Pathog* 15.8, e1007858. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1007858.

Burgel, P R et al. (2012). "High prevalence of azole-resistant Aspergillus fumigatus in adults with cystic fibrosis exposed to itraconazole". In: *Antimicrob Agents Chemother* 56.2, pp. 869–874. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.05077-11. URL: https://www.ncbi.nlm.nih.gov/pubmed/22123701.

Cadena, J, G R Thompson 3rd, and T F Patterson (2021). "Aspergillosis: Epidemiology, Diagnosis, and Treatment". In: *Infect Dis Clin North Am* 35.2, pp. 415–434. ISSN: 1557-9824 (Electronic) 0891-5520 (Linking). DOI: 10.1016/j.idc.2021.03.008. URL: https://www.ncbi.nlm.nih.gov/pubmed/34016284.

Cahill, D. P. et al. (Mar. 19, 1998). "Mutations of mitotic checkpoint genes in human cancers". In: *Nature* 392.6673, pp. 300–3. ISSN: 0028-0836 (Print) 0028-0836 (Linking). DOI: 10.1038/32688.

Camps, S M et al. (2012). "Discovery of a HapE mutation that causes azole resistance in Aspergillus fumigatus through whole genome sequencing and sexual crossing". In: *PLoS One* 7.11, e50034. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0050034. URL: https://www.ncbi.nlm.nih.gov/pubmed/23226235.

Chan, C. S. and D. Botstein (Nov. 1993). "Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast". In: *Genetics* 135.3, pp. 677–91. ISSN: 0016-6731 (Print) 0016-6731 (Linking). DOI: 10.1093/genetics/135.3.677.

Chang, Y C, A Khanal Lamichhane, and K J Kwon-Chung (2018). "Cryptococcus neoformans, Unlike Candida albicans, Forms Aneuploid Clones Directly from Uninucleated Cells under Fluconazole Stress". In: *mBio* 9.6. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mBio.01290-18. URL: https://www.ncbi.nlm.nih.gov/pubmed/30514783.

Cheeseman, I. M. and A. Desai (Jan. 2008). "Molecular architecture of the kinetochore-microtubule interface". In: *Nat Rev Mol Cell Biol* 9.1, pp. 33–46. ISSN: 1471-0080 (Electronic) 1471-0072 (Linking). DOI: 10.1038/nrm2310.

Chelius, C et al. (2020). "Dynamic Transcriptomic and Phosphoproteomic Analysis During Cell Wall Stress in Aspergillus nidulans". In: *Mol Cell Proteomics* 19.8, pp. 1310–1329. ISSN: 1535-9484 (Electronic) 1535-9476 (Print) 1535-9476 (Linking). DOI: 10.1074/mcp.RA119.001769. URL: https://www.ncbi.nlm.nih.gov/pubmed/32430394.

Chen, G. et al. (Jan. 29, 2012). "Hsp90 stress potentiates rapid cellular adaptation through induction of aneuploidy". In: *Nature* 482.7384, pp. 246–50. ISSN: 1476-4687 (Electronic) 0028-0836 (Print) 0028-0836 (Linking). DOI: 10.1038/nature10795.

Chen, J. et al. (Jan. 2005). "Mutations in the cyp51A gene and susceptibility to itraconazole in Aspergillus fumigatus serially isolated from a patient with lung aspergilloma". In: *J Antimicrob Chemother* 55.1, pp. 31–7. ISSN: 0305-7453 (Print) 0305-7453 (Linking). DOI: 10.1093/jac/dkh507.

Cheung, N., P. Nagra, and K. Hammersmith (July 2016). "Emerging trends in contact lens-related infections". In: *Curr Opin Ophthalmol* 27.4, pp. 327–32. ISSN: 1531-7021 (Electronic) 1040-8738 (Linking). DOI: 10.1097/ICU.0000000000000280.

Chowdhary, A., S. Kathuria, et al. (2012). "Clonal expansion and emergence of environmental multiple-triazole-resistant Aspergillus fumigatus strains carrying the TR(3)(4)/L98H mutations in the cyp51A gene in India". In: *PLoS One* 7.12, e52871. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0052871.

Chowdhary, A., C. Sharma, M. van den Boom, et al. (Nov. 2014). "Multi-azole-resistant Aspergillus fumigatus in the environment in Tanzania". In: *J Antimicrob Chemother* 69.11, pp. 2979–83. ISSN: 1460-2091 (Electronic) 0305-7453 (Linking). DOI: 10.1093/jac/dku259.

Chowdhary, A., C. Sharma, S. Kathuria, et al. (2015). "Prevalence and mechanism of triazole resistance in Aspergillus fumigatus in a referral chest hospital in Delhi, India and an update of the situation in Asia". In: *Front Microbiol* 6, p. 428. ISSN: 1664-302X (Print) 1664-302X (Electronic) 1664-302X (Linking). DOI: 10.3389/fmicb.2015.00428.

Cimini, D. et al. (Sept. 5, 2006). "Aurora kinase promotes turnover of kinetochore microtubules to reduce chromosome segregation errors". In: *Curr Biol* 16.17, pp. 1711–8. ISSN: 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2006.07.022.

Collier, S. A. et al. (Nov. 14, 2014). "Estimated burden of keratitis–United States, 2010". In: *MMWR Morb Mortal Wkly Rep* 63.45, pp. 1027–30. ISSN: 1545-861X (Electronic) 0149-2195 (Print) 0149-2195 (Linking).

Cornely, O A et al. (2007). "Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia". In: *N Engl J Med* 356.4, pp. 348–359. ISSN: 1533-4406 (Electronic) 0028-4793 (Linking). DOI: 10.1056/NEJMoa061094. URL: https://www.ncbi.nlm.nih.gov/pubmed/17251531.

Dagenais, T R and N P Keller (2009). "Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis". In: *Clin Microbiol Rev* 22.3, pp. 447–465. ISSN: 1098-6618 (Electronic) 0893-8512 (Print) 0893-8512 (Linking). DOI: 10.1128/CMR.00055-08. URL: https://www.ncbi.nlm.nih.gov/pubmed/19597008.

Dai, W. et al. (Jan. 15, 2004). "Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency". In: *Cancer Res* 64.2, pp. 440–5. ISSN: 0008-5472 (Print) 0008-5472 (Linking). DOI: 10.1158/0008-5472.can-03-3119.

Davies, A. E. and K. B. Kaplan (Apr. 19, 2010). "Hsp90-Sgt1 and Skp1 target human Mis12 complexes to ensure efficient formation of kinetochore-microtubule binding sites". In: *J Cell Biol* 189.2, pp. 261–74. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.200910036.

De Souza, C P et al. (2013). "Functional analysis of the Aspergillus nidulans kinome". In: *PLoS One* 8.3, e58008. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0058008. URL: https://www.ncbi.nlm.nih.gov/pubmed/23505451.

De Vroey, C (1979). "[Saphrophytic life of opportunistic fungi]". In: *Dermatologica* 159 (Suppl 1), pp. 47–55. ISSN: 0011-9075 (Print) 0011-9075 (Linking). URL: https://www.ncbi.nlm.nih.gov/pubmed/114433.

Deak, E. et al. (June 2011). "Aspergillus terreus accessory conidia are multinucleated, hyperpolarizing structures that display differential dectin staining and can induce heightened inflammatory responses in a pulmonary model of aspergillosis". In: *Virulence* 2.3, pp. 200–7. ISSN: 2150-5608 (Electronic) 2150-5594 (Print) 2150-5594 (Linking). DOI: 10.4161/viru.2.3.15799.

Denning, D. W., S. Park, et al. (May 2011). "High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease". In: *Clin Infect Dis* 52.9, pp. 1123–9. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/cir179.

Denning, D. W., S. A. Radford, et al. (Sept. 1997). "Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of Aspergillus fumigatus infection". In: *J Antimicrob Chemother* 40.3, pp. 401–14. ISSN: 0305-7453 (Print) 0305-7453 (Linking). DOI: 10.1093/jac/40.3.401.

Denning, D. W., K. Riniotis, et al. (Oct. 1, 2003). "Chronic cavitary and fibrosing pulmonary and pleural aspergillosis: case series, proposed nomenclature change, and review". In: *Clin Infect Dis* 37 Suppl 3, S265–80. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). DOI: 10.1086/376526.

Diaz-Guerra, T. M. et al. (Mar. 2003). "A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in Aspergillus fumigatus". In: *Antimicrob Agents Chemother* 47.3, pp. 1120–4. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.47.3.1120-1124.2003.

Dos Reis, T. F. et al. (Apr. 12, 2023). "A host defense peptide mimetic, brilacidin, potentiates caspofungin antifungal activity against human pathogenic fungi". In: *Nat Commun* 14.1, p. 2052. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/s41467-023-37573-y.

Dudakova, A et al. (2017). "Molecular Tools for the Detection and Deduction of Azole Antifungal Drug Resistance Phenotypes in Aspergillus Species". In: *Clin Microbiol Rev* 30.4, pp. 1065–1091. ISSN: 1098-6618 (Electronic) 0893-8512 (Print) 0893-8512 (Linking). DOI: 10.1128/CMR.00095-16. URL: https://www.ncbi.nlm.nih.gov/pubmed/28903985.

Edgerton, H, V Paolillo, and B R Oakley (2015). "Spatial regulation of the spindle assembly checkpoint and anaphase-promoting complex in Aspergillus nidulans". In: *Mol Microbiol* 95.3, pp. 442–457. ISSN: 1365-2958 (Electronic) 0950-382X (Print) 0950-382X (Linking). DOI: 10.1111/mmi.12871. URL: https://www.ncbi.nlm.nih.gov/pubmed/25417844.

Edlind, T. et al. (Oct. 2002). "Antifungal activity in Saccharomyces cerevisiae is modulated by calcium signalling". In: *Mol Microbiol* 46.1, pp. 257–68. ISSN: 0950-382X (Print) 0950-382X (Linking). DOI: 10.1046/j.1365-2958.2002.03165.x.

Edwards, F. et al. (Dec. 14, 2018). "BUB-1 promotes amphitelic chromosome biorientation via multiple activities at the kinetochore". In: *Elife* 7. ISSN: 2050-084X (Electronic) 2050-084X (Linking). DOI: 10.7554/eLife.40690.

Efimov, V P and N R Morris (1998). "A screen for dynein synthetic lethals in Aspergillus nidulans identifies spindle assembly checkpoint genes and other genes involved in mitosis". In: *Genetics* 149.1, pp. 101–116. ISSN: 0016-6731 (Print) 0016-6731 (Linking). DOI: 10.1093/genetics/149.1.101. URL: https://www.ncbi.nlm.nih.gov/pubmed/9584089.

Escobar, Natalia et al. (Apr. 2016). "Hide, Keep Quiet, and Keep Low: Properties That Make Aspergillus fumigatus a Successful Lung Pathogen". In: *Frontiers in Microbiology* 7. ISSN: 1664-302X. DOI: 10.3389/fmicb.2016.00438.

Falcone, M. et al. (May 2011). "Invasive aspergillosis in patients with liver disease". In: *Med Mycol* 49.4, pp. 406–13. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.3109/13693786.2010.535030.

Fan, X. et al. (Dec. 15, 2023). "Tandem gene duplications contributed to high-level azole resistance in a rapidly expanding Candida tropicalis population". In: *Nat Commun* 14.1, p. 8369. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/s41467-023-43380-2.

Fan, Y. et al. (June 4, 2021). "Genome-Wide Association Analysis for Triazole Resistance in Aspergillus fumigatus". In: *Pathogens* 10.6. ISSN: 2076-0817 (Print) 2076-0817 (Electronic) 2076-0817 (Linking). DOI: 10.3390/pathogens10060701.

Farkasovsky, M. and H. Kuntzel (Nov. 1995). "Yeast Num1p associates with the mother cell cortex during S/G2 phase and affects microtubular functions". In: *J Cell Biol* 131.4, pp. 1003–14. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). DOI: 10.1083/jcb.131.4.1003.

Fernius, J. and K. G. Hardwick (Nov. 2007). "Bub1 kinase targets Sgo1 to ensure efficient chromosome biorientation in budding yeast mitosis". In: *PLoS Genet* 3.11, e213. ISSN: 1553-7404 (Electronic) 1553-7390 (Print) 1553-7390 (Linking). DOI: 10.1371/journal.pgen.0030213.

Ferreira, M. E. et al. (May 2005). "The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus". In: *Med Mycol* 43 Suppl 1, S313–9. ISSN: 1369-3786 (Print) 1369-3786 (Linking). DOI: 10.1080/13693780400029114.

Fisher, M C and D W Denning (2023). "The WHO fungal priority pathogens list as a game-changer". In: *Nat Rev Microbiol* 21.4, pp. 211–212. ISSN: 1740-1534 (Electronic) 1740-1526 (Print) 1740-1526 (Linking). DOI: 10.1038/s41579-023-00861-x. URL: https://www.ncbi.nlm.nih.gov/pubmed/36747091.

Fisk, H. A., C. P. Mattison, and M. Winey (Apr. 2004). "A field guide to the Mps1 family of protein kinases". In: *Cell Cycle* 3.4, pp. 439–42. ISSN: 1538-4101 (Print) 1551-4005 (Linking).

Foley, E. A. and T. M. Kapoor (Jan. 2013). "Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore". In: *Nat Rev Mol Cell Biol* 14.1, pp. 25–37. ISSN: 1471-0080 (Electronic) 1471-0072 (Print) 1471-0072 (Linking). DOI: 10.1038/nrm3494.

Fraczek, M. G. et al. (July 2013). "The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in Aspergillus fumigatus". In: *J Antimicrob Chemother* 68.7, pp. 1486–96. ISSN: 1460-2091 (Electronic) 0305-7453 (Linking). DOI: 10.1093/jac/dkt075.

Fuhren, J. et al. (Oct. 2015). "High prevalence of azole resistance in Aspergillus fumigatus isolates from high-risk patients". In: *J Antimicrob Chemother* 70.10, pp. 2894–8. ISSN: 1460-2091 (Electronic) 0305-7453 (Linking). DOI: 10.1093/jac/dkv177.

Fumagalli, J. et al. (Feb. 1, 2022). "Ventilator-associated pneumonia among SARS-CoV-2 acute respiratory distress syndrome patients". In: *Curr Opin Crit Care* 28.1, pp. 74–82. ISSN: 1531-7072 (Electronic) 1070-5295 (Print) 1070-5295 (Linking). DOI: 10.1097/MCC.0000000000000908.

Funabiki, H., K. Kumada, and M. Yanagida (Dec. 2, 1996). "Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes". In: *EMBO J* 15.23, pp. 6617–28. ISSN: 0261-4189 (Print) 1460-2075 (Electronic) 0261-4189 (Linking).

Furukawa, T. et al. (Jan. 22, 2020). "The negative cofactor 2 complex is a key regulator of drug resistance in Aspergillus fumigatus". In: *Nat Commun* 11.1, p. 427. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/s41467-019-14191-1.

Garcia-Vidal, C. et al. (2015). "Causes of death in a contemporary cohort of patients with invasive aspergillosis". In: *PLoS One* 10.3, e0120370. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0120370.

Garribba, Lorenza and Stefano Santaguida (2022). "The Dynamic Instability of the Aneuploid Genome". In: *Frontiers in Cell and Developmental Biology* 10. ISSN: 2296634X. DOI: 10.3389/fcell.2022.838928.

Gassmann, R. et al. (Sept. 1, 2008). "A new mechanism controlling kinetochore-microtubule interactions revealed by comparison of two dynein-targeting components: SPDL-1 and the Rod/Zwilch/Zw10 complex". In: *Genes Dev* 22.17, pp. 2385–99. ISSN: 0890-9369 (Print) 1549-5477 (Electronic) 0890-9369 (Linking). DOI: 10.1101/gad.1687508.

Giacobbe, D. R. et al. (Mar. 2020). "Mould Infections of Traumatic Wounds: A Brief Narrative Review". In: *Infect Dis Ther* 9.1, pp. 1–15. ISSN: 2193-8229 (Print) 2193-6382 (Electronic) 2193-6382 (Linking). DOI: 10.1007/s40121-020-00284-8.

Glotzer, M. (Jan. 2009). "The 3Ms of central spindle assembly: microtubules, motors and MAPs". In: *Nat Rev Mol Cell Biol* 10.1, pp. 9–20. ISSN: 1471-0080 (Electronic) 1471-0072 (Print) 1471-0072 (Linking). DOI: 10.1038/nrm2609.

Goh, K. J. et al. (2017). "Sensitization to Aspergillus species is associated with frequent exacerbations in severe asthma". In: *J Asthma Allergy* 10, pp. 131–140. ISSN: 1178-6965 (Print) 1178-6965 (Electronic) 1178-6965 (Linking). DOI: 10.2147/JAA.S130459.

Gomes, M. Z. et al. (May 2014). "Effectiveness of primary anti-Aspergillus prophylaxis during remission induction chemotherapy of acute myeloid leukemia". In: *Antimicrob Agents Chemother* 58.5, pp. 2775–80. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.01527-13.

Gonczy, P. (May 2008). "Mechanisms of asymmetric cell division: flies and worms pave the way". In: *Nat Rev Mol Cell Biol* 9.5, pp. 355–66. ISSN: 1471-0080 (Electronic) 1471-0072 (Linking). DOI: 10.1038/nrm2388.

Goto, G H et al. (2011). "Bub1-mediated adaptation of the spindle checkpoint". In: *PLoS Genet* 7.1, e1001282. ISSN: 1553-7404 (Electronic) 1553-7390 (Print) 1553-7390 (Linking). DOI: 10.1371/journal.pgen.1001282. URL: https://www.ncbi.nlm.nih.gov/pubmed/21298086.

Groll, A. H., P. M. Shah, et al. (July 1996). "Trends in the postmortem epidemiology of invasive fungal infections at a university hospital". In: *J Infect* 33.1, pp. 23–32. ISSN: 0163-4453 (Print) 0163-4453 (Linking). DOI: 10.1016/s0163-4453(96)92700-0.

Groll, A. H. and T. J. Walsh (2001). "Uncommon opportunistic fungi: new nosocomial threats". In: *Clin Microbiol Infect* 7 Suppl 2, pp. 8–24. ISSN: 1198-743X (Print) 1198-743X (Linking). DOI: 10.1111/j.1469-0691.2001.tb00005.x.

Gsaller, F. et al. (Dec. 2016). "Correction: Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex". In: *PLoS Pathog* 12.12, e1006106. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1006106.

Gsaller, Fabio et al. (2016). "Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex". In: *PLOS Pathogens* 12.7. Publisher: Public Library of Science, e1005775. DOI: 10.1371/journal.ppat.1005775. URL: https://doi.org/10.1371/journal.ppat.1005775.

Gupta, A. et al. (2018). "Purification of kinetochores from the budding yeast Saccharomyces cerevisiae". In: *Methods Cell Biol* 144, pp. 349–370. ISSN: 0091-679X (Print) 0091-679X (Linking). DOI: 10.1016/bs.mcb.2018.03.023.

Hagiwara, D., T. Arai, et al. (Oct. 2018). "Non-cyp51A Azole-Resistant Aspergillus fumigatus Isolates with Mutation in HMG-CoA Reductase". In: *Emerg Infect Dis* 24.10, pp. 1889–1897. ISSN: 1080-6059 (Electronic) 1080-6040 (Print) 1080-6040 (Linking). DOI: 10.3201/eid2410.180730.

Hagiwara, D., H. Takahashi, et al. (Dec. 2014). "Whole-genome comparison of Aspergillus fumigatus strains serially isolated from patients with aspergillosis". In: *J Clin Microbiol* 52.12, pp. 4202–9. ISSN: 1098-660X (Electronic) 0095-1137 (Print) 0095-1137 (Linking). DOI: 10.1128/JCM.01105-14.

Hagiwara, D., A. Watanabe, et al. (2016). "Epidemiological and Genomic Landscape of Azole Resistance Mechanisms in Aspergillus Fungi". In: *Front Microbiol* 7, p. 1382. ISSN: 1664-302X (Print) 1664-302X (Electronic) 1664-302X (Linking). DOI: 10.3389/fmicb.2016.01382.

Handelman, M. and N. Osherov (2022). "Experimental and in-host evolution of triazole resistance in human pathogenic fungi". In: *Front Fungal Biol* 3, p. 957577. ISSN: 2673-6128 (Electronic) 2673-6128 (Linking). DOI: 10.3389/ffunb.2022.957577.

Harrison, B. D. et al. (Mar. 2014). "A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole". In: *PLoS Biol* 12.3, e1001815. ISSN: 1545-7885 (Electronic) 1544-9173 (Print) 1544-9173 (Linking). DOI: 10.1371/journal.pbio.1001815.

Hernando, E. et al. (July 20, 2001). "Molecular analyses of the mitotic checkpoint components hsMAD2, hBUB1 and hBUB3 in human cancer". In: *Int J Cancer* 95.4, pp. 223–7. ISSN: 0020-7136 (Print) 0020-7136 (Linking). DOI: 10.1002/1097-0215(20010720)95:4<223::aid-ijc1038>3.0.co;2-l.

Herrick, E. J. and M. F. Hashmi (2023). "Antifungal Ergosterol Synthesis Inhibitors". In: *StatPearls*. Treasure Island (FL).

Hill, J A et al. (2013). "Genetic and genomic architecture of the evolution of resistance to antifungal drug combinations". In: *PLoS Genet* 9.4, e1003390. ISSN: 1553-7404 (Electronic) 1553-7390 (Print) 1553-7390 (Linking). DOI: 10.1371/journal.pgen.1003390. URL: https://www.ncbi.nlm.nih.gov/pubmed/23593013.

Hodiamont, C J et al. (2009). "Multiple-azole-resistant Aspergillus fumigatus osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery". In: *Med Mycol* 47.2, pp. 217–220. ISSN: 1369-3786 (Print) 1369-3786 (Linking). DOI: 10.1080/13693780802545600. URL: https://www.ncbi.nlm.nih.gov/pubmed/19101840.

Hokken, M. W. J. et al. (July 30, 2023). "The Transcriptome Response to Azole Compounds in Aspergillus fumigatus Shows Differential Gene Expression across Pathways Essential for Azole Resistance and Cell Survival". In: *J Fungi (Basel)* 9.8. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof9080807.

Horta, M A C et al. (2022). "Examination of Genome-Wide Ortholog Variation in Clinical and Environmental Isolates of the Fungal Pathogen Aspergillus fumigatus". In: *mBio* 13.4, e0151922. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mbio.01519-22. URL: https://www.ncbi.nlm.nih.gov/pubmed/35766381.

Howard, S. J., D. Cerar, et al. (July 2009). "Frequency and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure". In: *Emerg Infect Dis* 15.7, pp. 1068–76. ISSN: 1080-6059 (Electronic) 1080-6040 (Print) 1080-6040 (Linking). DOI: 10.3201/eid1507.090043.

Howard, S. J., A. C. Pasqualotto, et al. (July 2013). "Major variations in Aspergillus fumigatus arising within aspergillomas in chronic pulmonary aspergillosis". In: *Mycoses* 56.4, pp. 434–41. ISSN: 1439-0507 (Electronic) 0933-7407 (Linking). DOI: 10.1111/myc.12047.

Howard, S. J., I. Webster, et al. (Nov. 2006). "Multi-azole resistance in Aspergillus fumigatus". In: *Int J Antimicrob Agents* 28.5, pp. 450–3. ISSN: 0924-8579 (Print) 0924-8579 (Linking). DOI: 10.1016/j.ijantimicag.2006.08.017.

Ippolito, M. et al. (Dec. 2021). "Personal protective equipment use by healthcare workers in intensive care unit during the early phase of COVID-19 pandemic in Italy: a secondary analysis of the PPE-SAFE survey". In: *Ther Adv Infect Dis* 8, p. 2049936121998562. ISSN: 2049-9361 (Print) 2049-937X (Electronic) 2049-9361 (Linking). DOI: 10.1177/2049936121998562.

Jabra-Rizk, M. A., W. A. Falkler, and T. F. Meiller (Jan. 2004). "Fungal biofilms and drug resistance". In: *Emerg Infect Dis* 10.1, pp. 14–9. ISSN: 1080-6040 (Print) 1080-6059 (Electronic) 1080-6040 (Linking). DOI: 10.3201/eid1001.030119.

Jez, Joseph (2021). *Encyclopedia of biological chemistry*. 3rd. San Diego: Elsevier.

Jia, L., B. Li, and H. Yu (Feb. 25, 2016). "The Bub1-Plk1 kinase complex promotes spindle checkpoint signalling through Cdc20 phosphorylation". In: *Nat Commun* 7, p. 10818. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/ncomms10818.

Jia, X. M. et al. (Jan. 2009). "RTA2 is involved in calcineurin-mediated azole resistance and sphingoid long-chain base release in Candida albicans". In: *Cell Mol Life Sci* 66.1, pp. 122–34. ISSN: 1420-9071 (Electronic) 1420-682X (Linking). DOI: 10.1007/s00018-008-8409-3.

Jin, F., M. Bokros, and Y. Wang (Mar. 2017). "Premature Silencing of the Spindle Assembly Checkpoint Is Prevented by the Bub1-H2A-Sgo1-PP2A Axis in Saccharomyces cerevisiae". In: *Genetics* 205.3, pp. 1169–1178. ISSN: 1943-2631 (Electronic) 0016-6731 (Print) 0016-6731 (Linking). DOI: 10.1534/genetics.116.195727.

Jumper, J. et al. (Aug. 2021). "Highly accurate protein structure prediction with AlphaFold". In: *Nature* 596.7873, pp. 583–589. ISSN: 1476-4687 (Electronic) 0028-0836 (Print) 0028-0836 (Linking). DOI: 10.1038/s41586-021-03819-2.

Juvvadi, P. R., D. C. Cole, et al. (Nov. 2, 2018). "Kin1 kinase localizes at the hyphal septum and is dephosphorylated by calcineurin but is dispensable for septation and virulence in the human pathogen Aspergillus fumigatus". In: *Biochem Biophys Res Commun* 505.3, pp. 740–746. ISSN: 1090-2104 (Electronic) 0006-291X (Print) 0006-291X (Linking). DOI: 10.1016/j.bbrc.2018.09.186.

Juvvadi, P. R., F. Lamoth, and W. J. Steinbach (Oct. 2014). "Calcineurin as a Multifunctional Regulator: Unraveling Novel Functions in Fungal Stress Responses, Hyphal Growth, Drug Resistance, and Pathogenesis". In: *Fungal Biol Rev* 28.2, pp. 56–69. ISSN: 1749-4613 (Print) 1878-0253 (Electronic) 1749-4613 (Linking). DOI: 10.1016/j.fbr.2014.02.004.

Juvvadi, P. R., S. C. Lee, et al. (Feb. 17, 2017). "Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach". In: *Virulence* 8.2, pp. 186–197. ISSN: 2150-5608 (Electronic) 2150-5594 (Print) 2150-5594 (Linking). DOI: 10.1080/21505594.2016.1201250.

Kamath, M. M. et al. (Oct. 2023). "The Aspergillus fumigatus UPR is variably activated across nutrient and host environments and is critical for the establishment of corneal infection". In: *PLoS Pathog* 19.10, e1011435. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1011435.

Kamthan, M. et al. (2014). "Characterization of a putative spindle assembly checkpoint kinase Mps1, suggests its involvement in cell division, morphogenesis and oxidative stress tolerance in Candida albicans". In: *PLoS One* 9.7, e101517. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0101517.

Kawashima, S. A. et al. (Jan. 8, 2010). "Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin". In: *Science* 327.5962, pp. 172–7. ISSN: 1095-9203 (Electronic) 0036-8075 (Linking). DOI: 10.1126/science.1180189.

Khateb, A. et al. (Apr. 18, 2023). "Aneuploidy Is Associated with Azole Resistance in Aspergillus fumigatus". In: *Antimicrob Agents Chemother* 67.4, e0125322. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/aac.01253-22.

Kim, J. et al. (Nov. 2012). "A defect in iron uptake enhances the susceptibility of Cryptococcus neoformans to azole antifungal drugs". In: *Fungal Genet Biol* 49.11, pp. 955–66. ISSN: 1096-0937 (Electronic) 1087-1845 (Print) 1087-1845 (Linking). DOI: 10.1016/j.fgb.2012.08.006.

Kim, T. (May 8, 2022). "Recent Progress on the Localization of PLK1 to the Kinetochore and Its Role in Mitosis". In: *Int J Mol Sci* 23.9. ISSN: 1422-0067 (Electronic) 1422-0067 (Linking). DOI: 10.3390/ijms23095252.

Kim, T. and A. Gartner (Feb. 17, 2021). "Bub1 kinase in the regulation of mitosis". In: *Anim Cells Syst (Seoul)* 25.1, pp. 1–10. ISSN: 1976-8354 (Print) 2151-2485 (Electronic) 1976-8354 (Linking). DOI: 10.1080/19768354.2021.1884599.

King, J., S. S. V. Henriet, and A. Warris (May 26, 2016). "Aspergillosis in Chronic Granulomatous Disease". In: *J Fungi (Basel)* 2.2. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof2020015.

Kitagawa, M. and S. H. Lee (2015). "The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis". In: *Front Cell Dev Biol* 3, p. 14. ISSN: 2296-634X (Print) 2296-634X (Electronic) 2296-634X (Linking). DOI: 10.3389/fcell.2015.00014.

Kiyomitsu, T. and S. Boerner (2021). "The Nuclear Mitotic Apparatus (NuMA) Protein: A Key Player for Nuclear Formation, Spindle Assembly, and Spindle Positioning". In: *Front Cell Dev Biol* 9, p. 653801. ISSN: 2296-634X (Print) 2296-634X (Electronic) 2296-634X (Linking). DOI: 10.3389/fcell.2021.653801.

Kline-Smith, S. L., S. Sandall, and A. Desai (Feb. 2005). "Kinetochore-spindle microtubule interactions during mitosis". In: *Curr Opin Cell Biol* 17.1, pp. 35–46. ISSN: 0955-0674 (Print) 1879-0410 (Electronic) 0955-0674 (Linking). DOI: 10.1016/j.ceb.2004.12.009.

Koehler, P et al. (2021). "Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance". In: *Lancet Infect Dis* 21.6, e149–e162. ISSN: 1474-4457 (Electronic) 1473-3099 (Print) 1473-3099 (Linking). DOI: 10.1016/S1473-3099(20)30847-1. URL: https://www.ncbi.nlm.nih.gov/pubmed/33333012.

Koide, S. et al. (2020). "Invasive Aspergillosis After Non-Fatal Drowning". In: *Int Med Case Rep J* 13, pp. 77–83. ISSN: 1179-142X (Print) 1179-142X (Electronic) 1179-142X (Linking). DOI: 10.2147/IMCRJ.S241234.

Kops, G. J., B. A. Weaver, and D. W. Cleveland (Oct. 2005). "On the road to cancer: aneuploidy and the mitotic checkpoint". In: *Nat Rev Cancer* 5.10, pp. 773–85. ISSN: 1474-175X (Print) 1474-175X (Linking). DOI: 10.1038/nrc1714.

Kosmidis, C and D W Denning (2015). "The clinical spectrum of pulmonary aspergillosis". In: *Thorax* 70.3, pp. 270–277. ISSN: 1468-3296 (Electronic) 0040-6376 (Linking). DOI: 10.1136/thoraxjnl-2014-206291. URL: https://www.ncbi.nlm.nih.gov/pubmed/25354514.

Kotak, S., C. Busso, and P. Gonczy (Oct. 1, 2012). "Cortical dynein is critical for proper spindle positioning in human cells". In: *J Cell Biol* 199.1, pp. 97–110. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.201203166.

Kowacs, P. A. et al. (Feb. 2004). "Central nervous system Aspergillus fumigatus infection after near drowning". In: *J Clin Pathol* 57.2, pp. 202–4. ISSN: 0021-9746 (Print) 1472-4146 (Electronic) 0021-9746 (Linking). DOI: 10.1136/jcp.2003.010066.

Krishnan Natesan, S. et al. (Nov. 2012). "In vitro-in vivo correlation of voriconazole resistance due to G448S mutation (cyp51A gene) in Aspergillus fumigatus". In: *Diagn Microbiol Infect Dis* 74.3, pp. 272–7. ISSN: 1879-0070 (Electronic) 0732-8893 (Linking). DOI: 10.1016/j.diagmicrobio.2012.06.030.

Kukurudz, R. J. et al. (Aug. 25, 2022). "Acquisition of cross-azole tolerance and aneuploidy in Candida albicans strains evolved to posaconazole". In: *G3 (Bethesda)* 12.9. ISSN: 2160-1836 (Electronic) 2160-1836 (Linking). DOI: 10.1093/g3journal/jkac156.

Lamarre, C. et al. (Sept. 16, 2008). "Transcriptomic analysis of the exit from dormancy of Aspergillus fumigatus conidia". In: *BMC Genomics* 9, p. 417. ISSN: 1471-2164 (Electronic) 1471-2164 (Linking). DOI: 10.1186/1471-2164-9-417.

Laniado-Laborin, R and M N Cabrales-Vargas (2009). "Amphotericin B: side effects and toxicity". In: *Rev Iberoam Micol* 26.4, pp. 223–227. ISSN: 1130-1406 (Print) 1130-1406 (Linking). DOI: 10.1016/j.riam.2009.06.003. URL: https://www.ncbi.nlm.nih.gov/pubmed/19836985.

Latge, J P and G Chamilos (2019). "Aspergillus fumigatus and Aspergillosis in 2019". In: *Clin Microbiol Rev* 33.1. ISSN: 1098-6618 (Electronic) 0893-8512 (Print) 0893-8512 (Linking). DOI: 10.1128/CMR.00140-18. URL: https://www.ncbi.nlm.nih.gov/pubmed/31722890.

Latge, J. P. (Apr. 1999). "Aspergillus fumigatus and aspergillosis". In: *Clin Microbiol Rev* 12.2, pp. 310–50. ISSN: 0893-8512 (Print) 1098-6618 (Electronic) 0893-8512 (Linking). DOI: 10.1128/CMR.12.2.310.

Latge, J. P. (Aug. 2001). "The pathobiology of Aspergillus fumigatus". In: *Trends Microbiol* 9.8, pp. 382–9. ISSN: 0966-842X (Print) 0966-842X (Linking). DOI: 10.1016/s0966-842x(01)02104-7.

Lazzarini, C et al. (2016). "Azole Resistance in Aspergillus fumigatus Clinical Isolates from an Italian Culture Collection". In: *Antimicrob Agents Chemother* 60.1, pp. 682–685. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.02234-15. URL: https://www.ncbi.nlm.nih.gov/pubmed/26552980.

Leontiou, I. et al. (July 22, 2019). "The Bub1-TPR Domain Interacts Directly with Mad3 to Generate Robust Spindle Checkpoint Arrest". In: *Curr Biol* 29.14, 2407–2414 e7. ISSN: 1879-0445 (Electronic) 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2019.06.011.

Lestrade, P. P. A. et al. (July 2019). "Triazole resistance in Aspergillus fumigatus: recent insights and challenges for patient management". In: *Clin Microbiol Infect* 25.7, pp. 799–806. ISSN: 1469-0691 (Electronic) 1198-743X (Linking). DOI: 10.1016/j.cmi.2018.11.027.

Levetin, E. (Feb. 2004). "An atlas of fungal spores". In: *J Allergy Clin Immunol* 113.2, pp. 366–8. ISSN: 0091-6749 (Print) 0091-6749 (Linking). DOI: 10.1016/j.jaci.2003.09.049.

Linden, J. W. van der, S. M. Camps, et al. (Aug. 2013). "Aspergillosis due to voriconazole highly resistant Aspergillus fumigatus and recovery of genetically related resistant isolates from domiciles". In: *Clin Infect Dis* 57.4, pp. 513–20. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). DOI: 10.1093/cid/cit320.

Linden, J. W. van der, A. Warris, et al. (2009). "[Azole-resistant invasive aspergillosis]". In: *Ned Tijdschr Geneeskd* 153, A765. ISSN: 1876-8784 (Electronic) 0028-2162 (Linking).

Lionakis, M S, R E Lewis, and D P Kontoyiannis (2018). "Breakthrough Invasive Mold Infections in the Hematology Patient: Current Concepts and Future Directions". In: *Clin Infect Dis* 67.10, pp. 1621–1630. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/ciy473. URL: https://www.ncbi.nlm.nih.gov/pubmed/29860307.

Liu, S. et al. (Apr. 2015). "Components of the calcium-calcineurin signaling pathway in fungal cells and their potential as antifungal targets". In: *Eukaryot Cell* 14.4, pp. 324–34. ISSN: 1535-9786 (Electronic) 1535-9778 (Print) 1535-9786 (Linking). DOI: 10.1128/EC.00271-14.

Liu, X. et al. (May 2011). "Invasive fungal infections in liver transplantation". In: *Int J Infect Dis* 15.5, e298–304. ISSN: 1878-3511 (Electronic) 1201-9712 (Linking). DOI: 10.1016/j.ijid.2011.01.005.

Lockhart, S. R. et al. (Sept. 2011). "Azole resistance in Aspergillus fumigatus isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene". In: *Antimicrob Agents Chemother* 55.9, pp. 4465–8. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00185-11.

Long, N et al. (2018). "Aspergillus fumigatus Afssn3-Afssn8 Pair Reverse Regulates Azole Resistance by Conferring Extracellular Polysaccharide, Sphingolipid Pathway Intermediates, and Efflux Pumps to Biofilm". In: *Antimicrob Agents Chemother* 62.3. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.01978-17. URL: https://www.ncbi.nlm.nih.gov/pubmed/29311083.

Maertens, J A et al. (2016). "Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial". In: *Lancet* 387.10020, pp. 760–769. ISSN: 1474-547X (Electronic) 0140-6736 (Linking). DOI: 10.1016/S0140-6736(15)01159-9. URL: https://www.ncbi.nlm.nih.gov/pubmed/26684607.

Maier, B. et al. (May 1, 2013). "The novel actin/focal adhesion-associated protein MISP is involved in mitotic spindle positioning in human cells". In: *Cell Cycle* 12.9, pp. 1457–71. ISSN: 1551-4005 (Electronic) 1538-4101 (Print) 1551-4005 (Linking). DOI: 10.4161/cc.24602.

Manavathu, E. K. et al. (Aug. 2000). "A comparative study of the in vitro susceptibilities of clinical and laboratory-selected resistant isolates of Aspergillus spp. to amphotericin B, itraconazole, voriconazole and posaconazole (SCH 56592)". In: *J Antimicrob Chemother* 46.2, pp. 229–34. ISSN: 0305-7453 (Print) 0305-7453 (Linking). DOI: 10.1093/jac/46.2.229.

Manfiolli, A. O. et al. (2019). "Aspergillus fumigatus High Osmolarity Glycerol Mitogen Activated Protein Kinases SakA and MpkC Physically Interact During Osmotic and Cell Wall Stresses". In: *Front Microbiol* 10, p. 918. ISSN: 1664-302X (Print) 1664-302X (Electronic) 1664-302X (Linking). DOI: 10.3389/fmicb.2019.00918.

Mann, P. A. et al. (Feb. 2003). "Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase". In: *Antimicrob Agents Chemother* 47.2, pp. 577–81. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.47.2.577-581.2003.

Martin-Vicente, A et al. (2020). "Overexpression of the Aspergillus fumigatus Small GTPase, RsrA, Promotes Polarity Establishment during Germination". In: *J Fungi (Basel)* 6.4. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof6040285. URL: https://www.ncbi.nlm.nih.gov/pubmed/33202962.

Mattos, E C, G Palmisano, and G H Goldman (2020). "Phosphoproteomics of Aspergillus fumigatus Exposed to the Antifungal Drug Caspofungin". In: *mSphere* 5.3. ISSN: 2379-5042 (Electronic) 2379-5042 (Linking). DOI: 10.1128/mSphere.00365-20. URL: https://www.ncbi.nlm.nih.gov/pubmed/32461274.

Mattos, E C, L P Silva, et al. (2020). "The Aspergillus fumigatus Phosphoproteome Reveals Roles of High-Osmolarity Glycerol Mitogen-Activated Protein Kinases in Promoting Cell Wall Damage and Caspofungin Tolerance". In: *mBio* 11.1. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mBio.02962-19. URL: https://www.ncbi.nlm.nih.gov/pubmed/32019798.

Mavridou, E. et al. (2013). "Composite survival index to compare virulence changes in azole-resistant Aspergillus fumigatus clinical isolates". In: *PLoS One* 8.8, e72280. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0072280.

Mayr, A. and C. Lass-Florl (Apr. 28, 2011). "Epidemiology and antifungal resistance in invasive Aspergillosis according to primary disease: review of the literature". In: *Eur J Med Res* 16.4, pp. 153–7. ISSN: 0949-2321 (Print) 2047-783X (Electronic) 0949-2321 (Linking). DOI: 10.1186/2047-783x-16-4-153.

McNally, F. J. (Jan. 21, 2013). "Mechanisms of spindle positioning". In: *J Cell Biol* 200.2, pp. 131–40. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.201210007.

McTaggart, L. R. et al. (Aug. 20, 2020). "Antifungal Susceptibility of Clinical Yeast Isolates from a Large Canadian Reference Laboratory and Application of Whole-Genome Sequence Analysis To Elucidate Mechanisms of Acquired Resistance". In: *Antimicrob Agents Chemother* 64.9. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00402-20.

Mead, M E et al. (2023). "COVID-19-Associated Pulmonary Aspergillosis Isolates Are Genomically Diverse but Similar to Each Other in Their Responses to Infection-Relevant Stresses". In: *Microbiol Spectr* 11.2, e0512822. ISSN: 2165-0497 (Electronic) 2165-0497 (Linking). DOI: 10.1128/spectrum.05128-22. URL: https://www.ncbi.nlm.nih.gov/pubmed/36946762.

Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, M. Cuenca-Estrella, et al. (July 2004). "Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of Aspergillus fumigatus are responsible for resistance in vitro to azole antifungal drugs". In: *Antimicrob Agents Chemother* 48.7, pp. 2747–50. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.48.7.2747-2750.2004.

Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, W. J. Melchers, et al. (June 2007). "A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations". In: *Antimicrob Agents Chemother* 51.6, pp. 1897–904. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.01092-06.

Meng, F. et al. (Feb. 9, 2024). "Aspergillus fumigatus-Derived Extracellular Vesicles Mitigate Fungal Keratitis by Modulating the Immune Cell Function". In: *ACS Infect Dis* 10.2, pp. 500–512. ISSN: 2373-8227 (Electronic) 2373-8227 (Linking). DOI: 10.1021/acsinfecdis.3c00454.

Min, Z. et al. (Aug. 2017). "Early-onset de novo invasive pulmonary aspergillosis in an orthotopic heart transplant recipient". In: *Lung India* 34.4, pp. 376–379. ISSN: 0970-2113 (Print) 0974-598X (Electronic) 0970-2113 (Linking). DOI: 10.4103/lungindia.lungindia_454_16.

Morelli, K. A., J. D. Kerkaert, and R. A. Cramer (Aug. 2021). "Aspergillus fumigatus biofilms: Toward understanding how growth as a multicellular network increases antifungal resistance and disease progression". In: *PLoS Pathog* 17.8, e1009794. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1009794.

Moreno-Velasquez, S. D. et al. (Oct. 2017). "Caspofungin-Mediated Growth Inhibition and Paradoxical Growth in Aspergillus fumigatus Involve Fungicidal Hyphal Tip Lysis Coupled with Regenerative Intrahyphal Growth and Dynamic Changes in beta-1,3-Glucan Synthase Localization". In: *Antimicrob Agents Chemother* 61.10. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00710-17.

Morin, X. and Y. Bellaiche (July 19, 2011). "Mitotic spindle orientation in asymmetric and symmetric cell divisions during animal development". In: *Dev Cell* 21.1, pp. 102–19. ISSN: 1878-1551 (Electronic) 1534-5807 (Linking). DOI: 10.1016/j.devcel.2011.06.012.

Mudrakola, H. V. et al. (Aug. 2022). "Autopsy study of fatal invasive pulmonary aspergillosis: Often undiagnosed premortem". In: *Respir Med* 199, p. 106882. ISSN: 1532-3064 (Electronic) 0954-6111 (Linking). DOI: 10.1016/j.rmed.2022.106882.

Muller, F. M., M. Seidler, and A. Beauvais (Apr. 2011). "Aspergillus fumigatus biofilms in the clinical setting". In: *Med Mycol* 49 Suppl 1, S96–S100. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.3109/13693786.2010.502190.

Musacchio, A. and E. D. Salmon (May 2007). "The spindle-assembly checkpoint in space and time". In: *Nat Rev Mol Cell Biol* 8.5, pp. 379–93. ISSN: 1471-0072 (Print) 1471-0072 (Linking). DOI: 10.1038/nrm2163.

Nascimento, A. M. et al. (May 2003). "Multiple resistance mechanisms among Aspergillus fumigatus mutants with high-level resistance to itraconazole". In: *Antimicrob Agents Chemother* 47.5, pp. 1719–26. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.47.5.1719-1726.2003.

Nywening, A. V. et al. (Dec. 2020). "Mechanisms of triazole resistance in Aspergillus fumigatus". In: *Environ Microbiol* 22.12, pp. 4934–4952. ISSN: 1462-2912 (Print) 1462-2912. DOI: 10.1111/1462-2920.15274.

Oda, N. et al. (2021). "Simultaneous treatment for M ycobacterium-avium complex lung disease and allergic bronchopulmonary aspergillosis: A case report". In: *Respir Med Case Rep* 34, p. 101488. ISSN: 2213-0071 (Print) 2213-0071 (Electronic) 2213-0071 (Linking). DOI: 10.1016/j.rmcr.2021.101488.

Orthaus, S. et al. (Jan. 4, 2008). "Assembly of the inner kinetochore proteins CENP-A and CENP-B in living human cells". In: *Chembiochem* 9.1, pp. 77–92. ISSN: 1439-7633 (Electronic) 1439-4227 (Linking). DOI: 10.1002/cbic.200700358.

Osawa, M. et al. (Apr. 2007). "Risk factors for invasive aspergillosis in living donor liver transplant recipients". In: *Liver Transpl* 13.4, pp. 566–70. ISSN: 1527-6465 (Print) 1527-6465 (Linking). DOI: 10.1002/lt.21099.

Paassen, J. van et al. (2016). "Emerging aspergillosis by azole-resistant Aspergillus fumigatus at an intensive care unit in the Netherlands, 2010 to 2013". In: *Euro Surveill* 21.30. ISSN: 1560-7917 (Electronic) 1025-496X (Linking). DOI: 10.2807/1560-7917.ES.2016.21.30.30300. URL: https://www.ncbi.nlm.nih.gov/pubmed/27541498.

Parums, D V (2022). "Editorial: The World Health Organization (WHO) Fungal Priority Pathogens List in Response to Emerging Fungal Pathogens During the COVID-19 Pandemic". In: *Med Sci Monit* 28, e939088. ISSN: 1643-3750 (Electronic) 1234-1010 (Print) 1234-1010 (Linking). DOI: 10.12659/MSM.939088. URL: https://www.ncbi.nlm.nih.gov/pubmed/36453055.

Patterson, T F, W R Kirkpatrick, et al. (2000). "Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. I3 Aspergillus Study Group". In: *Medicine (Baltimore)* 79.4, pp. 250–260. ISSN: 0025-7974 (Print) 0025-7974 (Linking). DOI: 10.1097/00005792-200007000-00006. URL: https://www.ncbi.nlm.nih.gov/pubmed/10941354.

Patterson, T F, G R Thompson 3rd, et al. (2016). "Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America". In: *Clin Infect Dis* 63.4, e1–e60. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/ciw326. URL: https://www.ncbi.nlm.nih.gov/pubmed/27365388.

Paul, S., D. Diekema, and W. S. Moye-Rowley (Dec. 2013). "Contributions of Aspergillus fumigatus ATP-binding cassette transporter proteins to drug resistance and virulence". In: *Eukaryot Cell* 12.12, pp. 1619–28. ISSN: 1535-9786 (Electronic) 1535-9778 (Print) 1535-9786 (Linking). DOI: 10.1128/EC.00171-13.

Paulussen, C. et al. (Mar. 2017). "Ecology of aspergillosis: insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species". In: *Microb Biotechnol* 10.2, pp. 296–322. ISSN: 1751-7915 (Electronic) 1751-7915 (Linking). DOI: 10.1111/1751-7915.12367.

Pennisi, R., P. Ascenzi, and A. di Masi (Oct. 16, 2015). "Hsp90: A New Player in DNA Repair?" In: *Biomolecules* 5.4, pp. 2589–618. ISSN: 2218-273X (Electronic) 2218-273X (Linking). DOI: 10.3390/biom5042589.

Pfaller, M. A. et al. (Aug. 2008). "In vitro survey of triazole cross-resistance among more than 700 clinical isolates of Aspergillus species". In: *J Clin Microbiol* 46.8, pp. 2568–72. ISSN: 1098-660X (Electronic) 0095-1137 (Print) 0095-1137 (Linking). DOI: 10.1128/JCM.00535-08.

Pham, C. D. et al. (Sept. 2014). "Passive surveillance for azole-resistant Aspergillus fumigatus, United States, 2011-2013". In: *Emerg Infect Dis* 20.9, pp. 1498–503. ISSN: 1080-6059 (Electronic) 1080-6040 (Print) 1080-6040 (Linking). DOI: 10.3201/eid2009.140142.

Pianalto, K. M. and J. A. Alspaugh (Oct. 2, 2016). "New Horizons in Antifungal Therapy". In: *J Fungi (Basel)* 2.4. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof2040026.

Pihet, M. et al. (June 2009). "Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis–a review". In: *Med Mycol* 47.4, pp. 387–97. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.1080/13693780802609604.

Pinto, M. et al. (2008). "Overexpression of the mitotic checkpoint genes BUB1 and BUBR1 is associated with genomic complexity in clear cell kidney carcinomas". In: *Cell Oncol* 30.5, pp. 389–95. ISSN: 1570-5870 (Print) 1875-8606 (Electronic) 1570-5870 (Linking). DOI: 10.3233/clo-2008-0439.

Pitayu-Nugroho, L. et al. (July 7, 2023). "Kinetochore component function in C. elegans oocytes revealed by 4D tracking of holocentric chromosomes". In: *Nat Commun* 14.1, p. 4032. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/s41467-023-39702-z.

Polakova, S. et al. (Feb. 24, 2009). "Formation of new chromosomes as a virulence mechanism in yeast Candida glabrata". In: *Proc Natl Acad Sci U S A* 106.8, pp. 2688–93. ISSN: 1091-6490 (Electronic) 0027-8424 (Print) 0027-8424 (Linking). DOI: 10.1073/pnas.0809793106.

Priebe, S. et al. (Apr. 2011). "FungiFun: a web-based application for functional categorization of fungal genes and proteins". In: *Fungal Genet Biol* 48.4, pp. 353–8. ISSN: 1096-0937 (Electronic) 1087-1845 (Linking). DOI: 10.1016/j.fgb.2010.11.001.

Qiao, J, W Liu, and R Li (2008). "Antifungal resistance mechanisms of Aspergillus". In: *Nihon Ishinkin Gakkai Zasshi* 49.3, pp. 157–163. ISSN: 1882-0476 (Electronic) 0916-4804 (Linking). DOI: 10.3314/jjmm.49.157. URL: https://www.ncbi.nlm.nih.gov/pubmed/18689963.

Ramage, G. et al. (Sept. 2001). "Biofilm formation by Candida dubliniensis". In: *J Clin Microbiol* 39.9, pp. 3234–40. ISSN: 0095-1137 (Print) 1098-660X (Electronic) 0095-1137 (Linking). DOI: 10.1128/JCM.39.9.3234-3240.2001.

Redemann, S. et al. (Aug. 20, 2010). "Membrane invaginations reveal cortical sites that pull on mitotic spindles in one-cell C. elegans embryos". In: *PLoS One* 5.8, e12301. ISSN: 1932-6203 (Electronic) 1932-6203 (Linking). DOI: 10.1371/journal.pone.0012301.

Ren, J. et al. (Mar. 15, 2017). "Fungicides induced triazole-resistance in Aspergillus fumigatus associated with mutations of TR46/Y121F/T289A and its appearance in agricultural fields". In: *J Hazard Mater* 326, pp. 54–60. ISSN: 1873-3336 (Electronic) 0304-3894 (Linking). DOI: 10.1016/j.jhazmat.2016.12.013.

Resendiz-Sharpe, A., K. Dewaele, et al. (Apr. 12, 2021). "Triazole-Resistance in Environmental Aspergillus fumigatus in Latin American and African Countries". In: *J Fungi (Basel)* 7.4. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof7040292.

Resendiz-Sharpe, A., R. Merckx, et al. (Dec. 2021). "Stable prevalence of triazole-resistance in Aspergillus fumigatus complex clinical isolates in a Belgian tertiary care center from 2016 to 2020". In: *J Infect Chemother* 27.12, pp. 1774–1778. ISSN: 1437-7780 (Electronic) 1341-321X (Linking). DOI: 10.1016/j.jiac.2021.08.024.

Richard, N. et al. (Oct. 24, 2018). "Human Bronchial Epithelial Cells Inhibit Aspergillus fumigatus Germination of Extracellular Conidia via FleA Recognition". In: *Sci Rep* 8.1, p. 15699. ISSN: 2045-2322 (Electronic) 2045-2322 (Linking). DOI: 10.1038/s41598-018-33902-0.

Ricke, R M et al. (2012). "Bub1 kinase activity drives error correction and mitotic checkpoint control but not tumor suppression". In: *J Cell Biol* 199.6, pp. 931–949. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.201205115. URL: https://www.ncbi.nlm.nih.gov/pubmed/23209306.

Ricke, R. M., K. B. Jeganathan, and J. M. van Deursen (June 13, 2011). "Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation". In: *J Cell Biol* 193.6, pp. 1049–64. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.201012035.

Rivero-Menendez, O et al. (2016). "Triazole Resistance in Aspergillus spp.: A Worldwide Problem?" In: *J Fungi (Basel)* 2.3. ISSN: 2309-608X (Electronic) 2309-608X (Linking). DOI: 10.3390/jof2030021. URL: https://www.ncbi.nlm.nih.gov/pubmed/29376938.

Rodrigues, M. L. (Sept. 18, 2018). "The Multifunctional Fungal Ergosterol". In: *mBio* 9.5. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mBio.01755-18.

Rodriguez-Tudela, J. L. et al. (July 2008). "Epidemiological cutoffs and cross-resistance to azole drugs in Aspergillus fumigatus". In: *Antimicrob Agents Chemother* 52.7, pp. 2468–72. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00156-08.

Rosa, R. H., D. Miller, and E. C. Alfonso (June 1994). "The changing spectrum of fungal keratitis in south Florida". In: *Ophthalmology* 101.6, pp. 1005–13. ISSN: 0161-6420 (Print) 0161-6420 (Linking). DOI: 10.1016/s0161-6420(94)31225-5.

Rouze, A. et al. (Jan. 4, 2022). "Invasive pulmonary aspergillosis among intubated patients with SARS-CoV-2 or influenza pneumonia: a European multicenter comparative cohort study". In: *Crit Care* 26.1, p. 11. ISSN: 1466-609X (Electronic) 1364-8535 (Print) 1364-8535 (Linking). DOI: 10.1186/s13054-021-03874-1.

Roy, B et al. (2022). "Aurora B phosphorylates Bub1 to promote spindle assembly checkpoint signaling". In: *Curr Biol* 32.1, 237–247 e6. ISSN: 1879-0445 (Electronic) 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2021.10.049. URL: https://www.ncbi.nlm.nih.gov/pubmed/34861183.

Rybak, J M, J R Fortwendel, and P D Rogers (2019). "Emerging threat of triazole-resistant Aspergillus fumigatus". In: *J Antimicrob Chemother* 74.4, pp. 835–842. ISSN: 1460-2091 (Electronic) 0305-7453 (Print) 0305-7453 (Linking). DOI: 10.1093/jac/dky517. URL: https://www.ncbi.nlm.nih.gov/pubmed/30561652.

Rybak, J M, W Ge, et al. (2019). "Mutations in hmg1, Challenging the Paradigm of Clinical Triazole Resistance in Aspergillus fumigatus". In: *mBio* 10.2. ISSN: 2150-7511 (Electronic). DOI: 10.1128/mBio.00437-19. URL: https://www.ncbi.nlm.nih.gov/pubmed/30940706.

Rybak, J. M. et al. (Sept. 2017). "Loss of C-5 Sterol Desaturase Activity Results in Increased Resistance to Azole and Echinocandin Antifungals in a Clinical Isolate of Candida parapsilosis". In: *Antimicrob Agents Chemother* 61.9. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00651-17.

Sah, S. K., J. J. Hayes, and E. Rustchenko (May 2021). "The role of aneuploidy in the emergence of echinocandin resistance in human fungal pathogen Candida albicans". In: *PLoS Pathog* 17.5, e1009564. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1009564.

Sardeshmukh, A. A. et al. (June 2023). "Mucormycosis and Aspergillosis Infection Following Maxillofacial Trauma: Case Report". In: *Indian J Otolaryngol Head Neck Surg* 75.2, pp. 1245–1249. ISSN: 2231-3796 (Print) 0973-7707 (Electronic) 2231-3796 (Linking). DOI: 10.1007/s12070-023-03492-z.

Seidler, M. J., S. Salvenmoser, and F. M. Muller (Nov. 2008). "Aspergillus fumigatus forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells". In: *Antimicrob Agents Chemother* 52.11, pp. 4130–6. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00234-08.

Selmecki, A, A Forche, and J Berman (2006). "Aneuploidy and isochromosome formation in drug-resistant Candida albicans". In: *Science* 313.5785, pp. 367–370. ISSN: 1095-9203 (Electronic) 0036-8075 (Print) 0036-8075 (Linking). DOI: 10.1126/science.1128242. URL: https://www.ncbi.nlm.nih.gov/pubmed/16857942.

– (2010). "Genomic plasticity of the human fungal pathogen Candida albicans". In: *Eukaryot Cell* 9.7, pp. 991–1008. ISSN: 1535-9786 (Electronic) 1535-9778 (Print) 1535-9786 (Linking). DOI: 10.1128/EC.00060-10. URL: https://www.ncbi.nlm.nih.gov/pubmed/20495058.

Selmecki, A, M Gerami-Nejad, et al. (2008). "An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1". In: *Mol Microbiol* 68.3, pp. 624–641. ISSN: 1365-2958 (Electronic) 0950-382X (Linking). DOI: 10.1111/j.1365-2958.2008.06176.x. URL: https://www.ncbi.nlm.nih.gov/pubmed/18363649.

Selmecki, A. M. et al. (Oct. 2009). "Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance". In: *PLoS Genet* 5.10, e1000705. ISSN: 1553-7404 (Electronic) 1553-7390 (Print) 1553-7390 (Linking). DOI: 10.1371/journal.pgen.1000705.

Semighini, C. P. et al. (Nov. 2011). "Deletion of Cryptococcus neoformans AIF ortholog promotes chromosome aneuploidy and fluconazole-resistance in a metacaspase-independent manner". In: *PLoS Pathog* 7.11, e1002364. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1002364.

Seyedmousavi, S. et al. (Nov. 2015). "Aspergillus and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease". In: *Med Mycol* 53.8, pp. 765–97. ISSN: 1460-2709 (Electronic) 1369-3786 (Linking). DOI: 10.1093/mmy/myv067.

Shah, A. and C. Panjabi (Mar. 1, 2014). "Allergic aspergillosis of the respiratory tract". In: *Eur Respir Rev* 23.131, pp. 8–29. ISSN: 1600-0617 (Electronic) 0905-9180 (Print) 0905-9180 (Linking). DOI: 10.1183/09059180.00007413.

Sharma, C. et al. (Nov. 2019). "Genomic perspective of triazole resistance in clinical and environmental Aspergillus fumigatus isolates without cyp51A mutations". In: *Fungal Genet Biol* 132, p. 103265. ISSN: 1096-0937 (Electronic) 1087-1845 (Linking). DOI: 10.1016/j.fgb.2019.103265.

Sherwin, D. and Y. Wang (Dec. 7, 2019). "The Opposing Functions of Protein Kinases and Phosphatases in Chromosome Bipolar Attachment". In: *Int J Mol Sci* 20.24. ISSN: 1422-0067 (Electronic) 1422-0067 (Linking). DOI: 10.3390/ijms20246182.

Shrestha, R. L. et al. (Apr. 5, 2021). "CENP-A overexpression promotes aneuploidy with karyotypic heterogeneity". In: *J Cell Biol* 220.4. ISSN: 1540-8140 (Electronic) 0021-9525 (Print) 0021-9525 (Linking). DOI: 10.1083/jcb.202007195.

Siemeister, Gerhard et al. (2017). "Abstract 287: BAY 1816032, a novel BUB1 kinase inhibitor with potent antitumor activity". In: *Cancer Research* 77.13, pp. 287–287. ISSN: 0008-5472. DOI: 10.1158/1538-7445.Am2017-287.

Silva, L. P. et al. (Sept. 16, 2020). "Putative Membrane Receptors Contribute to Activation and Efficient Signaling of Mitogen-Activated Protein Kinase Cascades during Adaptation of Aspergillus fumigatus to Different Stressors and Carbon Sources". In: *mSphere* 5.5. ISSN: 2379-5042 (Electronic) 2379-5042 (Linking). DOI: 10.1128/mSphere.00818-20.

Singh, A. and Y. J. Xu (Nov. 17, 2016). "The Cell Killing Mechanisms of Hydroxyurea". In: *Genes (Basel)* 7.11. ISSN: 2073-4425 (Print) 2073-4425 (Electronic) 2073-4425 (Linking). DOI: 10.3390/genes7110099.

Singh, P. et al. (Jan. 7, 2021). "BUB1 and CENP-U, Primed by CDK1, Are the Main PLK1 Kinetochore Receptors in Mitosis". In: *Mol Cell* 81.1, 67–87 e9. ISSN: 1097-4164 (Electronic) 1097-2765 (Print) 1097-2765 (Linking). DOI: 10.1016/j.molcel.2020.10.040.

Sionov, E., Y. C. Chang, et al. (July 2009). "Heteroresistance to fluconazole in Cryptococcus neoformans is intrinsic and associated with virulence". In: *Antimicrob Agents Chemother* 53.7, pp. 2804–15. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00295-09.

Sionov, E., H. Lee, et al. (Apr. 1, 2010). "Cryptococcus neoformans overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes". In: *PLoS Pathog* 6.4, e1000848. ISSN: 1553-7374 (Electronic) 1553-7366 (Print) 1553-7366 (Linking). DOI: 10.1371/journal.ppat.1000848.

Skrzypek, M S et al. (2017). "The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data". In: *Nucleic Acids Res* 45 (D1), pp. D592–D596. ISSN: 1362-4962 (Electronic) 0305-1048 (Print) 0305-1048 (Linking). DOI: 10.1093/nar/gkw924. URL: https://www.ncbi.nlm.nih.gov/pubmed/27738138.

Skrzypek, M. S. and J. Hirschman (Sept. 2011). "Using the Saccharomyces Genome Database (SGD) for analysis of genomic information". In: *Curr Protoc Bioinformatics* Chapter 1, pp. 1 20 1–1 20 23. ISSN: 1934-340X (Electronic) 1934-3396 (Print) 1934-3396 (Linking). DOI: 10.1002/0471250953.bi0120s35.

Slaven, J. W. et al. (Aug. 2002). "Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate". In: *Fungal Genet Biol* 36.3, pp. 199–206. ISSN: 1087-1845 (Print) 1087-1845 (Linking). DOI: 10.1016/s1087-1845(02)00016-6.

Slobbe, L. et al. (Dec. 15, 2008). "Outcome and medical costs of patients with invasive aspergillosis and acute myelogenous leukemia-myelodysplastic syndrome treated with intensive chemotherapy: an observational study". In: *Clin Infect Dis* 47.12, pp. 1507–12. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). DOI: 10.1086/591531.

Snelders, E., S. M. Camps, et al. (Sept. 2015). "Genotype-phenotype complexity of the TR46/Y121F/T289A cyp51A azole resistance mechanism in Aspergillus fumigatus". In: *Fungal Genet Biol* 82, pp. 129–35. ISSN: 1096-0937 (Electronic) 1087-1845 (Linking). DOI: 10.1016/j.fgb.2015.06.001.

Snelders, E., R. A. Huis In 't Veld, et al. (June 2009). "Possible environmental origin of resistance of Aspergillus fumigatus to medical triazoles". In: *Appl Environ Microbiol* 75.12, pp. 4053–7. ISSN: 1098-5336 (Electronic) 0099-2240 (Print) 0099-2240 (Linking). DOI: 10.1128/AEM.00231-09.

Snelders, E., A. Karawajczyk, et al. (June 2010). "Azole resistance profile of amino acid changes in Aspergillus fumigatus CYP51A based on protein homology modeling". In: *Antimicrob Agents Chemother* 54.6, pp. 2425–30. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.01599-09.

Sniegowski, P. D. and P. J. Gerrish (Apr. 27, 2010). "Beneficial mutations and the dynamics of adaptation in asexual populations". In: *Philos Trans R Soc Lond B Biol Sci* 365.1544, pp. 1255–63. ISSN: 1471-2970 (Electronic) 0962-8436 (Print) 0962-8436 (Linking). DOI: 10.1098/rstb.2009.0290.

Starr, D. A. et al. (Aug. 10, 1998). "ZW10 helps recruit dynactin and dynein to the kinetochore". In: *J Cell Biol* 142.3, pp. 763–74. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). DOI: 10.1083/jcb.142.3.763.

Steinbach, W. J. et al. (Nov. 2012). "Clinical epidemiology of 960 patients with invasive aspergillosis from the PATH Alliance registry". In: *J Infect* 65.5, pp. 453–64. ISSN: 1532-2742 (Electronic) 0163-4453 (Linking). DOI: 10.1016/j.jinf.2012.08.003.

Stemmann, O. et al. (June 25, 2002). "Hsp90 enables Ctf13p/Skp1p to nucleate the budding yeast kinetochore". In: *Proc Natl Acad Sci U S A* 99.13, pp. 8585–90. ISSN: 0027-8424 (Print) 1091-6490 (Electronic) 0027-8424 (Linking). DOI: 10.1073/pnas.082223899.

Straight, A F et al. (1996). "GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion". In: *Curr Biol* 6.12, pp. 1599–1608. ISSN: 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/s0960-9822(02)70783-5. URL: https://www.ncbi.nlm.nih.gov/pubmed/8994824.

Suijkerbuijk, S J et al. (2012). "The vertebrate mitotic checkpoint protein BUBR1 is an unusual pseudokinase". In: *Dev Cell* 22.6, pp. 1321–1329. ISSN: 1878-1551 (Electronic) 1534-5807 (Linking). DOI: 10.1016/j.devcel.2012.03.009. URL: https://www.ncbi.nlm.nih.gov/pubmed/22698286.

Sun, L. L. et al. (2023). "Aneuploidy enables cross-tolerance to unrelated antifungal drugs in Candida parapsilosis". In: *Front Microbiol* 14, p. 1137083. ISSN: 1664-302X (Print) 1664-302X (Electronic) 1664-302X (Linking). DOI: 10.3389/fmicb.2023.1137083.

Suzuki, A. et al. (Sept. 10, 2018). "A Kinesin-5, Cin8, Recruits Protein Phosphatase 1 to Kinetochores and Regulates Chromosome Segregation". In: *Curr Biol* 28.17, 2697–2704 e3. ISSN: 1879-0445 (Electronic) 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2018.08.038.

Tamborrini, D. et al. (Oct. 17, 2018). "Recruitment of the mitotic exit network to yeast centrosomes couples septin displacement to actomyosin constriction". In: *Nat Commun* 9.1, p. 4308. ISSN: 2041-1723 (Electronic) 2041-1723 (Linking). DOI: 10.1038/s41467-018-06767-0.

Tame, M. A. et al. (2014). "Astral microtubules control redistribution of dynein at the cell cortex to facilitate spindle positioning". In: *Cell Cycle* 13.7, pp. 1162–70. ISSN: 1551-4005 (Electronic) 1538-4101 (Print) 1551-4005 (Linking). DOI: 10.4161/cc.28031.

Tashiro, M. and K. Izumikawa (2016). "[The Current Status of Drug-resistant Aspergillus]". In: *Med Mycol J* 57.3, J103–12. ISSN: 1882-0476 (Electronic). DOI: 10.3314/mmj.16.001.

Tejerina, E. E. et al. (Aug. 2019). "Invasive aspergillosis in critically ill patients: An autopsy study". In: *Mycoses* 62.8, pp. 673–679. ISSN: 1439-0507 (Electronic) 0933-7407 (Linking). DOI: 10.1111/myc.12927.

Thompson, S. L., S. F. Bakhoum, and D. A. Compton (Mar. 23, 2010). "Mechanisms of chromosomal instability". In: *Curr Biol* 20.6, R285–95. ISSN: 1879-0445 (Electronic) 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2010.01.034.

Thorn, H. I. et al. (Feb. 13, 2024). "MOB-mediated regulation of septation initiation network (SIN) signaling is required for echinocandin-induced hyperseptation in Aspergillus fumigatus". In: *mSphere*, e0069523. ISSN: 2379-5042 (Electronic) 2379-5042 (Linking). DOI: 10.1128/msphere.00695-23.

Ting, D. S. J. et al. (Apr. 2021). "Infectious keratitis: an update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance". In: *Eye (Lond)* 35.4, pp. 1084–1101. ISSN: 1476-5454 (Electronic) 0950-222X (Print) 0950-222X (Linking). DOI: 10.1038/s41433-020-01339-3.

Todd, R T, A L Braverman, and A Selmecki (2018). "Flow Cytometry Analysis of Fungal Ploidy". In: *Curr Protoc Microbiol* 50.1, e58. ISSN: 1934-8533 (Electronic) 1934-8525 (Print) 1934-8525 (Linking). DOI: 10.1002/cpmc.58. URL: https://www.ncbi.nlm.nih.gov/pubmed/30028911.

Todd, R. T., A. Forche, and A. Selmecki (July 2017). "Ploidy Variation in Fungi: Polyploidy, Aneuploidy, and Genome Evolution". In: *Microbiol Spectr* 5.4. ISSN: 2165-0497 (Electronic) 2165-0497 (Linking). DOI: 10.1128/microbiolspec.FUNK-0051-2016.

Todd, R. T. and A. Selmecki (July 20, 2020). "Expandable and reversible copy number amplification drives rapid adaptation to antifungal drugs". In: *Elife* 9. ISSN: 2050-084X (Electronic) 2050-084X (Linking). DOI: 10.7554/eLife.58349.

– (2023). "Copy Number Variation and Allele Ratio Analysis in Candida albicans Using Whole Genome Sequencing Data". In: *Methods Mol Biol* 2658, pp. 105–125. ISSN: 1940-6029 (Electronic) 1064-3745 (Print) 1064-3745 (Linking). DOI: 10.1007/978-1-0716-3155-3_8.

Todd, R. T., N. Soisangwan, et al. (Jan. 4, 2023). "Antifungal Drug Concentration Impacts the Spectrum of Adaptive Mutations in Candida albicans". In: *Mol Biol Evol* 40.1. ISSN: 1537-1719 (Electronic) 0737-4038 (Print) 0737-4038 (Linking). DOI: 10.1093/molbev/msad009.

Tytell, J. D. and P. K. Sorger (Mar. 13, 2006). "Analysis of kinesin motor function at budding yeast kinetochores". In: *J Cell Biol* 172.6, pp. 861–74. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). DOI: 10.1083/jcb.200509101.

Ullmann, A J et al. (2007). "Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease". In: *N Engl J Med* 356.4, pp. 335–347. ISSN: 1533-4406 (Electronic) 0028-4793 (Linking). DOI: 10.1056/NEJMoa061098. URL: https://www.ncbi.nlm.nih.gov/pubmed/17251530.

Vader, G., R. H. Medema, and S. M. Lens (June 19, 2006). "The chromosomal passenger complex: guiding Aurora-B through mitosis". In: *J Cell Biol* 173.6, pp. 833–7. ISSN: 0021-9525 (Print) 1540-8140 (Electronic) 0021-9525 (Linking). DOI: 10.1083/jcb.200604032.

Vanoosthuyse, V. and K. G. Hardwick (July 28, 2009). "A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism". In: *Curr Biol* 19.14, pp. 1176–81. ISSN: 1879-0445 (Electronic) 0960-9822 (Print) 0960-9822 (Linking). DOI: 10.1016/j.cub.2009.05.060.

Varshney, N. and K. Sanyal (Aug. 2019). "Aurora kinase Ipl1 facilitates bilobed distribution of clustered kinetochores to ensure error-free chromosome segregation in Candida albicans". In: *Mol Microbiol* 112.2, pp. 569–587. ISSN: 1365-2958 (Electronic) 0950-382X (Linking). DOI: 10.1111/mmi.14275.

Verweij, P. E., R. J. M. Bruggemann, et al. (Jan. 2, 2020). "Influenza Coinfection: Be(a)ware of Invasive Aspergillosis". In: *Clin Infect Dis* 70.2, pp. 349–350. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/ciz391.

Verweij, P. E., A. Chowdhary, et al. (Feb. 1, 2016). "Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles?" In: *Clin Infect Dis* 62.3, pp. 362–8. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/civ885.

Verweij, P. E., G. H. Kema, et al. (Feb. 2013). "Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould Aspergillus fumigatus". In: *Pest Manag Sci* 69.2, pp. 165–70. ISSN: 1526-4998 (Electronic) 1526-498X (Linking). DOI: 10.1002/ps.3390.

Verweij, P. E., E. Mellado, and W. J. Melchers (Apr. 5, 2007). "Multiple-triazole-resistant aspergillosis". In: *N Engl J Med* 356.14, pp. 1481–3. ISSN: 1533-4406 (Electronic) 0028-4793 (Linking). DOI: 10.1056/NEJMc061720.

Vossen, M. L. et al. (Dec. 5, 2019). "CaMad2 Promotes Multiple Aspects of Genome Stability Beyond Its Direct Function in Chromosome Segregation". In: *Genes (Basel)* 10.12. ISSN: 2073-4425 (Electronic) 2073-4425 (Linking). DOI: 10.3390/genes10121013.

Walsh, T. J. et al. (Feb. 1, 2008). "Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America". In: *Clin Infect Dis* 46.3, pp. 327–60. ISSN: 1537-6591 (Electronic) 1058-4838 (Linking). DOI: 10.1086/525258.

Wang, H. C. et al. (Jan. 2018). "Prevalence, mechanisms and genetic relatedness of the human pathogenic fungus Aspergillus fumigatus exhibiting resistance to medical azoles in the environment of Taiwan". In: *Environ Microbiol* 20.1, pp. 270–280. ISSN: 1462-2920 (Electronic) 1462-2912 (Linking). DOI: 10.1111/1462-2920.13988.

Warkentien, T. et al. (Dec. 2012). "Invasive mold infections following combat-related injuries". In: *Clin Infect Dis* 55.11, pp. 1441–9. ISSN: 1537-6591 (Electronic) 1058-4838 (Print) 1058-4838 (Linking). DOI: 10.1093/cid/cis749.

Watanabe, Y. and T. S. Kitajima (Mar. 29, 2005). "Shugoshin protects cohesin complexes at centromeres". In: *Philos Trans R Soc Lond B Biol Sci* 360.1455, 515–21, discussion 521. ISSN: 0962-8436 (Print) 1471-2970 (Electronic) 0962-8436 (Linking). DOI: 10.1098/rstb.2004.1607.

Webb, B. J. et al. (Aug. 2018). "Epidemiology and Clinical Features of Invasive Fungal Infection in a US Health Care Network". In: *Open Forum Infect Dis* 5.8, ofy187. ISSN: 2328-8957 (Print) 2328-8957 (Electronic) 2328-8957 (Linking). DOI: 10.1093/ofid/ofy187.

White, A. J. et al. (Nov. 1, 2022). "Loss of Num1-mediated cortical dynein anchoring negatively impacts respiratory growth". In: *J Cell Sci* 135.21. ISSN: 1477-9137 (Electronic) 0021-9533 (Print) 0021-9533 (Linking). DOI: 10.1242/jcs.259980.

Wiederhold, N. P. et al. (Jan. 2016). "First Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in Aspergillus fumigatus Isolates in the United States". In: *J Clin Microbiol* 54.1, pp. 168–71. ISSN: 1098-660X (Electronic) 0095-1137 (Print) 0095-1137 (Linking). DOI: 10.1128/JCM.02478-15.

Wimbish, Robert T. and Jennifer G. DeLuca (Feb. 2020). "Hec1/Ndc80 Tail Domain Function at the Kinetochore-Microtubule Interface". In: *Frontiers in Cell and Developmental Biology* 8. ISSN: 2296-634X. DOI: 10.3389/fcell.2020.00043.

Winters, B. et al. (Nov. 2012). "Diagnostic errors in the intensive care unit: a systematic review of autopsy studies". In: *BMJ Qual Saf* 21.11, pp. 894–902. ISSN: 2044-5423 (Electronic) 2044-5415 (Linking). DOI: 10.1136/bmjqs-2012-000803.

Wu, C. J. et al. (Apr. 2020). "Multicenter Study of Azole-Resistant Aspergillus fumigatus Clinical Isolates, Taiwan(1)". In: *Emerg Infect Dis* 26.4, pp. 804–806. ISSN: 1080-6059 (Electronic) 1080-6040 (Print) 1080-6040 (Linking). DOI: 10.3201/eid2604.190840.

Wu, X., X. Xiang, and J. A. Hammer (Mar. 2006). "Motor proteins at the microtubule plus-end". In: *Trends Cell Biol* 16.3, pp. 135–43. ISSN: 0962-8924 (Print) 0962-8924 (Linking). DOI: 10.1016/j.tcb.2006.01.004.

Xiao, L. et al. (Feb. 2004). "Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from Aspergillus fumigatus and Candida albicans provide insights into posaconazole binding". In: *Antimicrob Agents Chemother* 48.2, pp. 568–74. ISSN: 0066-4804 (Print) 1098-6596 (Electronic) 0066-4804 (Linking). DOI: 10.1128/AAC.48.2.568-574.2004.

Yamawaki, S. et al. (2016). "Rice-Field Drowning-Associated Pneumonia in which Pseudomonas spp., Aspergillus fumigatus, and Cunninghamella sp. Are Isolated". In: *Intern Med* 55.7, pp. 825–9. ISSN: 1349-7235 (Electronic) 0918-2918 (Linking). DOI: 10.2169/internalmedicine.55.4454.

Yanagida, M. (Jan. 2000). "Cell cycle mechanisms of sister chromatid separation; roles of Cut1/separin and Cut2/securin". In: *Genes Cells* 5.1, pp. 1–8. ISSN: 1356-9597 (Print) 1356-9597 (Linking). DOI: 10.1046/j.1365-2443.2000.00306.x.

Yang, F., F. Teoh, et al. (Aug. 1, 2019). "Aneuploidy Enables Cross-Adaptation to Unrelated Drugs". In: *Mol Biol Evol* 36.8, pp. 1768–1782. ISSN: 1537-1719 (Electronic) 0737-4038 (Print) 0737-4038 (Linking). DOI: 10.1093/molbev/msz104.

Yang, F., L. Zhang, et al. (May 2017). "Tolerance to Caspofungin in Candida albicans Is Associated with at Least Three Distinctive Mechanisms That Govern Expression of FKS Genes and Cell Wall Remodeling". In: *Antimicrob Agents Chemother* 61.5. ISSN: 1098-6596 (Electronic) 0066-4804 (Print) 0066-4804 (Linking). DOI: 10.1128/AAC.00071-17.

Yona, A. H., I. Frumkin, and Y. Pilpel (Oct. 22, 2015). "A relay race on the evolutionary adaptation spectrum". In: *Cell* 163.3, pp. 549–59. ISSN: 1097-4172 (Electronic) 0092-8674 (Linking). DOI: 10.1016/j.cell.2015.10.005.

Zafar, H. et al. (Dec. 2019). "A titanic drug resistance threat in Cryptococcus neoformans". In: *Curr Opin Microbiol* 52, pp. 158–164. ISSN: 1879-0364 (Electronic) 1369-5274 (Print) 1369-5274 (Linking). DOI: 10.1016/j.mib.2019.11.001.

Zhang, J. et al. (Sept. 27, 2017). "Evolution of cross-resistance to medical triazoles in Aspergillus fumigatus through selection pressure of environmental fungicides". In: *Proc Biol Sci* 284.1863. ISSN: 1471-2954 (Electronic) 0962-8452 (Print) 0962-8452 (Linking). DOI: 10.1098/rspb.2017.0635.

Zhang, M. et al. (2010). "Invasive pulmonary and central nervous system aspergillosis following slops aspiration in a trauma patient". In: *Crit Care* 14.5, p. 442. ISSN: 1466-609X (Electronic) 1364-8535 (Print) 1364-8535 (Linking). DOI: 10.1186/cc9228.

Zheng, Z. et al. (Apr. 2013). "Evidence for dynein and astral microtubule-mediated cortical release and transport of Galphai/LGN/NuMA complex in mitotic cells". In: *Mol Biol Cell* 24.7, pp. 901–13. ISSN: 1939-4586 (Electronic) 1059-1524 (Print) 1059-1524 (Linking). DOI: 10.1091/mbc.E12-06-0458.

Zilberberg, M. D. et al. (May 17, 2019). "Burden of hospitalizations over time with invasive aspergillosis in the United States, 2004-2013". In: *BMC Public Health* 19.1, p. 591. ISSN: 1471-2458 (Electronic) 1471-2458 (Linking). DOI: 10.1186/s12889-019-6932-9.

Zmeili, O. S. and A. O. Soubani (June 2007). "Pulmonary aspergillosis: a clinical update". In: *QJM* 100.6, pp. 317–34. ISSN: 1460-2725 (Print) 1460-2393 (Linking). DOI: 10.1093/qjmed/hcm035.

# Vita

Ashley Nywening graduated magna cum laude with Honors from Belhaven University in Jackson, MS., where she earned her Bachelor's Degree in Biological Sciences with a Cellular and Molecular Emphasis and minor in both Chemistry and Fine Arts. She is currently a Ph.D. candidate in the Integrated Biomedical Sciences Program at UTHSC in Memphis, TN., and her focus is in Microbiology, Immunology, and Biochemistry. Her research focus is in the mode and mechanism of antifungal adaptation in the human pathogenic mold *Aspergillus fumigatus*.

She lives currently in Memphis, TN, with her family, two dogs named Timber and Luna, and a cat named Nigel. Ashley expects to receive her Ph.D. degree in May 2024.