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The Mi-2 Homolog Mit1 Actively Positions Nucleosomes Within Heterochromatin to Suppress Transcription

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Abstract

Eukaryotic DNA is packaged into the nucleus in complex with proteins that regulate access and utilization of the genetic material. These DNA and protein complexes form a dynamic structure known as chromatin. Nucleosomes are the repeating unit of chromatin, and consist of DNA wrapped around an octamer of histone proteins. Nucleosomes can then be modified and spatially arranged to facilitate processes such as transcription, DNA replication, and repair. A special transcriptionally repressive chromatin structure assembles onto gene-poor, repetitive regions of the genome known as constitutive heterochromatin. Mit1 is the putative chromatin remodeling subunit of the fission yeast Snf2/HDAC repressor complex (SHREC) and is known to repress transcription at regions of heterochromatin. However, how Mit1 modifies chromatin to silence transcription is largely unknown. Here we report that Mit1 mobilizes histone octamers in vitro Document Type: Doctoral Dissertation Name: Kevin M. Creamer Email Address: kevinmcreamer@gmail.com Title: The Mi-2 Homolog Mit1 Actively Positions Nucleosomes within Heterochromatin to Suppress Transcription Degree: Doctor of Philosophy Program (Major): Biomedical Sciences Concentration (Track): Cell Biology and Biochemistry Research Advisor: Janet F. Partridge, Ph.D. Advisor's Email: jpartri1@uthsc.edu Committee Members: Mark Bix, Ph.D. Paul K. Brindle, Ph.D. Linda M. Hendershot, Ph.D. Ronald N. Laribee, Ph.D. Keywords: chromatin remodeling, fission yeast, heterochromatin, Mit1, SHREC Availability: Embargoed for 12 months Graduation Date: May 2014 and requires ATP hydrolysis and conserved chromatin tethering domains including a previously unrecognized chromodomain to remodel nucleosomes and silence transcription. Loss of Mit1 remodeling activity results in nucleosome depletion at specific DNA sequences that display low intrinsic affinity for the histone octamer, but its contribution to antagonizing RNA Polymerase II access and transcription is not restricted to these sites. Genetic epistasis analyses demonstrate that SHREC subunits and the transcription coupled Set2 histone methyltransferase, which is involved in suppression of cryptic transcription at actively transcribed regions, cooperate to silence heterochromatic transcripts. In addition, we demonstrate that Mit1's remodeling activity contributes to SHREC function independently of Clr3's histone deacetylase activity on Lys14 of histone H3. We propose that chromatin remodeling by Mit1 cooperates with the Clr3 and other chromatin modifiers to stabilize heterochromatin structure and to prevent access to the transcriptional machinery.

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Kevin M. Creamer
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Eukaryotic DNA is packaged into the nucleus in complex with proteins that regulate access and utilization of the genetic material. These DNA and protein complexes form a dynamic structure known as chromatin. Nucleosomes are the repeating unit of chromatin, and consist of DNA wrapped around an octamer of histone proteins. Nucleosomes can then be modified and spatially arranged to facilitate processes such as transcription, DNA replication, and repair. A special transcriptionally repressive chromatin structure assembles onto gene-poor, repetitive regions of the genome known as constitutive heterochromatin. Mit1 is the putative chromatin remodeling subunit of the fission yeast Snf2/HDAC repressor complex (SHREC) and is known to repress transcription at regions of heterochromatin. However, how Mit1 modifies chromatin to silence transcription is largely unknown. Here we report that Mit1 mobilizes histone octamers *in vitro* and requires ATP hydrolysis and conserved chromatin tethering domains including a previously unrecognized chromodomain to remodel nucleosomes and silence transcription. Loss of Mit1 remodeling activity results in nucleosome depletion at specific DNA sequences that display low intrinsic affinity for the histone octamer, but its contribution to antagonizing RNA Polymerase II access and transcription is not restricted to these sites. Genetic epistasis analyses demonstrate that SHREC subunits and the transcription coupled Set2 histone methyltransferase, which is involved in suppression of cryptic transcription at actively transcribed regions, cooperate to silence heterochromatic transcripts. In addition, we demonstrate that Mit1's remodeling activity contributes to SHREC function independently of Clr3's histone deacetylase activity on Lys14 of histone H3. We propose that chromatin remodeling by Mit1 cooperates with the Clr3 and other chromatin modifiers to stabilize heterochromatin structure and to prevent access to the transcriptional machinery.

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LIST OF ABBREVIATIONS

ACF	ATP-dependent Chromatin-remodeling Factor
ADP	Adenosine Diphosphate
ARC	Argonaute siRNA Chaperone Complex
ATP	Adenosine Triphosphate
BAF	Brahma Homolog 1-associated Factors
CAF1	Chromatin Assembly Factor 1
CD	Chromodomain
CENP-A	Centromere Protein A
CHD	Chromodomain Helicase
ChIP	Chromatin Immunoprecipitation
CHRAC	Chromatin Accessibility Complex
CLRC	Clr4 Methyltransferase Complex
CREB	cAMP Response Element-binding Protein
DAPI	4′,6′-Diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DOC	Deoxycholate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FITC	Fluorescein Isothiocyanate
GST	Glutathione S-transferase
HDAC	Histone Deacetylase
HIRA	Histone Cell Cycle Regulation Defective Homolog A
HP1	Heterochromatin Protein 1
IGB	Integrated Genome Browser
ISWI	Imitation Switch
Kbp	Kilo-base Pair
Mit1	Mi-2-like Interacting with Clr3 Protein 1
MNase	Micrococcal Nuclease
NAP1	Nucleosome Assembly Protein 1
NCBI	National Center for Biotechnology Information
NFR	Nucleosome-free Region
NuPoP	Nucleosome Positioning Prediction Engine
NuRD	Nucleosome Remodeling Deacetylase
NURF	Drosophila Nucleosome Remodeling Factor
PAGE	Polyacrylamide Gel Electrophoresis
PBAF	Polybromo Brahma Homolog 1-associated Factors
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethyleneimine
PHD	Plant Homeo Domain
PMG	Pombe Minimal Glutamate
PMSF	Phenylmethylsulfonyl Fluoride

Q-PCR	Quantitative Polymerase Chain Reaction
RDRC	RNA-Dependent RNA Polymerase Complex
RITS	RNA-induced Initiation of Transcriptional Silencing Complex
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
RNA Pol II	Ribonucleic Acid Polymerase II
RPM	Revolutions Per Minute
RSC	Chromatin Structure Remodeling Complex
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SHREC	Snf2/Histone Deacetylase Repressor Complex
siRNA	Small Interfering Ribonucleic Acids
SSC	Saline-Sodium Citrate
SWI/SNF	Switching/Glucose Non-fermenting
TBE	Tris/Borate/EDTA
TBP	TATA-binding Protein
TBZ	Thiabendazole
TE	Tris/EDTA Buffer
TES	Tris/EDTA Buffer plus SDS
TGS	Transcriptional Gene Silencing
TLC	Thin Layer Chromatography
UV	Ultraviolet
YES	Yeast Extract with Supplements
5-FOA	5-fluoroorotic Acid

CHAPTER 1. INTRODUCTION

Packaging of Genetic Information into Chromatin

Eukaryotic genetic information is packaged by the assembly of histone proteins onto DNA to form nucleosomes. Nucleosomes most commonly consist of 147 base pairs of DNA wrapped around the histone octamer, itself formed by the association between two copies of each H3, H4, H2A, and H2B core histones. Nucleosomes are the repeating unit of chromatin, a modular structure that can be altered to regulate transcription, replication, and repair of the underlying DNA sequence. Chromatin is heterogeneous, an observation first made on the cytological level by Emil Heitz in the 1920's. Much research has been performed in the decades since then to understand how chromatin is assembled and modified to facilitate diverse usage of the genome. Current models suggest major themes in chromatin biology where chemical modification of histones and arrangement of nucleosomes on DNA regulate the accessibility and activity of various complexes and enzymes that act on chromatin.

Nucleosome Assembly

Rather than assembling into nucleosomes, core histones and DNA aggregate when incubated together under physiological conditions (1), suggesting nucleosome assembly is a coordinated process. Nucleosome reconstitutions performed *in vitro* utilizing a gradual decrease in ionic strength occur in a stepwise fashion, with binding of an H3/H4 tetramer to DNA directing nucleosome assembly with two additional H2A/H2B dimers (2). In the cell, coordination of nucleosome assembly is performed similarly by histone chaperones following DNA replication or processes that disrupt nucleosomes such as transcription and DNA repair. Unlike traditional chaperones, which promote proper protein folding, histone chaperones facilitate proper incorporation of histones onto DNA and do so by preventing non-productive interactions between the positively charged histones and the negatively charged backbone of DNA (3,4). Among histone chaperones are chromatin assembly factor 1 (CAF1) and Histone Cell Cycle Regulation Defective Homolog A (HIRA) which deposit H3/H4 tetramers onto DNA in a nucleosome intermediate called tetrasomes in replication and replication-independent pathways respectively (5-7). Incorporation of H2A/H2B dimers is then performed by other histone chaperones, most notably by nucleosome assembly protein 1 (NAP1) (4,8).

In addition to nucleosomes assembled with the canonical octamer of histones, most eukaryotes have a number of histone variants that aid in the diverse usage of chromatin, the best studied and evolutionarily conserved of which are CenH3, H3.3, H2A.Z, and H2A.X (9). CenH3 (also known as CENP-A) is similar to canonical H3 in the histone fold domain but has little or no conservation in the amino tail and functions to recruit kinetochore proteins that specify centromeres (10). Nucleosomes containing CenH3 are particularly atypical, as it has been demonstrated that incorporation of this protein switches the handedness in which DNA wraps around centromeric nucleosomes

(11). In contrast to the centromeric variant, H3.3 is much more similar to canonical histone H3 (often referred to as H3.1), typically differing by only a few amino acids depending on the species (9). Histone H3.1 is assembled into nucleosomes primarily during replication and repair by CAF1, while H3.3-containing nucleosomes are assembled independent of replication, such as during transcription, by HIRA (6). Yeast have only a single histone H3 (in addition to CenH3) that most resembles H3.3 and is incorporated both during and outside of DNA replication.

H2A also has two common variants. Incorporation of the similar H2A.Z protein into chromatin is known to affect transcription in a number of ways, including regulating chromatin remodeling, suppression of antisense transcription, and prevention of DNA methylation in gene promoters (12-14). H2A.X is distinguished by the presence of additional motifs in the C-terminus not found in H2A that can be phosphorylated by kinases. Phosphorylation of H2A.X (commonly known as γH2A.X) is most commonly associated with the response to double strand breaks and DNA repair (15), however H2A.X is also required for murine X chromosome inactivation, suggesting the usage of this variant may extend beyond the DNA damage response (16,17).

Post-translational Modification of Histones

Study of chemical modifications present on histones began with the discovery of acetylation on isolated calf thymus histones in the 1960's, and was soon implicated as a key regulator of transcription and eventually replication as well (18-20). Subsequent studies, particularly on the N-terminal tail of histone H4, revealed that the charge neutralization that occurs as a result of acetylation of lysine residues reduces the affinity of histones for the negatively-charged phosphate backbone of DNA (21). By altering the contacts made between histones and DNA, acetylation has been shown to be a key regulator of chromatin accessibility and compaction. Experiments from several model organisms and contexts revealed that chromatin that was hyperacetylated was more "open" and accessible while hypoacetylated chromatin tended to have a compact structure and was repressive toward processes like transcription (22). In addition to charge-based alterations in chromatin structure, histone acetylation was discovered to have additional roles in transcriptional activation by recruiting transcriptional activators through recognition by an evolutionarily conserved bromodomain (23), highlighting a more prevalent mechanism for alteration of chromatin by post-translational modifications as signals to other chromatin modifying complexes.

Several other chemical modifications found on non-histone proteins have also been detected on histones, which cluster in, but are not exclusive to, the histone amino tails that project from the octamer core including acetylation, methylation, phosphorylation, and ubiquitination among others (24). These modifications then either recruit or modulate the binding or activity of chromatin modifying complexes (25). The diverse set of moieties and amino acid modifications that can be added led to the development of the "histone code hypothesis," first proposed by Brian Strahl and David

Allis, which provided the basis for understanding how combinations of histone modifications can direct unique utilization of and access to chromatin (26).

Nucleosome Positioning

The impact of histones on the genetic information is influenced not only by modification but where they are located on genomic DNA. The understanding of where nucleosomes are found has been greatly advanced by genome-wide approaches in a number of organisms generating high resolution maps of nucleosome positioning in the cell. Where nucleosomes are located as well as how they are arranged in relationship to one another can have significant effects on processes like transcription. Chromatin presents a challenge to transcription as factors that initiate transcription, the TATA-binding protein (TBP), and the transcriptional machinery itself, are essentially unable to bind nucleosomal DNA (27). Furthermore, nucleosomes are a barrier to transcriptional processivity *in vitro* and in the cell (28,29).

In addition to their contribution to primary chromatin structure, periodicity and linker length between nucleosomes is thought to influence the formation of higher-order chromatin structures that can influence access to the underlying DNA sequences as well (30). Current models suggest the major factors in determining nucleosome positioning in the cell are DNA sequence, chromatin remodeling enzymes, transcription factors, and the transcriptional machinery (31).

Several key conclusions have been made from cell-free experiments testing the preference for nucleosome assembly on genomic DNA in the absence of other factors (32-35). First, nucleosomes assemble with preference for certain sequence characteristics (see below). Secondly, *in vitro* reconstitutions recapitulate some of the nucleosome positioning patterning observed in the cell, suggesting this observation is biologically relevant. However, greater than 95% of genomic eukaryotic DNA does not differ significantly from scrambled DNA sequence in terms of affinity for the histone octamer and is therefore unlikely to directly contribute to positioning preferences for the majority of nucleosomes (36).

Affinity of the histone octamer for a given 147 base pair sequence can vary greatly and is most related to the ability of DNA to bend around to make proper contacts with the histone octamer (37). A prominent feature of high affinity DNA sequences is the rotational phasing of A/T dinucleotides positioned so they are making contacts with the histone octamer every 10 base pairs of the helical repeat as DNA wraps around the octamer core (35,38). Rather than phased nucleotides, homopolymeric sequences, such as poly(dA:dT) tracts, are particularly refractory to nucleosome assembly due to their rigid structure. (33,39,40). Cells seem to use this to their advantage, as A/T-rich sequences that intrinsically disfavor nucleosome occupancy are common features at many eukaryotic promoters and have biological effects on transcription (33,41,42).

While intrinsic affinity for the histone octamer explains some aspects of nucleosome positioning, many others aspects of nucleosome patterning do not appear to be governed by these properties. In order to achieve the functional patterning of nucleosomes observed in the cell, eukaryotes have evolved numerous special helicase-like enzymes to facilitate movement of histone octamers on DNA (43). These so-called chromatin remodeling enzymes are SNF2 family ATPases and are molecular motors that directly influence nucleosome positioning in the cell. Transcription factors, DNA repair and replication machinery, and RNA Polymerase complexes all recruit chromatin remodeling enzymes to chromatin to facilitate movement of nucleosomes resulting in changes in accessibility to the underlying DNA or patterning of nucleosome positions (31). Chromatin remodeling enzymes are covered in greater detail below.

ATP-dependent Chromatin Remodeling Enzymes

Chromatin remodeling enzymes are molecular motors required for the packaging of chromatin in eukaryotic cells. Specifically, chromatin remodeling enzymes are diverse DNA translocases that utilize the energy of ATP hydrolysis to alter the contacts between DNA and histones. This generalized activity can then be modulated to yield a number of specific outcomes including repositioning or eviction of the histone octamer from DNA, exchange of specific histones, and unwrapping of DNA from around the nucleosome (44).

Mechanism of Histone Octamer Mobilization

Perhaps the most prominent feature, at least *in vitro*, of chromatin remodeling factors is the ability to "slide" histone octamers along DNA. In order to mobilize nucleosomes, chromatin remodeling factors must disrupt over one hundred contacts between the histone octamer and DNA as revealed in the crystal structure of the nucleosome core particle (45). Though several models have been proposed for how nucleosome mobilization occurs (46), recent in depth, single-molecule approaches studying the remodeling activity of ISWI-family remodeling enzymes have revealed a mechanism of DNA translocation (47). In this model, the energy provided by hydrolyzing one molecule of ATP to ADP is used to translocate one base pair of DNA toward the exit side of the nucleosome. The movement of DNA with respect to the histone octamer disrupts histone-DNA contacts, and after seven one base pair translocations these contacts become distorted such that three base pairs of DNA enter and then exit the nucleosome, a cycle which is repeated as histone octamers "slide" along DNA. While this mechanism is likely shared by other chromatin remodeling enzymes as well, the specific nature of nucleosome mobilization in terms of directionality, kinetics, and functionality *in vivo* is determined by the unique chromatin interacting motifs of individual remodelers and additional subunits of chromatin modifying complexes (48),(49).

Organization and Activities of Chromatin Remodeling Enzymes

Following the discovery and characterization of the first chromatin remodeling factor, the SWI/SNF complex in 1994 (50), enzymes with Snf2-related catalytic cores have been classified into four major evolutionarily conserved families (SWI/SNF, ISWI, CHD, and INO80) based on their related structures and activities (43,46).

The large, multisubunit SWI/SNF complex of *Saccharomyces cerevisiae* was the first identified and is perhaps the best studied remodeling complex. Named for its role in regulating mating type switching and usage of metabolites (sucrose nonfermenting), SWI/SNF is recruited to chromatin by transcription factors and regulates approximately 5% of all genes in budding yeast. (51-53). Yeast, and most higher organisms as well, have two similar SWI/SNF-related complexes, the second of which is known as RSC (remodels structure of chromatin) in yeast and regulates an even greater number of genes than does ySWI/SNF (44,54). Human homologs of ySWI/SNF and RSC (hBAF and hPBAF) also regulate transcription, particularly during development, and have considerably diversified their complex architectures and functions through evolution (55). First identified in the *Drosophila melanogaster* SWI/SNF homolog, these complexes characteristically have histone acetylation recognizing bromodomains which are important for their function (23).

ISWI remodeling complexes (known as ACF, NURF, and CHRAC in most metazoans) were first identified as small complexes that mobilize nucleosomes to allow for binding of transcription factors (56,57). In contrast with SWI/SNF complexes which are known to disrupt nucleosomes (58), ISWI complexes are associated with assembly and organization of nucleosomes and are perhaps best known for their ability to space or regularly phase nucleosomes (59,60). ISWI remodeling complexes are often associated with the activation of transcription, but are also known to associate with heterochromatin, leading researchers to postulate their involvement with the formation of specialized chromatin structures (61).

INO80/SWR1 family remodelers are similar to SWI/SNF family enzymes, but structurally differ by inclusion of large spacer regions within their catalytic core (46). SWR1 is known to catalyze the exchange of the H2A.Z, a histone variant known to have roles in regulating transcription and barrier function, into chromatin (62). INO80 is a factor involved in the response to DNA damage and is recruited by γH2A.X to evict histones and facilitate repair (63).

The CHD family of remodeling enzymes are structurally similar to SWI/SNF family enzymes, but also contain chromodomains. Metazoan genomes encode several CHD family remodeling enzymes to regulate transcription. A subset (CHD3/4), are subunits of larger NuRD complexes which also contain a PHD finger chromatin binding motif and associate with histone deacetylases (see below). Of CHD family members, Chd1 is the only member of this family conserved from yeast to mammals and is recruited by the transcriptional machinery to aid transcriptional elongation. (64-66).

Fission Yeast Heterochromatin Assembly and Function

All eukaryotes have developed epigenetic mechanisms to temporarily or constitutively silence regions of their genomes, though how these organisms accomplish this task may differ between organisms or depending on the genomic context. While facultative heterochromatin assembles on genes that may be activated in other developmental or signal response contexts, constitutive heterochromatin is more permanent in nature and typically forms on repetitive DNA elements and transposable elements (67,68). Repetitive DNA sequences are a prominent feature of centromeres and telomeres in many organisms (67). Heterochromatin plays an essential role for the proper function of each of these regions. For instance, the heterochromatin that assembles near chromosome ends is known to control telomere length by preventing degradation of chromosome ends and repressing the double-strand DNA break repair pathways at these regions (69). Pericentromeric heterochromatin is essential for proper centromere function as it serves as a recruiting platform for cohesion and may aid orienting centromeres during chromosome segregation (70-73).

The genetic tractability and conservation in pathways that regulate the structure and functions of chromatin have made the fission yeast *Schizosaccharomyces pombe* an attractive model organism for studying many epigenetic processes, most notably heterochromatin assembly. Nearly two decades ago it was found that transgenes inserted into fission yeast centromeres were epigenetically repressed similar to position effect silencing previously observed in *Drosophila melanogaster* (74). Subsequent genetic and biochemical approaches in *S. pombe* have been important for determining the molecular basis for transcriptional silencing. Heterochromatin assembles at three large regions in *S. pombe*, at the centromeres, telomeres, and mating type region which all share some sequence homology (72,75-77) (**Figure 1-1**). In addition to the centromeric and telomeric functions described above, heterochromatin serves to properly maintain mating identity by repressing recombination and silencing transcription at the mating type region (76,78).

Clr4-mediated Methylation of Histone H3 Directs Heterochromatin Assembly

Heterochromatin in fission yeast can largely be defined by the presence of methylation on lysine 9 of histone H3 (79). In higher organisms, H3K9 methylation is a mark frequently associated with transcriptional silencing, however, differential and context specific methylation of Lys9 on histone H3 can specify different functional outcomes. For instance in mammals H3K9 methylation is detected in actively transcribed chromatin and is associated with transcriptional elongation (80). In contrast, H3K9 methylation is found almost exclusively in transcriptionally silent regions of constitutive heterochromatin in fission yeast. Furthermore, H3K9 methylation is performed by a single enzyme in fission yeast cryptic loci regulator 4 (Clr4)(81). In contrast, most metazoans have three different methyltransferases which specifically methylate Lys9 on histone H3: Su(var)3-9, SETDB1, and G9a (82). *S. pombe* lack methylation on lysine 27 on histone H3 and DNA methylation, signals frequently associated with heterochromatin
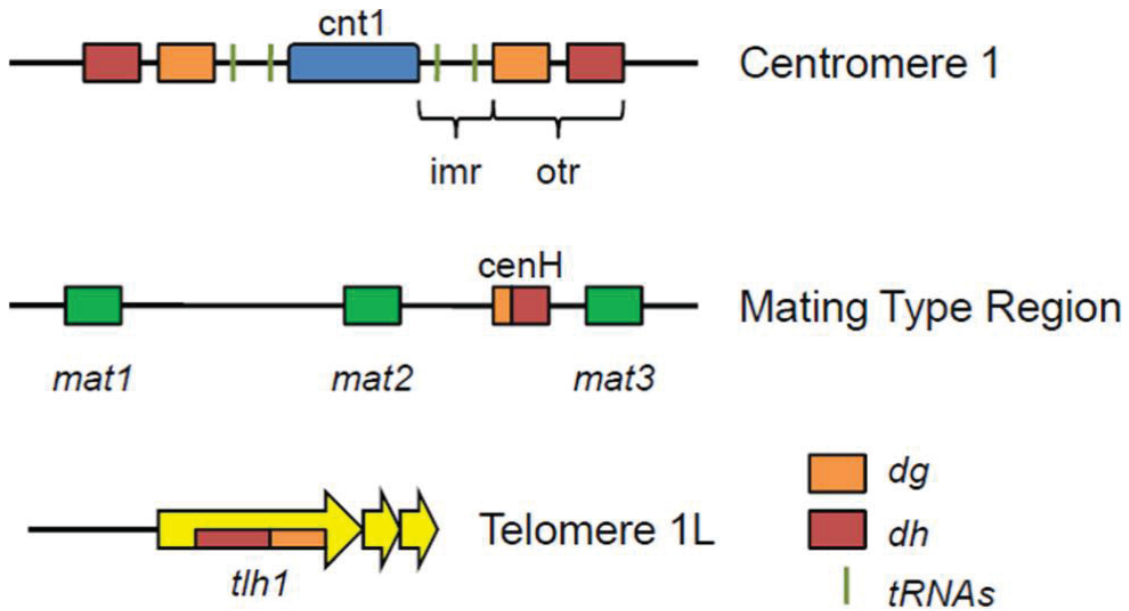
Figure 1-1. **Sites of heterochromatin assembly in fission yeast.**
Schematic of the three major regions where heterochromatin assembles in *S. pombe*.
Transcriptionally silent heterochromatin assembles at repetitive *dg* and *dh* outer repeat
(otr) sequences surrounding the central core and inner most repeats (imr) of centromeres
as well as the mating type region on chromosome 2 and at telomeres. The mating type
region and left telomere on chromosome 1 each contain sequences with homology to the
centromeric *dg* and *dh* elements.
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Partridge. 2011. RITS-connecting transcription, RNA interference, and heterochromatin
assembly in fission yeast. Wiley Interdiscip. Rev. RNA 2:632-46.

and transcriptional silencing in many higher organisms (82,83). The relatively simplistic nature of the pathways surrounding H3K9 methylation has made *S. pombe* a valuable model organism to study constitutive heterochromatin assembly and function.

Clr4 associates with Rik1, Cul4, Dos1, and Dos2 proteins to form the Clr4 methyltransferase complex, CLRC (84-86). While homology suggests these additional subunits are involved in the transfer of ubiquitin and it has been demonstrated that purified CLRC has ubiquitin ligase activity *in vitro*, the relevant target of this activity is unknown (87). Although the precise contribution of these proteins is unclear, they are essential for the recruitment and function of Clr4 in heterochromatin assembly.

Methylation on Lys9 of histone H3 signals the recruitment of proteins containing chromodomains that specifically recognize this mark including HP1-like proteins Swi6 and Chp2, RNA interference factor Chp1, as well as Clr4 itself (88-91). Swi6 physically associates with cohesin and is essential for proper recruitment of this complex to centromeres to facilitate proper chromosome segregation (70,92). Furthermore, Swi6 associates with and contributes to the recruitment of a number of silencing factors including the histone deacetylase Clr6 and HIRA complex, which promotes nucleosome occupancy within regions of heterochromatin (93-95). Chp2 is best known for its association with the SHREC complex (see below), while Chp1 links H3K9 methylation to post-transcriptional silencing of heterochromatic transcripts by the RNA interference machinery (see below).

RNA Interference in *S. pombe*

Heterochromatin in fission yeast is largely inert to transcription through transcriptional gene silencing (TGS) mechanisms that inhibit RNA polymerase II recruitment and function, however, these regions, particularly pericentric repeats, are transcribed at low levels (96,97). The few transcripts that do emanate from regions of heterochromatin are silenced post-transcriptionally by the RNA interference pathway. Interestingly, genes involved in the RNA interference pathway are required to maintain high levels of H3K9 methylation at centromeric repeats and are essential for proper chromosome segregation (89,98). Work from several groups has shown that rather than simple degradation byproducts, small RNA generated by the RNAi pathway are important for targeting and reinforcing H3K9 methylation at the genomic locations from which they are derived.

Many of the molecular details for the RNA pathway have been described in order to explain this interesting paradox that centromeric repeats must be transcribed in order to be silenced. While RNAi in fission yeast is a cyclical process (**Figure 1-2**), the first step in the generation of siRNA involves the generation of double stranded RNA (dsRNA). It has been suggested that dsRNAs that feed into the RNAi pathway can be formed through intrinsic folding of a longer centromeric repeat transcript or other means (99,100), though the significance of this has yet to be elucidated entirely. More commonly, dsRNA are generated through the action of an RNA-dependent RNA polymerase (Rdp1), which,
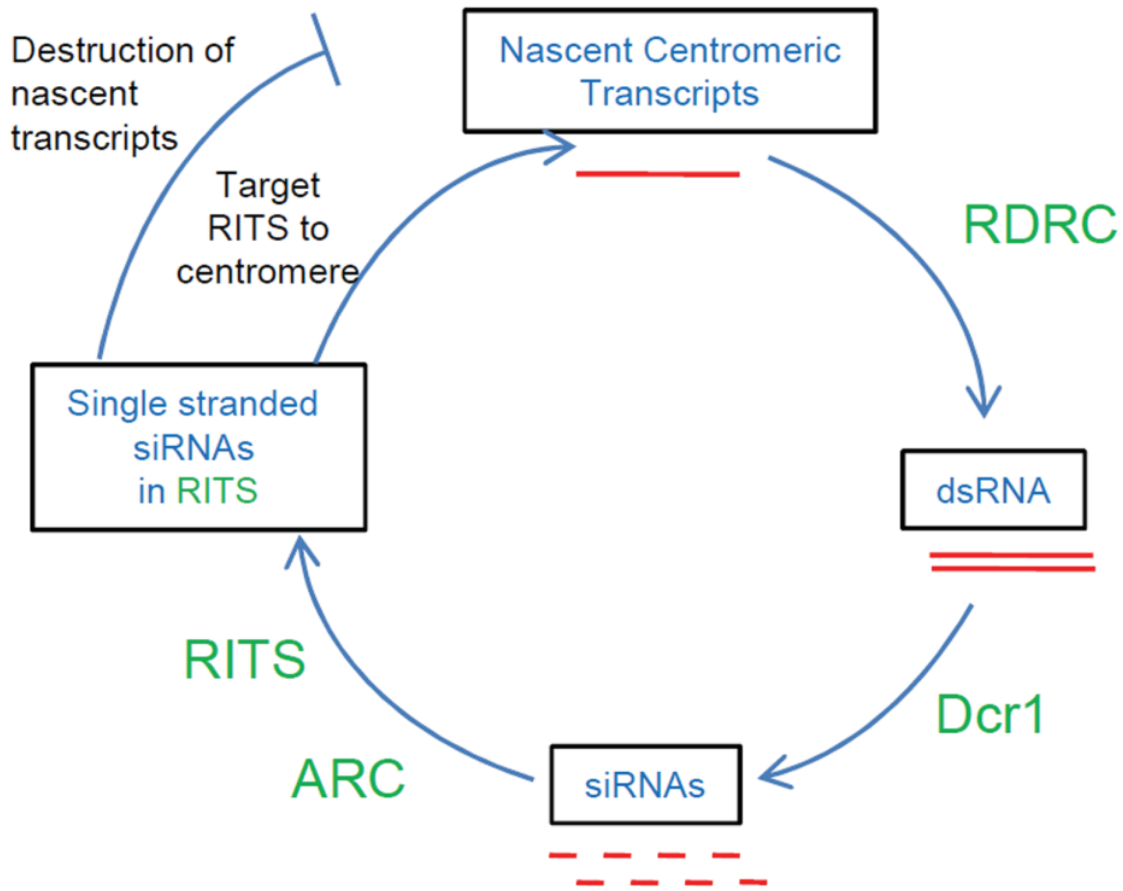
Figure 1-2. **The RNA cycle of heterochromatin assembly.**
Transcripts originating from the centromeric outer repeats are degraded by a RNA
interference pathway. Centromeric transcripts are converted into double stranded RNA
by the RNA-depentent RNA polymerase complex (RDRC) before endonuclease "slicing"
into small siRNAs by Dicer (Dcr1). These siRNAs are then loaded sequentially into the
ARC and RITS complexes and serve to target the RITS complex back to homologous
DNA sequences by base-pair interactions.
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Partridge. 2011. RITS-connecting transcription, RNA interference, and heterochromatin
assembly in fission yeast. Wiley Interdiscip. Rev. RNA 2:632-46.

together with Hrr1 and Cid12 proteins make up the RNA-dependent RNA polymerase complex (RDRC) (101). These dsRNAs are then cleaved by the activity of an RNAse III endonuclease, Dicer (Dcr1) (101,102).

Products of Dicer's endonuclease are small dsRNA of approximately 22 base pairs known as siRNA (103). These siRNA are then "loaded" into the ARC complex, consisting of Argonaute (Ago1), an enzyme with RNA endonuclease, or "slicer" activity, as well as Argonaute-binding proteins 1 and 2 (Arb1/2) (104,105). Following endonucleolytic digestion of one strand of the double-stranded siRNA, the "passenger" strand, Ago1 associates with a different set of proteins, Tas3 and Chp1, forming the RITS (RNA induced initiation of transcriptional silencing) complex (106). RITS associates with heterochromatin in two ways: base-pair interactions between the single stranded RNA associating with Ago1 and recognition of H3K9 methylation by Chp1 chromodomain (91,105,107). Stable association of RITS with nascent transcripts facilitates their degradation via Ago1 slicer activity.

Fission yeast RNAi occurs primarily in *cis*, aided by physical interaction between the RDRC and RITS (101). Furthermore, RITS is thought to interact with the H3K9 methyltransferase complex CLRC through a bridge protein known as Stc1 (108). The physical interaction between these complexes and simultaneous targeting by siRNA base-pair interactions with nascent transcripts as well as binding to methylated K9 by Chp1 is thought to provide a positive-feedback mechanism for targeting Clr4 methytransferase activity to regions of heterochromatin (**Figure 1-3**).

The apparent opposing nature of the requirement for transcription in order to assemble heterochromatin structure was further explained by the discovery that transcription of centromeric repeats is temporally regulated. Transcription of centromeric repeats and processing of these nascent transcripts into siRNA occurs largely during S phase following replication of the underlying DNA sequences (109,110). Hallmark characteristics of heterochromatin, including H3K9 methylation and Swi6 are transiently disrupted during this time, presumably by the process of replication itself, allowing for a brief window to allow transcription. Rather than having a continuous block on transcription, the results of these studies suggest that centromeric chromatin is dynamic. While the heterochromatin that assembles on these sequences normally functions as a barrier to transcription, the transient transcription that occurs following dissolution of the chromatin state during replication feeds into the RNAi pathway which ultimately aids in the reassembly of heterochromatin.

RNA interference is not unique to fission yeast and was first discovered in the worm *C. elegans* (111). Furthermore, RNAi is required for centromeric heterochromatin formation in *D. melanogaster* and there is considerable conservation between the factors that mediate RNA interference in these diverse organisms (112). Human RNA interference pathways exist as a means for regulating transcription as well, but the relationship between this process and chromatin modification is less clear. This may be, in part, because many higher organisms, including humans, lack the canonical RNA-dependent RNA polymerase found in worms and fission yeast (113).
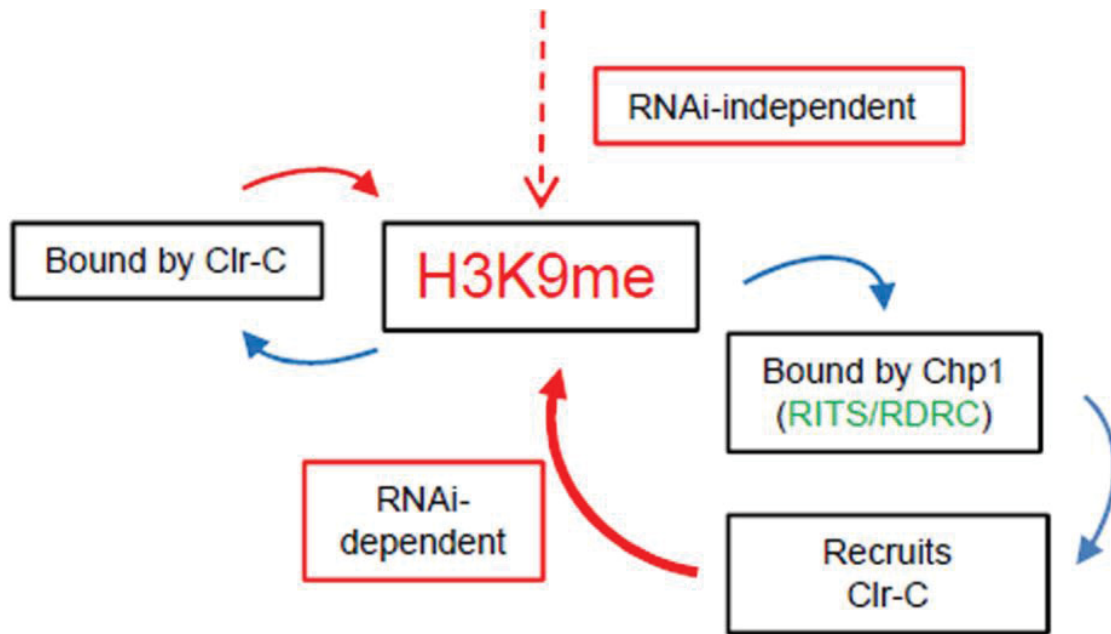
Figure 1-3.   The H3K9me cycle.
Methylation of Lys9 on histone H3 is directed by RNAi-dependent and independent
pathways targeting the methyltransferase activity of Clr4 (red arrows), a subunit of the
ClrC complex. H3K9 methylation is then maintained by a positive feedback loop
facilitated by the recognition of this modification by the RNAi factor Chp1 as well as
Clr4 itself.
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The SHREC Complex

SHREC Is a Transcription Silencing Complex

The Snf2/HDAC-containing repressor complex SHREC was purified in an effort to identify factors associated with Clr3, a known histone deacetylase with roles in regulating gene expression and heterochromatin silencing (114). The core SHREC complex consists of Clr3, the zinc-finger protein Clr1, a Clr2 protein which bears little resemblance to other chromatin modifiers, and Mit1, a putative chromatin remodeling enzyme. While Clr1, Clr2, and Clr3 were identified in a similar fashion several years prior in genetic screens for their role in transcriptional silencing at the mating type region (115-117), little was known about the function of Clr1 and Clr2 in heterochromatin silencing prior to their co-purification with Clr3. In contrast, the homology of Clr3 with previously characterized histone deacetylases in other organisms had led to the identification of acetylated Lys14 on histone H3 as a major target for Clr3 activity in the cell which was presumed to be a primary function of Clr3 in silencing (118).

Shortly after the publication of these findings another group reported purification of a similar complex, only including the association with the HP1-like protein Chp2 as well (93). In this study, the Chp2 and Mit1 subunits of SHREC co-purified exclusively with N-terminal fragments of Clr1, while only C-terminal fragments of Clr1 were detected by mass spectrometry analysis of Clr2 and Clr3 purifications. These findings led to the proposed architecture depicted in **Figure 1-4**.

The association of Chp2 with SHREC subunits was proposed to provide a clear link between H3K9me3 that typifies heterochromatin and the histone deacetylase and potential chromatin remodeling activity of SHREC (93). In addition, SHREC associates with other proteins including Ccq1, a telomere associated protein (114), and Seb1 which associates with centromere derived non-coding RNA (119) that may contribute to their association with heterochromatin. Furthermore, prior to the identification of associated factors, Clr3 was found to be recruited to the mating type regions by sequence-specific DNA binding proteins, the ATF/CREB-like proteins Atf1/Pcr1 (120).

SHREC is associated with all major regions of heterochromatin and cells lacking subunits of SHREC or that have inactivating point mutations in catalytic Clr3 and Mit1 subunits have increased acetylation on H3K14 at these loci and are increasingly accessible to RNA Pol II, leading to increased levels of their transcription (93,114). Marks of heterochromatin, such as the extent of methylation on Lys9 of histone H3 and Swi6 recruitment, are reduced and unable to spread efficiently throughout the mating type region in the absence of SHREC activity (93,120). Genetic analysis in these studies suggest that SHREC makes contributions to heterochromatin assembly and silencing that are partially redundant and act in parallel to RNAi.
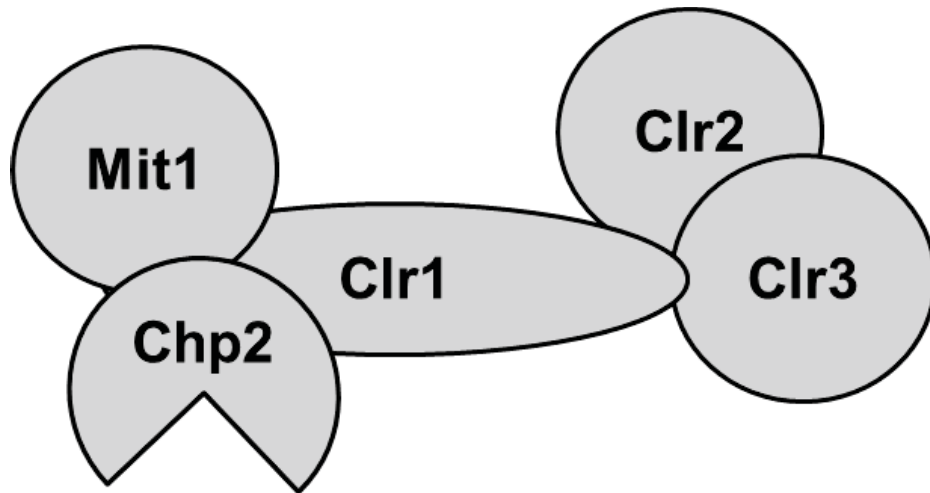
Figure 1-4.   The SHREC complex.
Architecture of the SHREC complex as previously proposed based on mass spectrometry
of purified SHREC subunits (93). Note that is not known whether Chp2 and Mit1 interact
directly or through the N-terminus of Clr1. Similarly, the nature of Clr2-Clr3 interactions
have not been determined, except that they associate with the C-terminus of Clr1.

SHREC Is Required to Maintain Histone Occupancy in Regions of Heterochromatin

Several studies have implicated SHREC in having a role in regulating nucleosome positioning and chromatin dynamics within heterochromatin. Early locus-specific studies using nuclease digestion followed by Southern blotting demonstrated that cells expressing catalytically inactive Clr3 or Mit1 had nucleosome occupancy changes in the mating type region (114). Subsequent tiling array-based mapping of nucleosomes in regions of heterochromatin confirmed this result, as well as identified additional regions within the mating type region, centromeres, and telomeres that depended on SHREC for proper nucleosome occupancy, though several loci required only the activity of Clr3 (121). Despite being required for efficient centromeric silencing, nucleosome occupancy was notably unaffected at centromeres in cells expressing a catalytically deficient Mit1 in this study.

A separate study also found that the Clr3 subunit of SHREC was required to maintain nucleosome occupancy at regions of heterochromatin in a pathway parallel to Asf1/HIRA and Clr6 (Complex II) histone deacetylase activity (94). In a subsequent publication, the same group found that Clr3 deacetylase activity is important for limiting histone turnover, giving a possible explanation for how Clr3 contributes to the stability of heterochromatin (122).

SHREC and NuRD Are Similar in Composition and Function

SHREC is similar in composition to the nucleosome remodeling and deacetylase (NuRD) complexes found in higher organisms, but previously thought to be absent in yeast. NuRD has been purified in a number of species from flies to human and provocatively couples two chromatin modifying activities in chromatin remodeling by chromodomain-helicase-DNA-binding proteins 3 and 4 (CHD3/4, also known as Mi-2α/β) and histone deacetylation by histone deacetylases 1 and 2 (HDAC1/2) (123-126). NuRD also has several interchangeable non-catalytic subunits which mediate association of the complex with chromatin and transcription factors including methyl-CpG-binding domain proteins 2 and 3 (MBD2/3), metastasis-associated genes 1,2 and 3 (MTA1/2/3), and retinoblastoma-binding proteins 4 and 7 (RBBP4/7) (127). NuRD is thought for be cable of inducing densely compact, hypoacetylated chromatin refractory to transcription. Consistent with this activity, the biological role of NuRD is most frequently associated with negative regulation of gene expression, although the complex has been implicated in induction of transcription in some instances (128-130). Transcriptional programming by NuRD is required for normal development and lineage commitment of embryonic stem cells (131). Furthermore, NuRD is known to be important for maintaining the pluripotency of stem cells by repressing the transcription of certain target genes (132).

The principal similarities between SHREC and NuRD are found between the chromatin remodeling enzymes Mit1 and CHD3/4(Mi-2) as well as Clr3 and HDAC1/2 histone deacetylases. There is little obvious or reported conservation between the other

subunits of SHREC and NuRD Furthermore, NuRD is not thought to interact with any HP1 isoforms, while SHREC associates with HP1-like protein Chp2.

Purified NuRD or CHD3/4 in isolation has nucleosome sliding activity *in vitro*, and this activity has been shown to require the conserved tandem PHD and chromodomains. The PHD domains of CHD3/4 in various organisms have been shown to bind unmethylated H3K4 and H3K9 methylation, while CHD3/4 chromodomains are thought to be atypical and bind DNA. Prior to this study, the *in vitro* activity of Mit1 was unstudied and sequence analysis of Mit1 suggested only a single uncharacterized PHD finger with no chromodomains.

Clr3 and HDAC1/2 are Rdpd3/Hda1-like lysine deacetylases (133). However, while acetylated H3K14 is thought to be the primary target of Clr3 based on western blot analysis and ChIP experiments comparing wild type to *clr3Δ* cells, HDAC1/2 deletion has been reported to result in slight to moderate increases in the acetylation of many targets including lysines 4, 8, 9, 14, and 56 on histone H3 as well as lysines 5, 8, 12, and 16 on histone H4 (118,134,135). In addition, HDAC1/2 have important non-histone targets as well (136), though no such targets have been identified for Clr3 to date. The chromatin remodeling and histone deacetylase activities of NuRD are thought to be synergistic, as ATP stimulates HDAC1 deacetylation on nucleosomal subtrates (123). Similarly, deletion of *mit1*$^+$ or other subunits of SHREC results in a similar increase in H3K14 acetylation in regions of heterochromatin as *clr3Δ*, though it is not known whether this effect is direct (114).

While the majority of studies focus on the gene regulation aspect of NuRD function, recent work has revealed additional roles for NuRD related to heterochromatin. NuRD has been found to associate with centromeric heterochromatin during S phase and has a role in maintaining chromatin structure there (137,138). In a separate landmark study, Pegoraro et al. found NuRD associates with the nuclear filament protein lamin A and proposed that the disruption of this interaction may be the underlying cause for the premature ageing syndromes that occur in patients expressing a truncated form of the protein (139). Chromatin-related phenotypes associated with natural and diseased cellular ageing include general genomic instability, reduced foci of heterochromatic marks, and increased transcription of satellite repeats normally silenced by heterochromatin (140-143). Interestingly, knock down of NuRD subunits recapitulates many of these phenotypes, revealing a previously unascribed role for NuRD in regulating heterochromatin assembly and function in the context of ageing.

Aims of This Study

While the contribution of nucleosome positioning to the regulation of transcription has been relatively well characterized, the arrangement of nucleosomes in regions of constitutive heterochromatin is less well understood. The NuRD-related SHREC complex has a known role in silencing transcription in fission yeast, but the molecular details of how it accomplishes this task are unclear. The principal aim of this

study is to study the putative chromatin remodeling activity of the Mit1 subunit of SHREC. By combining *in vitro* biochemical analysis of Mit1 activity and characterization of the requirement for this activity in positioning nucleosomes within regions of heterochromatin, a model is proposed where Mit1 actively mobilizes nucleosomes within regions of heterochromatin to prevent the formation of nucleosome depleted sites and suppress access to the transcriptional machinery.

CHAPTER 2.   METHODOLOGY

S. pombe strains were grown and manipulated using common laboratory procedures (144). Strains, plasmids, and oligonucleotides used in this study are listed in the Appendix.

Affinity Purification of Mit1

Fission yeast with an endogenous *mit1$^+$* deletion and episomally expressing 3XFLAG-Mit1-3XV5 fusion protein under the control of the full strength *nmt1$^+$* promoter were grown to mid-log phase in 8 liters of PMG minimal media (145) lacking uracil to maintain the plasmid. PMG is Edinburgh minimal medium with glutamate. Cells were harvested by centrifugation and subject to mechanical lysis using a Krups Tipo 203 coffee grinder in the presence of dry ice for 5 minutes. Extracts were made by the addition of 2.5ml Extraction Buffer A [50mM Tris-HCl pH 7.5, 300mM NaCl, 8mM EDTA, 0.2%NP-40, 10% glycerol, 0.1mM PMSF, and a protease inhibitor cocktail (Roche)] for every gram of cell pellet and incubation at 4°C with rotation for 60 minutes before ultracentrifugation at 25,000 RPM for 30min using a TLA-120.2 rotor to remove debris. Cleared lysate was adjusted to 150mM NaCl, 4mM EDTA, and 0.1% NP-40 before binding on column to 250µl anti-FLAG M2 affinity gel (Sigma). Bound proteins were washed with 10ml Wash Buffer [10mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% NP-40, 10% glycerol] three times and eluted 3 times with 4ml Elution Buffer [10mM Tris-HCl, 150mM NaCl, 10% glycerol] containing 0.05% NP-40, 0.1mM PMSF, and 0.25mg/ml 3XFLAG peptide for 30 minutes each elution. The eluate was incubated with 100µl anti-V5 affinity gel (Sigma) for 3 hours at 4°C with rotation, washed with 3 times with 5 ml Wash Buffer, and again eluted 5 times with 80µl Elution Buffer lacking protease inhibitor and NP-40, but containing 0.5mg/ml 3XV5 peptide. Elutions were analyzed by SDS-PAGE and Western blotting with antibodies against the FLAG and V5 epitopes. Mit1 is found primarily in the second and third elutions under these conditions.

ATP Hydrolysis Assay

ATP hydrolysis was performed essentially as previously described (146). A total reaction volume of 10µl containing normalized amounts of Mit1 (approximately 2.5nM), mutant, or mock purifications was incubated with 0.75µl [α-^{32}P]ATP (3000Ci/mmol, 10mCi/µl, PerkinElmer) in buffer containing final concentrations of 20mM Tris-HCl pH 7.5, 70mM KCl, 15mM NaCl, 6.5mM MgCl$_2$, 0.8mM EDTA, 1mM DTT, 0.016% NP-40, 1% glycerol, 0.1mM unlabeled ATP, and 30ng/µl plasmid DNA (p199-1). After 30 minutes at 30°C, reactions were stopped by addition of 0.5µl of 0.5M EDTA and placed on ice.

Reactions were then spotted (0.5 µl) onto PEI-cellulose thin-layer chromatography plates (Sigma) and resolved in 0.75M KH$_2$PO$_4$ (pH 3.5). Plates were

dried and exposed to a phosphor screen. Screens were analyzed by phoshor imaging (Molecular Dynamics STORM 860). In instances where results were quantified, amount of enzyme was adjusted to result in approximately 10-15% hydrolysis and densitometry was performed (Imagequant™ 3.0, GE Healthcare), subtracting a reaction without protein as background.

Nucleosome Remodeling Assays

[32]P-dCTP incorporated PCR product was generated using primers that amplify products with distinctly localized nucleosome positioning sequences (i.e. 70N0, 70N70, and 0N0) from a plasmid containing the 601 nucleosome positioning sequence (p199-1) as a template (147). Mononucleosomes were prepared by first mixing approximately eqimolar amounts of histone octamer (human, recombinant, NEB) and the specific labeled PCR product. A typical reconstitution would consist of a reaction containing 25pmol PCR product, 5µM H2A/H2B dimer, 2.5µM H3/H4 tetramer, and 2M NaCl in 10µl total volume (80µl final). Assembly was performed by stepwise dilution of salt concentration from 2M to 0.25M (30 minutes each at 2M, 1.48M, 1.0M, 0.6M, and 0.25M NaCl) using 10mM Tris-HCl pH 8.0 at room temperature and material was stored at 4°C.

Nucleosome mobilization was performed by incubating purified remodeler with labeled mononucleosomes (see figure legends for concentrations used in each experiment) in 20µl reactions containing final concentrations of 10mM Tris-HCl pH 7.5, 50mM NaCl, 5mM MgCl2, 0.5mM DTT, 50ng/µl BSA, and 5mM ATP for 60 minutes at 30°C. Reactions were stopped by the addition of plasmid DNA (p199-1) to 300ng/µl and glycerol to 6%. Samples were incubated on ice for 10 minutes before electrophoresis on pre-run, 20mm, 0.5X TBE, 5% native polyacrylamide gels at 200V for approximately four hours. Gels were dried and exposed to phoshor screens prior to imaging.

Calf Thymus Histone Pull-down

Pulldowns were performed as previously described (148) with some modification. Calf thymus histones (Worthington Biochemical) at 5µg per reaction were incubated with 2µg GST or GST fusion protein in 1ml Calf Thymus Histone Binding Buffer [20mM Tris-HCl pH 7.5, 750mM NaCl, 0.75% NP-40, 0.5mM DTT] at 4°C for 4 hours. After binding to glutathione-agarose for one hour, beads were washed 3 times with Calf Thymus Histone Binding Buffer with 5 minutes rotation for each wash. Bound proteins were then analyzed by SDS-PAGE and western blotting using antibodies against GST (GE Healthcare 27-4577-01), H3 (Abcam ab1791), H2B (Millipore 07-371), H3K4me3 (Active Motif 39160), H3K9me2 (Active Motif 39239), and H3K36me3 (Epigentek A-4042).

Binding to Modified Histone Peptide Array

Binding specificity of GST fusion proteins and antibodies was determined using a peptide microarray including 384 unique histone tail modification combinations (MODified™ Histone Peptide Array, Active Motif). First, arrays were blocked with 10ml Blocking Buffer [10mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% Tween-20, 5% powdered milk] for 1 hour on orbital shaker. Arrays were incubated with GST fusion protein (120nM) or anti-H3K9me3 (Millipore 07-442, 1 in 1000 dilution) in 10ml Array Binding Buffer [10mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% Tween-20, 10μM ZnCl$_2$] for one hour at room temperature with gentle rotation. Arrays were washed three times in Array Binding Buffer for 5 minutes. Arrays bound by GST fusions were then incubated one hour with anti-GST (1 in 5000 dilution, GE Healthcare) in 10 ml Array Binding Buffer (GE Healthcare) and again washed. Binding was then visualized by incubating with the appropriate secondary antibody (1 in 5000 dilution) conjugated to HRP in 10ml Array Binding Buffer followed by chemiluminescence detection reagent (Pierce) and imaging using a Gel Doc™ (Bio-Rad) camera.

Biotinylated DNA Pull-down

Pulldowns were performed by incubating 3μg GST or GST fusion protein with 20pmols biotin or 601 NPS generated by PCR with biotinylated or unlabeled primers in 0.5ml Pull-down Buffer [20mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40, 1mM DTT] for 2.5 hours at 4°C with rotation. Streptavidin Sepharose (GE Healthcare) beads were added and samples were incubated for an additional hour before washing three times with 1ml Pull-down Buffer. Associated proteins were then analyzed by SDS-PAGE and Western blotting with an antibody against GST (GE Healthcare 27-4577-01).

Electrophoretic Mobility Shift Assay

Indicated amounts of GST or GST fusion proteins were incubated with a radiolabelled PCR product (50ng) or an equivalent amount of DNA following nucleosome reconstitution in 20μl EMSA Binding Buffer [10mM Tris-HCl pH 7.5, 50mM NaCl, 5mM MgCl$_2$, 0.5mM DTT, 50ng/μl BSA, 5% glycerol] and incubated for 30 minutes at 4°C. Samples were then loaded onto a pre-run (200V for 30 minutes) 20mm, 0.5X TBE, 4.5% polyacrylamide gel and electrophoresed at 200V for 4 hours at 4°C in 0.5X TBE buffer. Gels were dried and exposed to a phosphor screen and analyzed (Molecular Dynamics STORM 860).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (149). Exponentially growing fission yeast (45ml at 6.6 x 10^6 cells/ml) were fixed by the addition of 5ml 10% (ChIP of histones and histone modifications) or 30% (ChIP of non-

histone proteins) paraformaldehyde and incubated for 18 minutes at room temperature with gentle rotation. Fixation was stopped by the addition of 5ml 2.5M glycine and incubating for 5 minutes with gentle rotation. Cell pellets were washed three times with 25ml ice cold PBS and centrifugation at 3,000 RPM for three minutes in a table top centrifuge. Samples were again suspended in PBS and transferred to a 2ml beadbeating tube before centrifugation at 8,000 RPM for 3 minutes. After removal of PBS, cell pellets were frozen on dry ice.

Fixed cells were lysed by the addition of 400µl ChiP Lysis Buffer [50mM HEPES-NaOH pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% deoxycholate (DOC)] and beadbeated for 90 seconds two times with 5 minutes on ice in between each round. Lysates were recovered by "piggyback" centrifugation into a 1.5ml microcentrifuge tube. Cells were further lysed and chromatin was sheared by probe sonication for three rounds of 20 seconds on maximum power. Lysates were cleared of debris by centrifugation at maximum speed in a refrigerated benchtop centrifuge. To limit non-specific binding, lysates were pre-cleared by incubation with 50µl washed Protein A Sepharose beads (GE Healthcare) for one hour at 4°C with rotation. Beads were removed by centrifugation for 1 minute at 8,000 RPM in a refrigerated benchtop centrifuge.

Half of each lysate was used for ChIP by incubation with the appropriate amount of antibody overnight at 4°C with rotation. The following morning, 25µl washed Protein A beads were added to the lysates and incubated for another hour at 4°C with rotation. Proteins bound to the beads were washed twice with 1ml ChIP Lysis Buffer, once with 1ml 0.5M Lysis Buffer [50mM HEPES-NaOH pH 7.5, 0.5M NaCl, 1mM EDTA, 1% Triton X-100, 0.1% DOC], once with 1ml ChIP Wash Buffer [10mM Tris-HCl pH 8.0, 250mM LiCl, 1mM EDTA, 0.5% Igepal CA-630, 0.5% DOC], and once with 1ml TE for 5 minutes each at room temperature with rotation.

Proteins on the washed beads were digested and decrosslinked first by addition of 250µl TE with additional 0.5% SDS and incubated overnight at 65°C. Proteinase K was added to a concentration 0.8mg/ml along with TE to 500µl total volume and samples were incubated for 1 hour at 37°C. DNA was then cleaned up using the Wizard DNA Clean-Up System (Promega). Samples were then analyzed by quantitative real-time PCR.

Antibodies used were anti-Histone H3 C-terminus (Abcam ab1791), anti-RNA Pol II (Covance MMS-126R), anti-H3K9me2 (Abcam ab1220), anti-Chp1 (Abcam ab18191), anti-Swi6 (Thermo Scientific PA1-4977), and anti-GFP (Invitrogen A11122). Primers used for real-time PCR analysis are listed in the Appendix.

Co-immunoprecipitation of Mit1-3XV5 and 6XFLAG-Chp2

Extracts from 1×10^8 exponentially growing fission yeast cells were prepared by addition of 0.5ml Co-IP Lysis Buffer [10mM HEPES-NaOH pH 7.5, 150mM NaCl, 0.1% NP-40, 1mM EDTA, 1mM EGTA, 20% glycerol, 1mM PMSF, and a protease inhibitor cocktail (Roche)] and grinding with a mortar and pestle while frozen using liquid

nitrogen. Extracts were subject to ultracentrifugation to remove debris before incubation with 50µl anti-V5 affinity gel (Sigma) for two hours. Beads were washed three times with 1ml Co-IP Lysis Buffer and bound proteins were eluted with 20µl 0.4mg/ml 3XV5 peptide in Co-IP lysis buffer, four times for 30 minutes each. Co-immunoprecipitated proteins were then analyzed by Western blot with antibodies against V5 (Serotec) and FLAG (Sigma).

Chromosome Segregation Analysis

Analysis of chromosome segregation defects by immunofluorescence miscroscopy was performed as previously described (150). Cultures of 25ml yeast grown to exponential growth phase in YES media at 25°C were fixed for 30 minutes with a final concentration of 3.8% paraformaldehyde. Cells were spin at 3,000RPM for 5 minutes in a table top centrifuge before being washed twice with 15ml and once with 1ml ice cold PEM Buffer [100mM Pipes pH 6.9, 1mM EDTA, 1mM $MgSO_4$, pH to 6.9 with NaOH]. Cell walls were then disrupted by incubation in 1mL PEM buffer with additional 1mg/ml zymolyase and 1.2M sorbitol for 90 minutes at 36°C with rotation. Cells were spun down gently in a benchtop centrifuge before being resuspended in 200µl PEM buffer with supplemented with 1.2M sorbitol and 1% Trition-X100 for 5 minutes at room temperature. Cells were then washed three times with 1ml PEM buffer and then incubated for 30 minutes with rotation in PEM buffer supplemented with 1% bovine serum albumin, 0.1% sodium azide, and 100mM lysine (PEMBAL). Resuspended cells (100µl) were then incubated overnight at 4°C with 1µl anti-tubulin antibody (Keith Gull).

The next day cells were washed three times with 1ml PEMBAL before incubation in 100µl PEMBAL with 1µl conjugated FITC secondary antibody four hours at room temperature with rotation. Cells were then washed once with 1ml PEMBAL and twice with 1ml PBS. To visualize DNA, cells were resuspended in 30µl PBS containing 8µg/ml 4′,6′-Diamidino-2-phenylindole (DAPI). Samples were then cytospun onto polylysine-coated slides and visualized with a Zeiss Axioskop II microscope with appropriate color filters. Cells were scored as being in anaphase based on cell shape and visualization of microtubules and DNA (DAPI). Chromsome missegregation was scored by the visualization of lagging chromosomes during cell division.

Artificial Minichromosome Loss Assay

Maintenance of artificial minichromosomes was monitored as previously described (72). Cells were maintained on PMG media lacking leucine to facilitate retention of a linear minichromosome and suspended in PMG media with no additives at a concentration of 7.5 x 10^3 cells/ml before spreading 100µl of onto non selective YES agar plates containing approximately 12% of required adenine. The amount of added adenine was subject to batch variation the amount of adenine contained in the yeast extract used to make YES media. For these experiments, YES media was supplemented with only 1µg/L of adenine. Plates were incubated for 5 days at 32°C and then allowed to

"develop" color another 3 days at 4°C. Loss of the minichromosome is monitored by deficiency of an adenine biosynthesis pathway gene provided by the minichromosome which results in red colored cells. Approximately one thousand colonies for each genotypes were scored for color for each experiment. Colonies that were half or one-fourth red were scored as having lost the minichromosome.

Fluorescence Microscopy

Cells were fixed as described in chromosome segregation analysis. Samples were then cytospun onto polylysine-coated slides and visualized with a Zeiss Axioskop II microscope with appropriate color filter. Images were taken using a Coolsnap HQ camera (Photometrics).

In Vivo Nucleosome Scanning Assay

Mononucleosomal DNA was prepared as previously described (151) with some modification. Log-phase cultures of *S. pombe* (2.5×10^8 cells) grown in 50ml YES media were fixed for 20 minutes in 0.5% paraformaldehyde. Fixation was stopped by the addition of glycine to 125mM and pellets were washed three times with 25ml ice cold PBS. Cell pellets were resuspended in 2ml Pre-incubation Buffer [20mM citric acid, 20mM Na_2HPO_4, 40mM EDTA, 30mM β-mercaptoethanol] and incubated for 10 minutes at 30°C. Cells were permeabilized by the addition of 1ml Sorbitol/Tris Buffer [1M sorbitol, 10mM β-mercaptoethanol, 50mM Tris-HCl pH 7.5] with zymolyase (MP Biomedicals) added to 1mg/ml and incubation at 30°C for 30min with shaking. After washing twice with 1ml Sorbitol/Tris buffer lacking zymolyase, spheroplasts were digested with micrococcal nuclease (Worthington Biochemicals) at 256U/ml in 0.5ml NP-40 Buffer [1M sorbitol, 50mM NaCl, 5mM $MgCl_2$, 1mM $CaCl_2$, 0.75% NP-40, 0.5mM spermidine, 10mM Tris-HCl pH 7.5] for 25 minutes at 36°C. Reactions were stopped by the addition of 65μl Stop Buffer [5% SDS, 100mM EDTA]. RNA was removed from the samples by incubation with RNase A for 90 minutes at 37°C. Crosslinking was reversed and proteins removed by incubation overnight at 65°C in the presence of 1mg/ml Proteinase K. After phenol-chloroform extraction, DNA was ethanol precipitated and mononucleosomal DNA was isolated by gel extraction of a band corresponding to approximately 150 base pairs of DNA as visualized by addition of ethidium bromide prior to 1.2% agarose gel electrophoresis. TE buffer (0.5ml) was added to the isolated gel fragments which were then crushed and subjected to three freeze-thaw cycles on dry ice and incubation in a 50°C water bath for 20 minutes with occasional vortexing. Samples were phenol-chloroform extracted and ethanol precipitated.

Isolated mononucleosomal and input genomic DNA prepared without the addition of micrococcal nuclease and without agarose gel isolation were subjected to quantitative PCR and analyzed using primers listed in the Appendix before being normalized to amplification by a primer set in the open reading frame of *adh1*[+].

In Vitro Chromatin Reconstitution and Nucleosome Mapping

Nucleosomes were assembled on a 2Kbp PCR product including the mating type region *REII* element by salt step down procedure beginning with 10µg DNA, 7.5µg histone octamer (NEB), and 2M NaCl in a 10µl reaction. Reconstitutions (5 µg DNA equivalent) were then incubated in 0.24 ml MNase Digestion Buffer [10mM Tris-HCl pH 7.5, 150mM NaCl, 2.5mM MgCl$_2$, 2.5mM CaCl$_2$] with or without 100U/ml MNase for 10 minutes at 30°C. Reactions were stopped by the addition of SDS to 0.5% and EDTA to 25mM. Proteinase K was added and samples were incubated at 37°C overnight. Following phenol-chloroform extraction, mononucleosomal DNA from MNase digested samples was isolated by 1.2% agarose gel electrophoresis and analyzed by quantitative PCR relative to undigested input DNA.

In Silico Prediction of Nucleosome Occupancy

Sequences were analyzed by the NuPoP Nucleosome Positioning Prediction Engine (4th order) algorithm located at (http://nucleosome.stats.northwestern.edu) and the Online Nucleosomes Position Prediction by Genomic Sequence Version 3.0 algorithm located at (http://genie.weizmann.ac.il/software/nucleo_prediction.html).

Transcript Analysis by High-resolution Tiling Array and RT-PCR

RNA was prepared using a hot phenol extraction method (152). Fission yeast grown to 2 x 10^6 cells/ml in 25 ml YES media were resuspended in 0.5 ml TES Buffer [50mM Tris-HCl pH 7.5, 10mM EDTA, 100mM NaCl, 0.5% SDS made in DEPC H$_2$O] followed by addition of an equal volume phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.7) and incubated one hour at 65°C with vortexing for 10 seconds every 15 minutes. The aqueous phase was isolated after centrifugation at 13,000 RPM for 5 minutes and samples were again phenol-chloroform extracted and ethanol precipitated. Dry RNA pellets were suspended in 50µl DEPC treated H$_2$O. Trace amounts of DNA were removed by incubating 100µg RNA as analyzed by a spectrophotometer with 1.5µl TURBO™ DNase (Ambion) in 100µl reactions. DNasing was performed once at 37°C and 1.5 DNase was again added and incubated another 30 minutes prior to removal of the DNAse with 25µl inactivation beads as recommended by the supplier. RNA was further cleaned up using an RNeasy® Mini Kit (Sigma), eluting twice with the sample 50µl volume of DEPC H$_2$O.

For high-resolution mapping of transcriptional changes, reverse transcribed cDNA was hybridized to the Affymetrix *S. pombe* Tiling 1.0FR array (20 base pair resolution) and mapped to the *S. pombe* genome (2004 version), which was visualized using Integrated Genome Browser (IGB). Microarray data can be accessed at the NCBI Gene Expression Omnibus, accession number GSE46649.

Quantitative PCR analysis of transcripts was performed on reverse transcribed cDNA using Quantifast SYBR green (Qiagen) and an Eppendorf Mastercycler machine. Primers used for analysis are listed in the Appendix.

Northern Analysis of siRNA

Analysis of siRNA was performed as previously described (153). Exponentially growing fission yeast (50ml at 8 x 10^6 cells/ml) were cultured in YES media in a shaking incubator at 25°C. Cell pellets were washed with 1ml TE buffer before being resuspended in 0.6ml siRNA Extraction Buffer [50mM Tris-HCl pH 7.5, 10mM EDTA, 100mM NaCl, 1% SDS made in DEPC treated H_2O]. An equal volume of phenol-chloroform (pH 4.7) was added and cells were lysed by beat beating for four minutes. After centrifugation the aqueous phase was again phenol-chloroform extracted. RNA in the aqueous phase was then ethanol precipitated by adding three volumes ice cold ethanol and immediately spinning at 13,000 RPM in a refrigerated benchtop centrifuge. The resulting pellet was air dried before being resuspended in 0.4ml DEPC treated H_2O. After the addition of 50µl 5M NaCl and 100µl 40% polyethylene glycol (average molecular weight of 8,000), samples were incubated for 30 minutes on ice before centrifugation at 13,000 RPM for 20 minutes at 4°C. Small RNA obtained in the supernatant were then precipitated by the addition of one-tenth volume 3M sodium acetate (pH 5.6) and three volumes ice cold ethanol and incubation overnight at -20°C. The following day samples were subject to centrifugation for 30 minutes at 13,000 RPM and 4°C. Pellets were washed once with 0.5ml 95% ethanol before being allowed to air dry and resuspended in 30µl 50% formamide. An equal volume of 2X Small RNA Sample Buffer [10mM EDTA, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue prepared in deionized formamide] was added.

Samples were electrophoresed at 300V through a pre-run 20mm, 8% polyacrylamide gel (National Diagnostics) containing 1X TBE and 0.75M urea. Gels were then soaked for 10 minutes sodium phosphate buffer (pH 7.0) followed by 20X saline-sodium citrate (SSC) buffer for 10min. RNA was then blotted by transfer to a Hybond-NX (GE Healthcare) neutral nylon membrane by flow of 20X SSC up through the gel using paper towels as a wick. RNA was then UV-crosslinked (approximately 1200mJ) to the membrane.

Northern blots we performed by incubating 25ng denatured radiolabelled PCR products in 10ml PerfectHyb hybridization buffer (Sigma) overnight at 42°C. Blots were then washed twice with buffer containing 2X SSC and 0.2% SDS at 42°C. The membrane was then exposed to a phosphor screen and imaged (Molecular Dynamics STORM 860).

CHAPTER 3.   RESULTS

Mit1 Is Required for Efficient Silencing at Regions of Heterochromatin

Mi-2 remodelers play important roles in control of euchromatic gene expression (154,155). In contrast, Mit1 is primarily known as a heterochromatin silencing factor but several reports implicate Mit1 in euchromatic control. Like other components of SHREC, Mit1 is primarily localized to regions of heterochromatin, but it is also enriched at some euchromatic loci (156). Mit1 has also been reported to have genome-wide roles in nucleosome positioning, and Mit1 loss resulted in expression changes of over 200 genes (157). Although the genome-wide changes in nucleosome positioning previously linked to Mit1 activity have recently been refuted and attributed to other CHD remodelers (158), it remained possible that Mit1, like Mi-2 remodelers, could influence gene expression.

To address whether Mit1 regulates gene expression, we analyzed global transcription changes caused by $mit1^+$ deletion using a genome-wide high resolution tiling array (**Figure 3-1A** and **3-1B**). We performed analysis using duplicate biological replicates of cDNA prepared from WT and $mit1\Delta$ cells, and specified that hits show at least a 2-fold difference in expression between $mit1\Delta$ and WT with significance $p<0.05$ between the replicates. Using these parameters, we observed elevated levels of transcripts in regions normally suppressed by constitutive heterochromatin including subtelomeres and centromeres in $mit1\Delta$ and relatively few changes in coding regions. Quantitative real time PCR (Q-PCR) confirmed elevation in transcripts from heterochromatic loci in $mit1\Delta$ (**Figure 3-1C**) (159). Q-PCR analysis was also performed on the nine euchromatic sites on chromosome 1 that our array analysis identified as differentially regulated (sites A-I). Most of these sites are in intergenic rather than coding regions and transcript levels were either very low or undetectable (**Figure 3-1D** and **Table 3-1**), although one transcript showed 2-fold down-regulation in $mit1\Delta$. We also used Q-PCR to reanalyze transcripts from several genes previously identified as strongly regulated by Mit1 (157). We found that these genes were similarly unaffected by $mit1\Delta$ (**Figure 3-1D**). We conclude that Mit1 is primarily involved in regulating transcription at regions of heterochromatin and does not have a widespread or significant role in the regulation of steady-state gene expression within euchromatin.

It is presently unclear how the SHREC complex acts to silence heterochromatic transcripts. SHREC has been proposed to act downstream of H3K9 methylation in silencing, but SHREC components have also been shown to be important for heterochromatin stability and spreading (78,156,160-164). ChIP experiments reveal that histones in heterochromatic regions retain methylation at Lys9 on histone H3 in $mit1\Delta$ cells and that Swi6 is not delocalized from heterochromatin foci (**Figure 3-2A** and **3-2B**) (156,159,165). Deletion of $mit1^+$ does not dramatically disrupt the role of heterochromatin to retain fidelity of chromosome segregation, as we do not observe the lagging chromosome defect seen on deletion of heterochromatin factors in cells lacking Mit1 (**Table 3-2**). Cells harboring $mit1\Delta$ do not efficiently maintain artificial

Figure 3-1.   Mit1 regulates transcription at regions of heterochromatin.
(A) Genome wide analysis of transcriptional changes in *mit1Δ* cells. Genomic regions that have a 2-fold or greater change in expression in *mit1Δ* cells compared to wild type were identified. Transcript changes were identified by comparing the hybridization of cDNA from both strain backgrounds (n=2) to a high-resolution tiling array and filtering for regions that differed with greater than 100 base pair runs and less than 150 base pair gaps (p<.05).
(B) Mit1 promotes silencing at subtelomeric regions. Comparison of hybridization signals at the left telomere of chromosome 1 on a high resolution microarray of cDNA prepared from *mit1Δ* and wild type cells.
(C) Confirmation of Mit1's role in heterochromatic silencing by Q-PCR. Q-PCR analysis of elevated transcripts in *mit1Δ* and *clr4Δ* cells relative to wild type at regions of heterochromatin normalized to a euchromatic control gene, *adh1$^+$*.
(D) Impact of *mit1Δ* on euchromatic gene expression. Quantitative PCR analysis of transcriptional changes in *mit1Δ* relative to wild type strains for regions of change identified by tiling array. Also included are Q-PCR analyses of transcripts for four genes whose expression has previously been identified as being regulated by *mit1$^+$* (*mcp4$^+$*, *slx1$^+$*, *coq10$^+$*, and *SPAC18g6.09c*).

A

5.6Mb   4.6Mb   2.5Mb

mit1

Chromosome 1   **Chromosome 2**   **Chromosome 3**

A   BC   D   E F   G   H   I

>2 fold Down
p<0.05

>2 fold Up
p<0.05

1   2   3   4   5   1   2   3   4   1   2

B

Left Telomere 1

Signal *mit1Δ*/WT

0   5   10   15   20 Kb

C

Transcripts Relative to *adh1+*/ WT ($\log_2$)

■ *clr4Δ*
▪ *mit1Δ*

cen dg   cen dh   tel1   tlh1   MTR cenH

D

Transcripts Relative to *adh1+*/ WT ($\log_2$)

undetectable   undetectable   undetectable   undetectable   undetectable   undetectable

A   B   C   D   E   F   G   H   I   *mcp4+*   *slx1+*   *coq10+*   *SPAC18g6.09c*

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Table 3-1.   **Changes in transcripts identified by tiling array in *mit1*Δ cells.**

Site	Region of change		Change in *mit1Δ**
	Start	**End**	
A	75,234	75,525	UP
B	893,234	893,387	DOWN
C	1,047,607	1,047,795	DOWN
D	1,642,326	1,642,508	UP
E	2,662,194	2,662,362	UP
F	2,934,006	2,939,830	DOWN
G	3,227,318	3,227,488	UP
H	3,813,267	3,813,448	DOWN
I	4,619,879	4,620,086	DOWN

*as identified by high-resolution tiling array, see **Figure 3-1A**
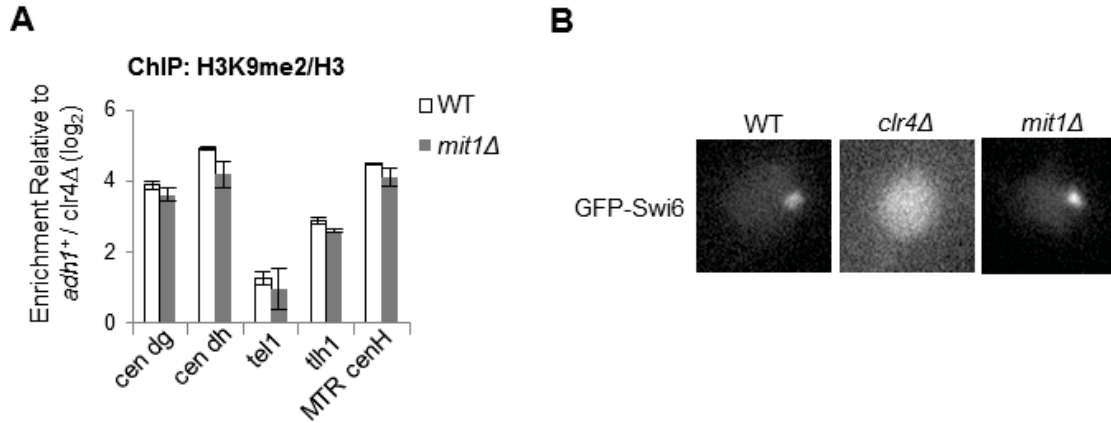
Figure 3-2.   Analysis of heterochromatin by ChIP.
(A) ChIP experiment using an antibody against H3K9me2 normalized to ChIP signal obtained using an antibody against the C-terminus of histone H3 assaying for changes in enrichment at various regions of heterochromatin relative to wild type and a control locus, $adh1^+$. Data are plotted on a $\log_2$ scale and represent the mean of duplicate experiments, with error bars reflecting the SEM (n=2).
(B) Fluorescence microscopy of cells expressing GFP tagged Swi6. Note that in $clr4\Delta$ cells Swi6 is dispersed throughout the nucleus.

Table 3-2.   **Chromosome segregation analysis in *mit1Δ* cells.**

Genotype	# Anaphase cells screened	# Cells with lagging chromosomes
wild type	100	0
mit1Δ	100	0
clr4Δ	98	15

minichromosomes (**Figure 3-3**), suggesting that while deletion of *mit1⁺* is not sufficient to cause overt defects in chromosome segregation, rare defects are possible.

Consistent with our transcript analyses, RNA Pol II is enriched at sites of heterochromatin in *mit1Δ* cells (**Figure 3-4A**) (159). Interestingly, the levels of RNA Pol II recruitment are similar between *mit1Δ* and *clr4Δ* cells (which completely lack heterochromatin), even though the accumulation of heterochromatic transcripts in *mit1Δ* cells is reduced compared with *clr4Δ*. These data suggest that silencing pathways, such as co-transcriptional silencing by RNAi, are functional in *mit1Δ* cells, and act to reduce steady state levels of heterochromatic transcripts (166-168). Consistent with this, RITS appears to be localized normally in cells lacking *mit1⁺* (**Figure 3-4B**) and centromeric siRNAs are present at elevated levels in cells lacking *mit1⁺* (159). These observations support a model where Mit1/SHREC acts to silence transcription at the level of regulating RNA Pol II accessibility, while other silencing mechanisms that depend on H3K9 methylation remain intact.

Mit1 Requires its ATPase Activity and Conserved Chromatin Tethering Domains to Silence Transcription

The behavior of ATP-dependent chromatin remodelers can be influenced greatly by proteins that associate with the remodeler as well as by the sequences that surround the highly conserved catalytic core (169). In addition to a Snf2-like ATPase domain, Mit1 has a zinc-finger-like plant homeodomain (PHD) (156). Through further sequence analysis, we also identified a loosely conserved (Pfam E-value 0.72), but tryptophan-containing chromodomain (CD) (**Figure 3-5A**). Both PHD and CD domains are well recognized as chromatin interaction motifs (170,171) and are commonly found in chromatin modifying enzymes including Mi-2 family remodelers (172).

We interrogated whether these domains are required for the silencing function of Mit1, which is required to repress the transcription of a centromeric reporter gene (*otr1R::ura4⁺*) (156). We asked whether cells episomally expressing Mit1 mutated in its ATPase domain (Mit1^{K587A})*,* or deleted for either the PHD finger (Mit1^{ΔPHD}) or the chromodomain (Mit1^{ΔCD}), could complement the centromeric silencing defect of *mit1Δ* cells. Expression of wild type Mit1 efficiently silenced the centromeric *ura4⁺* reporter gene in *mit1Δ* cells, allowing cells to grow on media containing 5-FOA, which is toxic to cells expressing *ura4⁺* (**Figure 3-5B**). Mit1^{K587A} expressing cells were unable to silence this reporter (156). We found that cells expressing Mit1^{ΔPHD} or Mit1^{ΔCD} were also unable to silence the centromeric reporter, suggesting that these putative chromatin interaction motifs are important for heterochromatic silencing by Mit1.

The Chromodomain of Mit1 Binds DNA

The Mi-2 subfamily of CHD remodelers found in higher organisms are known to utilize their tandem PHD and chromo domains for histone tail and DNA binding
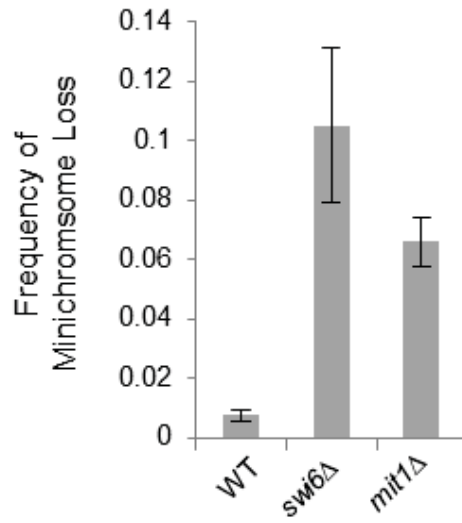
Figure 3-3.   Cells lacking Mit1 do not efficiently retain minichromsoomes.
Rate of loss of minichromosomes during cell division. See materials and methods for
methodology. Error bars represent SEM (n=2).
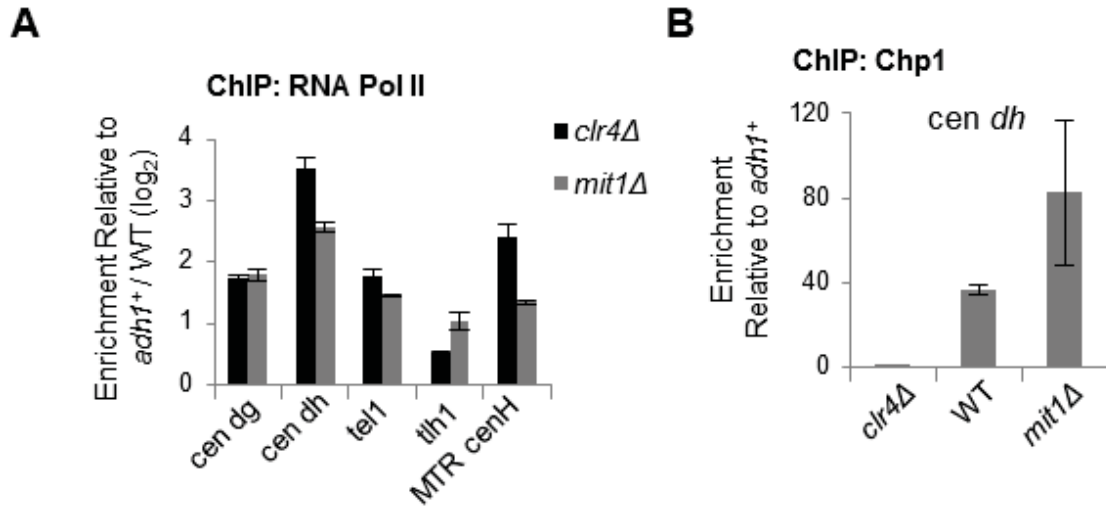
Figure 3-4.   Analysis of Pol II and Chp1 enrichment at regions of heterochromatin.

(A) ChIP experiment using an antibody against RNA Pol II for changes in enrichment at various regions of heterochromatin relative to wild type and a control locus, *adh1$^+$*. Data are plotted on a log$_2$ scale and represent the mean of duplicate experiments, with error bars reflecting the SEM (n=2).

(B) ChIP experiment using an antibody against Chp1comparing enrichment at cen *dh* relative to a control locus, *adh1$^+$*. Data represent the mean of duplicate experiments, with error bars reflecting the SEM (n=2).
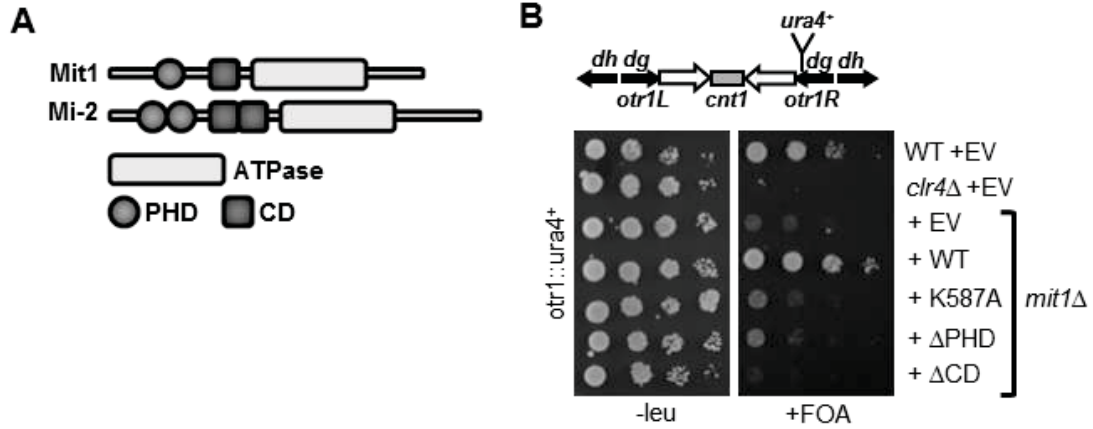
Figure 3-5.   Mit1 requires conserved domains and activities for centromeric silencing.
(A) Mit1 has conserved PHD and CD domains. Schematic representation of SpMit1 and Mi-2 conserved motifs including the plant homeodomain (PHD), chromodomain (CD), and SWI/SNF-like ATPase domains.
(B) The PHD and CD domains contribute to silencing activity of Mit1. Serial dilution spotting assay on media lacking leucine (to maintain expression plasmids) with or without 5'-fluoro-orotic acid (FOA) of strains containing a centromeric *ura4⁺* reporter gene (otr1::*ura4⁺*). Plates were incubated at 32°C. EV is empty vector.

respectively (173,174). Although Mit1 harbors only a single PHD finger and a loosely conserved chromodomain, we wondered whether these motifs might serve similar roles.

To test this hypothesis we monitored the ability of Mit1's CD to associate with DNA. First, we tested binding of a recombinant GST fusion protein (**Figure 3-6A**) of Mit1's CD domain to DNA in solution. GST-CD, but not GST alone, bound to biotin-labeled dsDNA and was captured by streptavidin agarose (**Figure 3-6B**). GST-CD did not bind to biotin alone nor was the apparent pull-down the result of insoluble aggregation by GST-CD/DNA complexes, since no complex associated with streptavidin beads in the absence of biotin on the DNA.

To determine whether the chromodomain could bind nucleosomal DNA, we used electrophoretic mobility shift assays (**Figure 3-6C**). GST-CD (lanes 4 and 5) specifically caused a slower migrating shift of a 147 base pair sequence (0N0) radiolabeled dsDNA following native PAGE relative to DNA incubated with GST (lanes 2 and 3) or to the probe alone (lane 1), indicative of DNA binding activity. Nucleosomes were reconstituted onto 0N0 as mononucleosomes such that they lacked free DNA ends, and were tested for association with Mit1 CD. Shifts were seen on incubation with nucleosomal DNA (compare lanes 9 and 10 with lanes 6, 7 and 8), suggesting that the association of Mit1's chromodomain with DNA is not significantly altered by the histone octamer. Unlabeled competitor DNA greatly diminished the abundance of the slower migrating DNA smear, indicative of association rather than non-specific retention in the wells (**Figure 3-6D**). Additionally, no DNA binding was observed when the GST-PHD fusion was tested for association with DNA in EMSA (**Figure 3-6D**).

The PHD Domain of Mit1 Binds Histone H3

To test whether Mit1's PHD domain associates with histones we assayed the ability of GST-PHD fusion proteins to bind calf thymus histones. As previously demonstrated for the ING2 PHD domain (175), we found that GST-PHD (Mit1) binds histone H3 in preference to other histones (**Figure 3-7**). Several studies of Mi-2 PHD fingers have shown sensitivity of H3 tail interactions to different methyl marks (176-178). We interrogated the modification status of histone H3 bound by GST-PHD (Mit1), and showed that it was enriched for methyl marks of transcriptional activity: H3K4me3 and H3K36me3, but curiously lacked the heterochromatic hallmark, H3K9me2. This indicates that binding of Mit1 PHD to histone H3 may be inhibited by the H3K9me2 modification, which was a surprising result, given that the SHREC complex is recruited to regions of heterochromatin (156). Attempts to further dissect GST-PHD binding specificity were hampered since we were unable to demonstrate specific binding to a modified histone tail peptide array or to peptides in solution (**Figure 3-8**). We speculate that Mit1's PHD domain in isolation may have low affinity for histone H3 or requires a more extensive interaction interface with histone H3 than peptides provide.

We tested whether loss of Mit1 or Mit1's chromatin association domains specifically impaired Mit1's association with SHREC. While it remains a possibility that
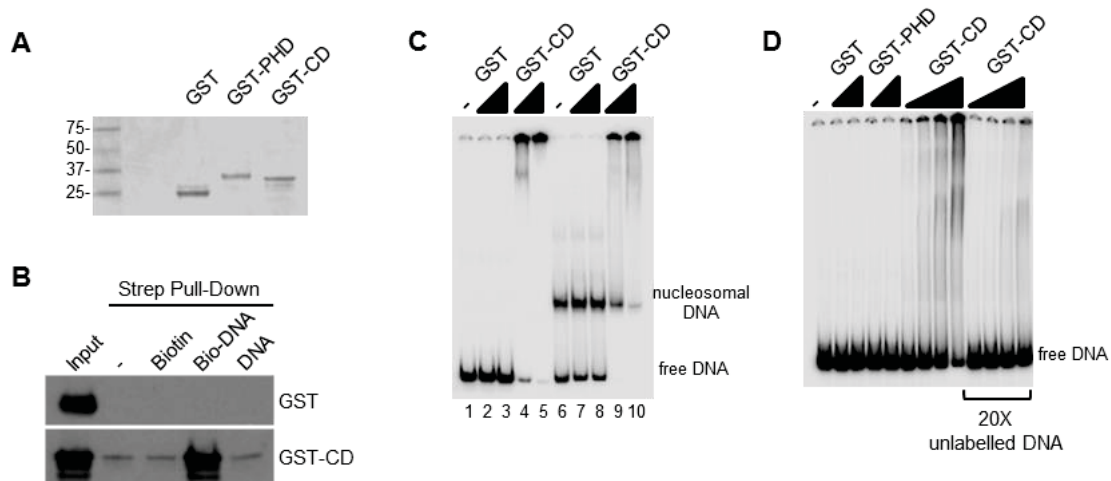
Figure 3-6.   Mit1's chromodomain binds DNA *in vitro.*
(A) Purification of recombinant GST fusions of domains of Mit1. SDS-PAGE and coomassie staining of purified recombinant GST, GST-PHD[Mit1], and GST-CD[Mit1] fusion proteins.
(B) Mit1's CD domain can bind DNA in vitro. Biotinylated double stranded DNA or an equivalent amount of biotin or unlabeled DNA was incubated with recombinant GST or GST fused to Mit1 chromodomain. The DNA and associated proteins were captured by streptavidin beads and analyzed by Western blot.
(C) Mit1 CD can bind nucleosomal DNA. EMSA to compare the binding of Mit1 chromodomain to free DNA and nucleosomal DNA. Radiolabelled 147 base pair Widom 601 nucleosome positioning sequence (50ng, lanes 1-5) or mononucleosomes without free DNA ends reconstituted by salt dilution on the same sequence (50ng DNA equivalent, lanes 6-10) were incubated with GST (2μg, 4μg) or GST-CD[Mit1] (2μg, 4μg) fusion protein.
(D) Competition EMSA with cold DNA. EMSA demonstrates that Mit1's CD but not PHD domain interacts with DNA. Radiolabelled 217 base pair PCR product (50ng, 70N0) was incubated with buffer, GST (2μg, 4μg), GST-PHD (2μg, 4μg), GST-CD (0.5μg, 1μg, 2μg, 4μg), or GST-CD co-incubated with 1μg of unlabelled 70N0 PCR product.
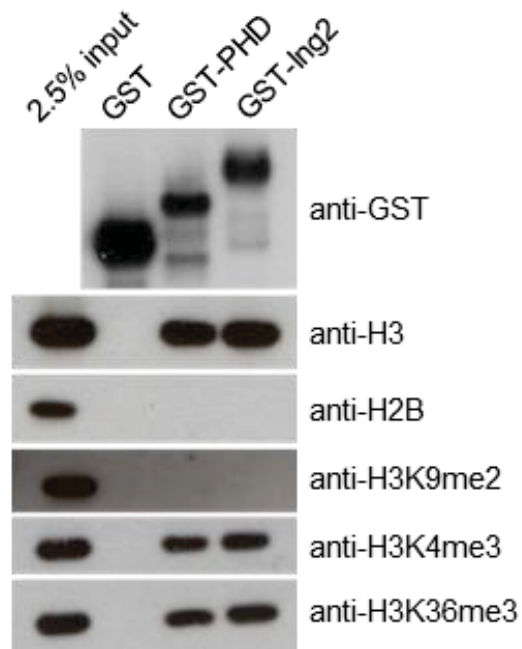
Figure 3-7.   Mit1's PHD domain binds histone H3 *in vitro*.
Calf thymus histone pull-down experiment comparing the ability of GST, GST-PHD[Mit1] and GST-PHD[Ing2] proteins to bind to histones. Histone association was monitored by immunoblotting using antibodies specific for different histones or histone modifications.

Figure 3-8.   Test for Mit1 PHD finger binding specificity.
(A) Modified histone peptide microarray probed with GST-PHD^{Ing2}. Binding was visualized by probing with anti-GST antibody and secondary antibody conjugated to HRP. Location of the H3K4me3 peptide is annotated. The majority of other strong signals are H3K4 methylated peptides in combination with other modifications.
(B) Modified histone peptide microarray incubated with Mit1 PHD domain fused to GST and processed as in (A). No preferential binding was observed.
(C) Modified histone peptide microarray incubated with anti-H3K9me3 and secondary antibody conjugated to HRP. Location of the H3K9me3 peptide is annotated.

A

GST-PHD^{ING2}   H3K4me3
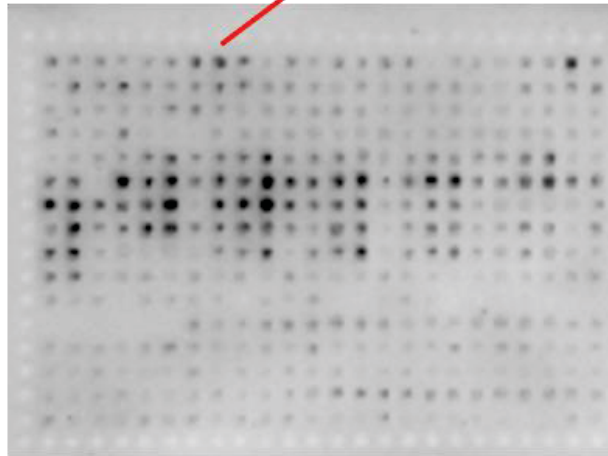
B

GST-PHD^{Mit1}

C

anti-H3K9me3   H3K9me3
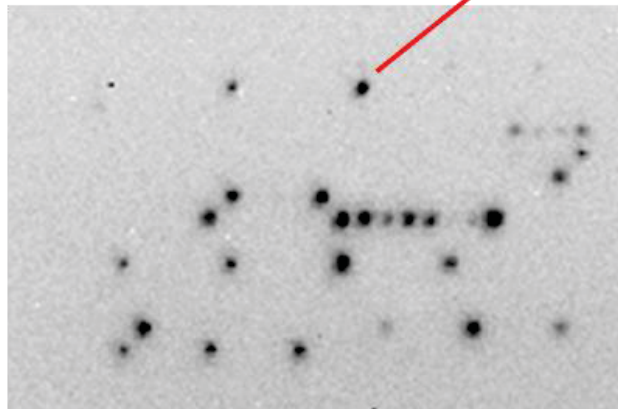
these deletions may impact additional Mit1 interactions, Mit1$^{\Delta PHD}$ and Mit1$^{\Delta CD}$ maintain association with Chp2 (**Figure 3-9A**), indicating that Chp2's ability to bind Mit1 is retained in these mutants. SHREC can presumably still be targeted to heterochromatin via Chp2's recognition of H3K9 methylation, although we have been unable to directly ChIP Mit1 at these regions. Interestingly, we found that Chp2 association with centromeric repeats is partially dependent on Mit1 and specifically on its PHD and CD domains (**Figure 3-9B**). Furthermore, cells expressing Mit1 with the catalytic K587A mutation show no defect in Chp2 association with cen *dh*, suggesting that Mit1 domains which interact with elements of chromatin *in vitro* contribute to the association of Chp2 with chromatin in the cell.

Mit1 Is an ATP-dependent Nucleosome Remodeling Factor

Mit1 has a highly conserved Snf2-related ATPase domain which suggests Mit1 may modulate the interaction between DNA and histones to reposition nucleosomes on DNA (179). However, to date there has been no demonstration that Mit1 is a bona fide chromatin remodeler. To determine if Mit1 has chromatin remodeling activity, we first purified Mit1 from fission yeast. We found that Mit1 is expressed at low levels and is labile under standard purification conditions. To purify sufficient Mit1 we employed a double epitope tagging strategy incorporating different tags at either end of the protein and overexpressed Mit1 from a full strength *nmt1$^+$* promoter. Sequential affinity purification allowed isolation of full length Mit1 (**Figure 3-10**). Mass spectrometry analysis did not identify co-purification of other subunits of SHREC under these conditions (**Table 3-3**), likely due to the considerable overexpression and dual tagging strategy that was employed to minimize purification of Mit1 breakdown products.

To determine whether Mit1 has *in vitro* chromatin remodeling activity, we performed a series of mononucleosome sliding assays which take advantage of the difference in mobility between nucleosomes positioned at the ends and center of short DNA fragments when resolved in native polyacrylamide gels (180). First, we assembled mononucleosomes by salt dialysis onto a 217 base pair DNA fragment upon which deposition of the octamer is directed to the DNA end by the 601 nucleosome positioning sequence (181) (70N0, **Figure 3-11A**). We then incubated the positioned nucleosomes with purified Mit1, or ScISW2 as a positive control, in the presence or absence of ATP. ISW2 is known to mobilize histone octamers from the end to center of short DNA fragments (182). Similar to ScISW2, Mit1 caused an ATP-dependent mobilization of the nucleosome (**Figure 3-11B**). Remodeling by Mit1 was dose-dependent (**Figure 3-12A**), as titration of Mit1 generated a species that migrated between bands observed on incubation with no ATP (lane 5) or with saturating Mit1 and ATP (lane 4), which is likely to be a position intermediate (lane 3). We note that there was no evidence of octamer eviction, as we observed no increase in free DNA caused by Mit1 remodeling. We further tested whether Mit1 could facilitate histone octamer disassembly by co-incubating Mit1 with recombinant Nap1 and ATP. The chromatin remodeling complex RSC has been shown to evict nucleosomes under similar conditions (183). Again, under
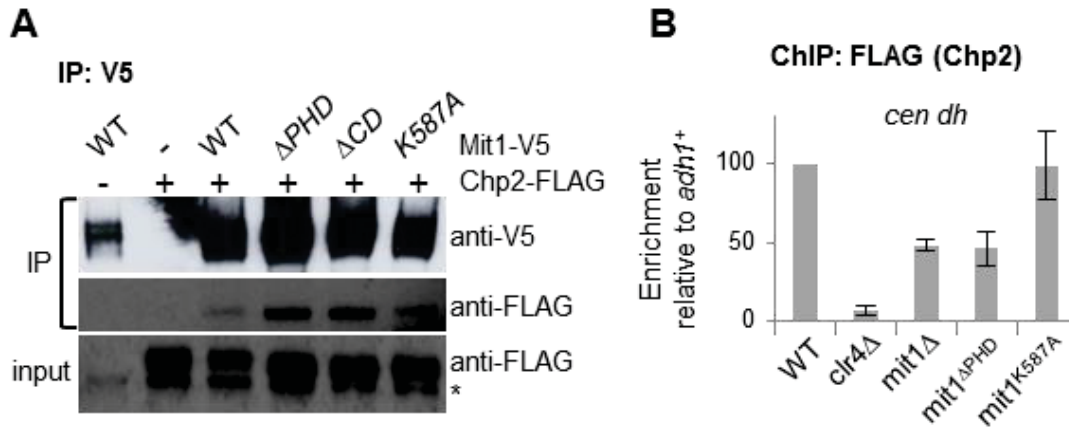
Figure 3-9.   Mit1's PHD domain is not required for Chp2 association.
(A) Mit1 PHD and CD domains are not required to maintain association with Chp2.
Immunoprecipitation of wild type 3XV5 tagged Mit1, Mit1$^{\Delta PHD}$, and Mit1$^{\Delta CD}$, Mit1^{K587A}
in strains expressing 6XFLAG-Chp2 and blotting against V5 and FLAG epitopes. Note
that the SDS-PAGE gels used in these experiments do not effectively resolve wild type
Mit1 from Mit1$^{\Delta PHD}$ and Mit1$^{\Delta CD}$. Asterisk indicates a non-specific band.
(B) Centromeric association of Chp2 is partially dependent on Mit1. ChIP of Chp2
association with centromeres relative to a euchromatic locus in the indicated strain
backgrounds. Error bars reflect the SEM (n=4).
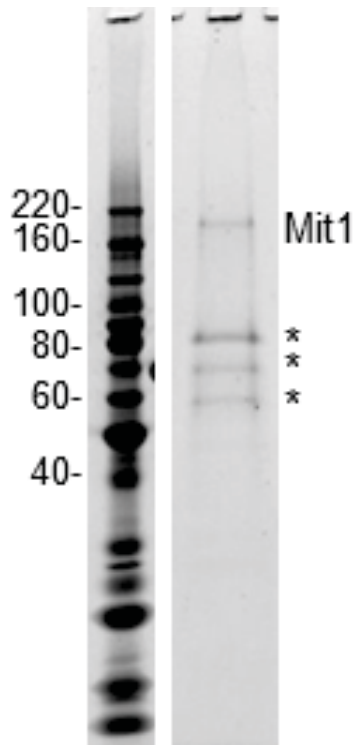
Figure 3-10.   Tandem purification of Mit1.
An aliquot of the final purification of 3XFLAG-Mit1-3XV5 expressed in *mit1Δ* cells was
analyzed by SDS-PAGE and SYPRO® Ruby staining. Mit1 was identified as the
approximately 170kDa band with 49% sequence coverage by mass spectrometry.
Asterisks represent Mit1 breakdown products and heat shock proteins.

Table 3-3.   **Summary of peptides identified by mass spectrometry following Mit1 purification.**

Band	Protein	# Peptides*	MW (kDa)
1 (190 kDa)	Mit1	59	163
2 (85 kDa)	Ssa2	32	70
2	Bip1	13	73
2	Mit1	10	163
3 (70 kDa)	Ssa2	18	70
3	Hsp60	13	62
3	Ivl1	9	73
3	Mit1	8	163
4 (60 KDa)	Ssa2	18	70
4	Rvb2	12	52
4	Rpt2	9	50
4	Mit1	7	163
4	SPBC543.02c	7	53
4	Rvb1	7	50

*Proteins listed were determined by an arbitrary cut off of 7 peptides
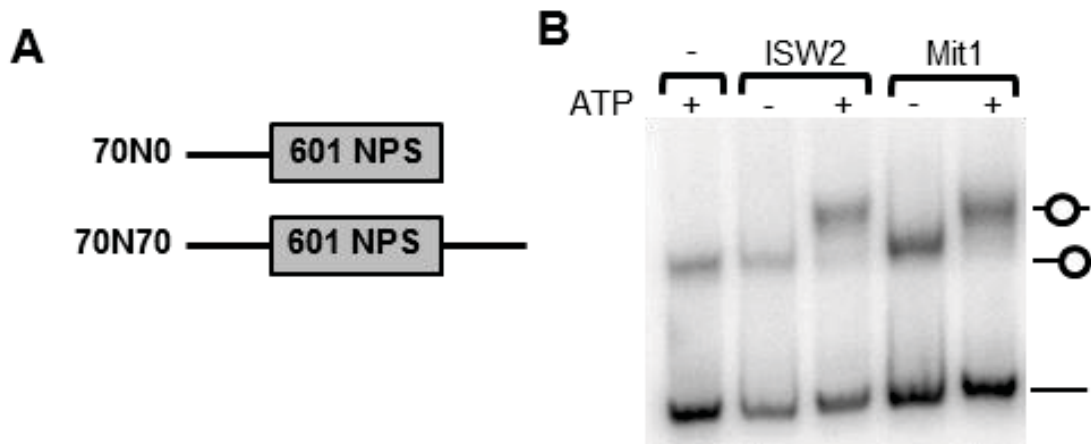
Figure 3-11. Mit1 is a chromatin remodeling factor.
(A) Schematic representation of short mononucleosomes reconstituted for remodeling experiments. Nucleosomes were positioned by the Widom 601 nucleosome positioning sequence.
(B) Mit1 can remodel a mononucleosomal substrate. End-positioned (70N0) mononucleosomes (30nM) were incubated with ScISW2 (5.0nM) or SpMit1 (2.5nM) in the presence or absence of ATP and resolved on a native 5% polyacrylamide gel.
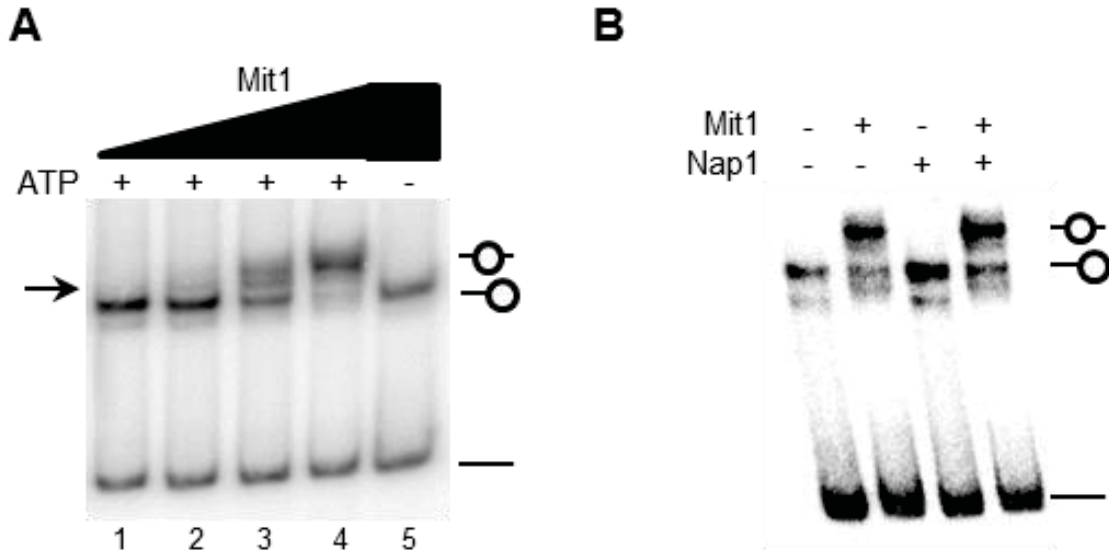
Figure 3-12. Mit1 mobilizes, but does not evict mononucleosomes.
(A) Titration of Mit1 protein reveals intermediate remodeling events. Nucleosomes were remodeled as in **Figure 3-11B** with a titration of Mit1 (0.3nM, 0.6nM, 1.25nM and 2.5nM).
(B) Remodeling in the presence of Nap does not result in nucleosome eviction. Nucleosomes were incubated with or without Mit1 (2.5nM) or recombinant hNap1 (.25μg, Active Motif) as indicated.

these conditions we saw no increase in the abundance of free DNA (**Figure 3-12B**), suggesting that nucleosome eviction is not a prominent feature of Mit1 remodeling.

We tested whether the chromatin remodeling activity of Mit1 was linked to ATP hydrolysis. Mit1 incubated with a non-hydrolysable ATP analog, ATPγS, did not change the mobility of an end positioned nucleosome (**Figure 3-13A**). We also purified a predicted ATPase dead Mit1^{K587A} (mutated in the ATP-binding site of the catalytic core K587A), and confirmed that it could not hydrolyze ATP (**Figure 3-13B**, (156)). This also demonstrated that our purified material was free of contaminating ATPases. Importantly, Mit1^{K587A} showed no sliding activity and could not mobilize an end-positioned nucleosome (**Figure 3-13C**). These results confirm that Mit1 is indeed an ATP-dependent nucleosome remodeling factor.

Next, we addressed whether the remodeling activity of Mit1 was directional, since many other chromatin remodeling enzymes show directional *in vitro* nucleosome mobilization. Some remodelers such as ISWI mobilize histone octamers to the ends of short DNA fragments while others, including Mi-2 and several CHD family remodelers preferentially slide nucleosomes toward the center (172,184). On incubation with saturating amounts of Mit1, essentially all end positioned nucleosomes were mobilized to the center of the DNA as evidenced by a discrete slower migrating species (**Figures 3-11B**, **3-12A** and **3-13C**), suggesting that remodeling by Mit1 is non-random and directional. To further probe the directionality of remodeling by Mit1, we incubated Mit1 with a centrally positioned mononucleosome (70N70, **Figure 3-11A**). Consistent with a preference for moving histone octamers away from DNA ends, Mit1 did not move the 70N70 positioned nucleosome (**Figure 3-14**). Taken together, these data demonstrate Mit1 is a directional (end to center) ATP-dependent chromatin remodeling factor that shares *in vitro* nucleosome remodeling characteristics with other Mi-2 family enzymes.

Mit1's Chromatin Tethering Domains Promote Remodeling Activity Independent of Effects on ATPase Activity

We have demonstrated that Mit1's CD can bind to DNA, and that the PHD finger binds to histone H3, and that both domains contribute to Mit1's silencing function. We next sought to determine mechanistically how loss of Mit1's chromatin interaction domains impact Mit1 function. First, using purified proteins (**Figure 3-15A**) we tested the ability of the mutant proteins to mobilize end positioned nucleosomes. We found that Mit1^{ΔPHD} and Mit1^{ΔCD} have reduced ability to mobilize nucleosomes, reflected in the incomplete shift of end positioned mononucleosomes to a slower migrating (centered) species in native PAGE (**Figure 3-15B**). Importantly, Mit1^{ΔPHD} and Mit1^{ΔCD} have not lost their directional specificity but have approximately 8-fold reduced mobilization activity, since addition of more protein overcomes the mobilization defect for these mutants, but not for the K587A mutant (**Figure 3-15C**).

These experiments demonstrate that removal of Mit1's DNA (CD) or H3 (PHD) tethering domains results in a reduced ability to remodel nucleosomes. While their
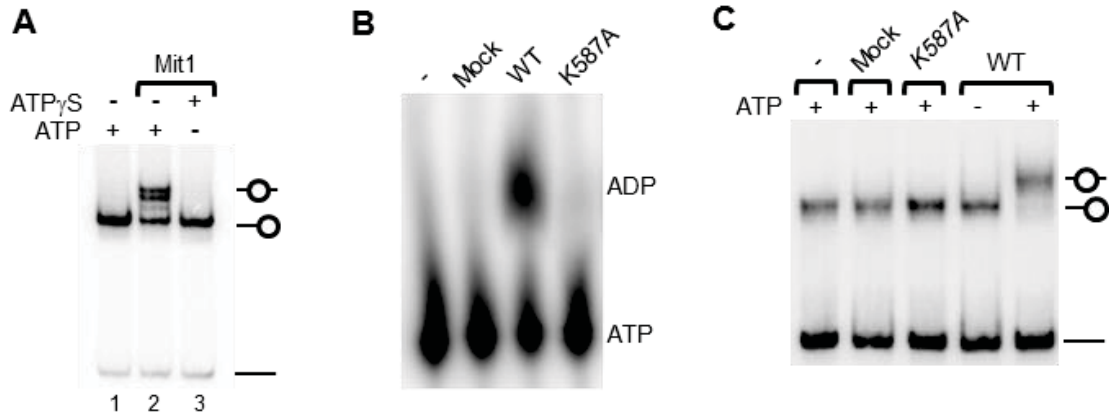
Figure 3-13.   Remodeling by Mit1 requires ATP hydrolysis.
(A) Mit1 utilizes ATP hydrolysis to remodel a mononucleosomal substrate. Nucleosomes were remodeled as in **Figure 3-11B** on addition of ATP, but not on addition of the non-hydrolysable analogue, ATP-γS.
(B) Mit1, but not Mit1^{K587A} can hydrolyze ATP. ATP hydrolysis assay comparing the activity of wild type Mit1 relative to Mit1^{K587A}, a mock purification, and a buffer-only control.
(C) Mit1^{K587A} cannot mobilize nucleosomes. Nucleosome mobilization assays comparing octamer mobilization by wild type Mit1, Mit1^{K587A} and mock purifications.
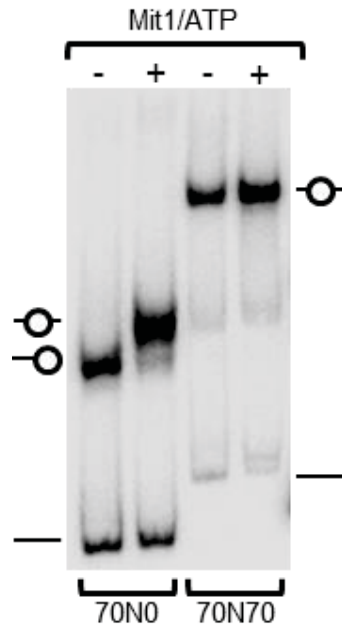
Figure 3-14.   Octamer mobilization by Mit1 is directional.
Nucleosome mobilization assay comparing the remodeling of end-positioned (70N0) and center-positioned (70N70) nucleosomes incubated with or without Mit1 (2.5nM) and ATP.
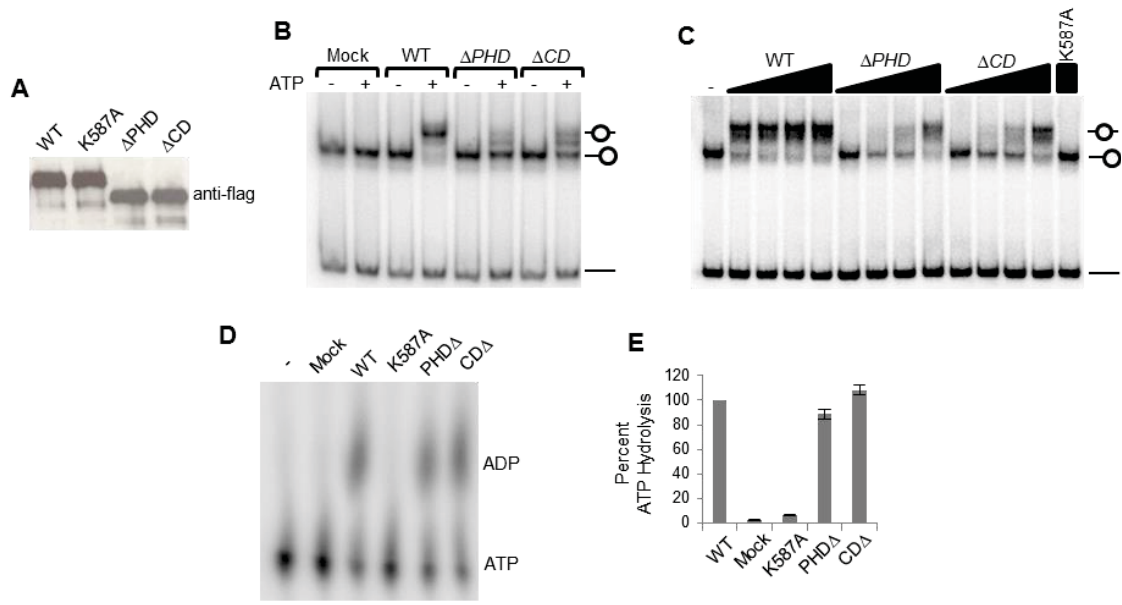
Figure 3-15.   Remodeling by Mit1 requires ATP hydrolysis.
(A) Western blot of purified 3XFLAG-Mit1-3XV5 proteins.
(B) Mit1 PHD and CD domains contribute to nucleosome mobilization activity.
Nucleosome mobilization assays as performed comparing mobilization by purified wild
type Mit1, Mit1$^{\Delta PHD}$, and Mit1$^{\Delta CD}$ proteins (2.5nM), as well as eluate from a mock
purification.
(C) Higher concentrations of PHD or CD mutant Mit1 proteins overcome the
mobilization defect. End-positioned (70N0) mononucleosomes (30nM) were remodeled
with buffer or increasing concentrations of purified Mit1 (1.5nM, 3.0nM, 6.0nM,
12.0nM), Mit1$^{\Delta PHD}$ (1.5nM, 3.0nM, 6.0nM, 12.0nM), Mit1$^{\Delta CD}$ (1.5nM, 3.0nM, 6.0nM,
12.0nM), and Mit1^{K587A} (12.0nM).
(D) PHD and CD mutant Mit1 proteins retain ATP hydrolysis activity. ATP hydrolysis
assay as performed in **Figure 3-13B** comparing the activity of purified Mit1 mutants to
wild type as well as buffer only and mock purifications.
(E) Semi-quantitative comparison of Mit1 mutant ATPase activity. A low level of
purified proteins was used so that hydrolysis of ATP to ADP was approximately 10-15%
for wild type Mit1. Reactions were quantified by densitometry following TLC (see
methods) and the buffer only reaction was subtracted as background before making all
reactions relative to the hydrolysis observed using purified wild type Mit1. Error bars
represent standard error of the mean (n=3).

requirement for octamer mobilization is not absolute *in vitro,* in the context of chromatin these interactions may be essential to maintain efficient interaction with nucleosomes or may modulate how nucleosomes are remodeled, since cells expressing Mit1 lacking these domains have similar silencing defects to Mit1^{K587A} or *mit1Δ* cells (**Figure 3-5B**). The remodeling defect did not arise from a reduced ability of the mutant proteins to hydrolyze ATP, since Mit1^{ΔPHD} and Mit1^{ΔCD} hydrolyze ATP at levels similar to wild type Mit1 (**Figure 3-15D** and **3-15E**). This suggests that the Mit1 PHD and chromo domains are not required for ATPase activity of the complex, but are necessary to promote chromatin association and remodeling by SHREC.

Mit1 Contributes to the Formation of a Nucleosome Free Region on an Intrinsically Unfavorable Site at *REII*

Our data show that Mit1 is required to prevent RNA pol II access to regions of heterochromatin, and that Mit1 is able to mobilize nucleosomes. One mechanism by which heterochromatin can restrict RNA polymerase II accessibility is through altering nucleosomal occupancy to prevent access of transcription factors to their target sequences. Heterochromatin prevents the appearance of nucleosome-free regions (NFRs) at various locations in silenced regions of the genome, and nucleosome occupancy at some of these sites has been shown to depend on Mit1/SHREC (156,160,161,185). Interestingly, the sites that most depend on Mit1 do not map to sites of transcription initiation or to regions particularly enriched for RNA Pol II. Mit1 also did not significantly suppress the formation of any nucleosome–free regions within centromeres (185) despite increased levels of Pol II and elevated transcripts originating from the *dg* and *dh* repeats in *mit1Δ* cells (**Figures 3-1C** and **3-4A**).

We hypothesized that the DNA sequence may contribute to the location of nucleosome free region formation in *mit1Δ* cells. To test this idea, we analyzed these regions with two *in silico* nucleosome prediction algorithms that predict nucleosome occupancy based on DNA sequence characteristics (186,187) We found that some sites that depend on Mit1 for prevention of nucleosome free regions appear to be particularly refractory to octamer occupancy, and lie near positions predicted to have relatively well positioned nucleosomes, particularly at *REII* in the mating type region and at telomere 2R (**Figure 3-16A** and **3-16B**). The predicted low affinity of these sequences is likely due to low G/C content and high percentage of DNA with 5-mers of exclusively A/T nucleotides (**Figure 3-16C** and **3-16D**), both of which disfavor nucleosome formation (187,188). This would suggest that Mit1 may play a critical role in moving nucleosomes onto DNA sequences that are energetically unfavorable for nucleosome positioning.

We used an in vivo PCR-based nucleosome scanning method to confirm the existence of a NFR at *REII* that forms in *clr4Δ* and *mit1Δ* cells. In short, mononucleosomal DNA was prepared following micrococcal nuclease digestion of chromatin prepared from wild-type and mutant cells, and was subject to Q-PCR with primers that amplify 18 overlapping fragments spanning the region. Using this assay, regions occupied by nucleosomes will be well-represented in the DNA sample, whereas

Figure 3-16. Mit1 prevents the formation of a NFR on an intrinsically unfavorable site at *REII*.

(A,B) Schematic of the fission yeast mating type region and right telomere of chromosome 2. Predicted nucleosome occupancy using the Nucleosome Positioning Prediction Engine (NuPoP) for these regions is plotted below. Grey ovals indicate previously identified regions of nucleosome occupancy changes in *mit1Δ*.

(C,D) Sequence analysis of nucleosome depleted regions. Sequence was analyzed for G/C content and presence of A/T tracts and reported as the percentage of nucleotides in 100 base pair windows that are either G or C or within A/T tracts defined as five or more consecutive A or T nucleotides.

(E) *In vivo* nucleosome scanning of *REII* at the mating type locus. Nucleosome scanning experiment comparing the relative protection of the region surrounding the *REII* silencing element from digestion by micrococcal nuclease in wild type, *mit1Δ*, and *clr4Δ* backgrounds. Mononucleosomal DNA was analyzed by Q-PCR and normalized to amplification within *adh1$^+$* and compared to wild type. Data plotted on a log2 scale, error bars represent SEM (n=2).

(F) *In vitro* nucleosome scanning at *REII* using reconstituted chromatin. Nucleosome scanning experiment using mononucleosomal DNA isolated from *in vitro* reconstitution of nucleosomes onto *REII* region synthetic DNA.

(G) Mutant Mit1 proteins cannot suppress NFR formation at *REII* in *mit1Δ* cells. Nucleosome scanning experiment performed as in **Figure 3-16E**, comparing wild type cells transformed with empty vector (dashed line) to *clr4Δ* or *mit1Δ* cells transformed with empty vector and *mit1Δ* cells transformed with vectors expressing Mit1, Mit1^{K587A}, Mit1^{ΔPHD}, and Mit1^{ΔCD}. Data plotted on a log$_2$ scale, error bars represent SEM (n=2).

A Mating Type Region

Predicted Occupancy

B Right Telomere 2

Predicted Occupancy

C % G/C Content

% A/T Tracts

D % G/C Content

% A/T Tracts

E *In vivo* Nucleosome Scanning at REII

WT
clr4Δ
mit1Δ

F *In vitro* Reconstitution at REII

G *In vivo* Nucleosome Scanning at REII Region

clr4Δ + EV

mit1Δ + EV

mit1Δ + WT

mit1Δ + K587A

mit1Δ + ΔPHD

mit1Δ + ΔCD

52
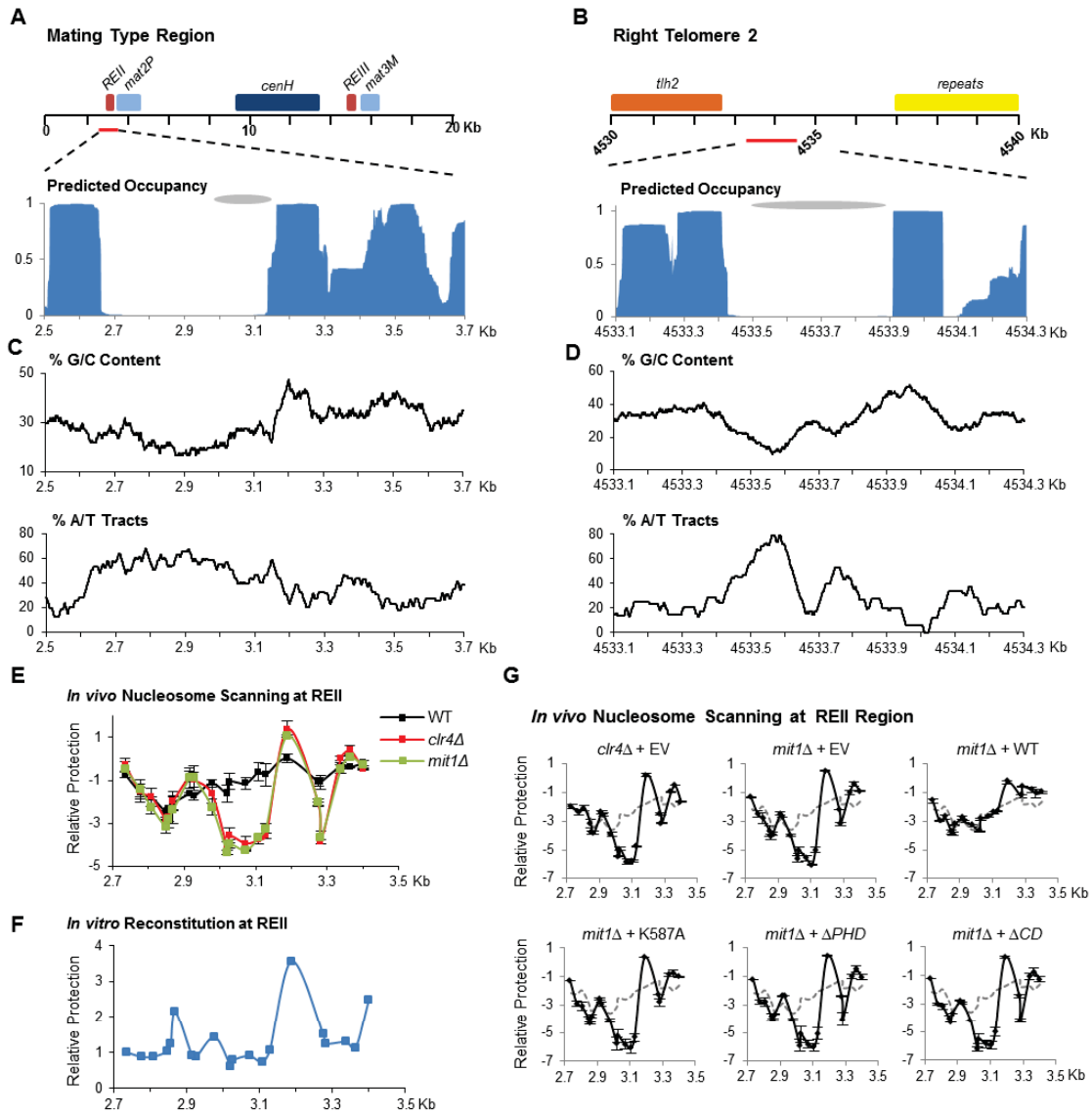
regions with low nucleosome occupancy or poorly positioned nucleosomes will amplify less. In wild-type cells, evenly dispersed and poorly positioned nucleosomes span this region (**Figure 3-16E**). In contrast, in *clr4Δ* and *mit1Δ* cells, a NFR clearly forms and is evident as a strong trough in amplification over the *REII* sequence. In contrast to the "fuzzy" nucleosome occupancy found in wild-type cells at this locus, a strongly positioned nucleosome is observed in the absence of silencing factors at the downstream sequence adjacent to the nucleosome free region at 3.2 Kb. The position of this nucleosome relative to the NFR corresponds to a sharp transition in the GC content of the underlying DNA sequence.

To investigate the influence of sequence on nucleosome occupancy, we assembled chromatin *in vitro* by salt dilution onto synthetic DNA including the *REII* region. Nucleosome occupancy in this cell free reconstitution system greatly resembled both the predicted occupancy, and interestingly, the occupancy observed in *clr4Δ* and *mit1Δ* cells (**Figure 3-16F**). These results suggest that silencing factors act to override the contribution of DNA sequence to nucleosome positioning at this site.

Next, we assayed whether Mit1 or Mit1 chromatin interaction mutants could correct NFR formation at *REII* in *mit1Δ* cells. *In vivo* nucleosome scanning assays were performed on *mit1Δ* cells transformed with vectors expressing wild type or mutant Mit1 proteins (**Figure 3-16G**). These experiments revealed that while episomally expressed wild type Mit1 could complement for nucleosome occupancy at *REII* in *mit1Δ* cells, all three mutant Mit1 proteins (Mit1^{K587A}, Mit1^{ΔPHD} and Mit1^{ΔCD}) were unable to eliminate the NFR. These data demonstrate that the PHD and CD motifs of Mit1 that are required for efficient octamer mobilization *in vitro* are also essential for the nucleosome positioning function of Mit1 *in vivo*.

Mit1 Functions Synergistically with Set2 for Maintenance of Transcriptional Silencing

Given that the PHD domain of Mit1 associates preferentially with K4 and K36, but not K9 methyl marked histone H3, we sought to determine whether we could place Mit1 in a genetic pathway linking it to methyltransferase activity. In fission yeast, K4 methylation is mediated by Set1 and is not required for heterochromatin silencing (189). Set2, which mediates H3K36 methylation, is believed to make minor contributions to heterochromatin silencing and methylates histones within centromeric repeats when heterochromatin is disrupted during S phase (109,190). To test whether Mit1 functions in the same pathway as Set2 to repress aberrant transcription, we assessed genetic epistasis. We found that Set2 strongly synergizes with Mit1 to block accumulation of centromeric and subtelomeric transcripts (**Figure 3-17**), with transcript levels approaching those found in *clr4Δ* cells that completely lack heterochromatin. In contrast combining deletions of *mit1* and *set1* resulted in no additive or synergistic effects, which may indicate that Mit1 and Set1 function within the same pathway or that there is no synthetic genetic interaction. We also found that *set2Δ* showed synthetic interactions with deletions of other SHREC components (**Figure 3-18**). Together these data suggest that Set2
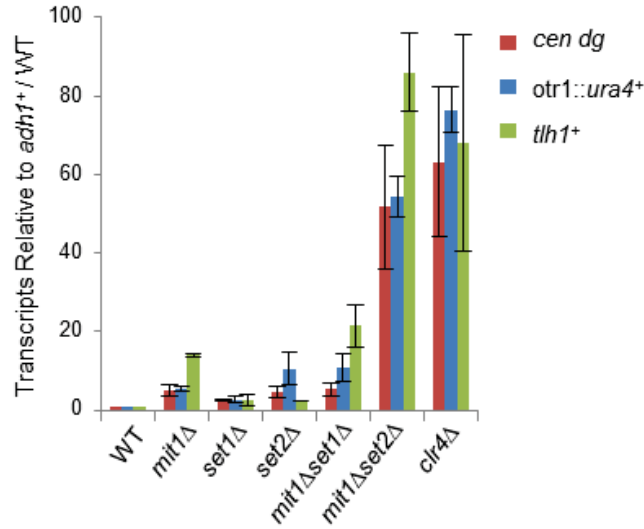
Figure 3-17.   Mit1 and Set2 synergize to silence heterochromatin transcripts.
Quantitative real time PCR analysis of transcripts from a centromeric transgene
(otr1::*ura4*⁺), centromeric repeats (cen *dg*), and subtelomeres (*tlh1*⁺) in cDNA prepared
from the indicated strain backgrounds. Transcript levels were normalized to *adh1*⁺
transcripts and to transcripts in WT cells. Error bars represent SEM (n=2).
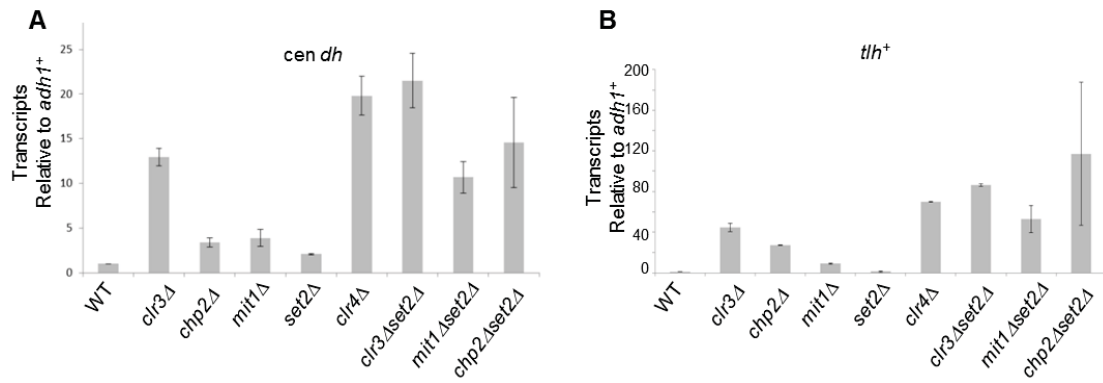
Figure 3-18. Transcript analysis comparing strains with deletions of individual SHREC subunits to *SHRECΔ*/*set2Δ* double mutant strains.
Quantitative real time PCR analysis of transcripts from (A) centromeric repeats (cen *dh*), and (B) subtelomeres (*tlh1*⁺) in cDNA prepared from the indicated strain backgrounds. Transcript levels were normalized to *adh1*⁺ transcripts and to transcripts in WT cells. Error bars represent SEM (n=2).

functions in conjunction with Mit1 and SHREC to suppress transcript accumulation from regions of heterochromatin.

Mit1 is not thought to significantly contribute to removal of nucleosome free regions within centromere 1 (185). Given the strong synthetic interaction between Set2 and Mit1 for silencing of centromeric transcripts, we tested whether the combined loss of both factors results in altered nucleosome occupancy within centromeric sequences. We focused on analyzing a region of centromeric *dh* sequence that includes a Clr4-dependent NFR (185). *In vivo* PCR based nucleosome scanning was performed on a 0.7Kb region, using 26 sets of real time PCR primers. In agreement with previous microarray based studies, cells lacking *clr4* show a clear NFR centered at 3755.6 Kb on chromosome 1 (**Figure 3-19**, (185)). We found that *mit1Δ* cells show reduced nucleosome occupancy at this site, and cells lacking *set2* also have a somewhat decreased signal. Importantly, the *mit1Δset2Δ* double mutant shows a greater defect in nucleosome occupancy than does the *mit1Δ* mutant. Thus our analysis has uncovered a role for Mit1 in helping prevent NFR formation at a centromeric locus, and this phenotype is exacerbated in the *mit1Δset2Δ* compound mutant.

The large increase in centromeric transcript accumulation in *mit1Δset2Δ* cells prompted us to investigate whether post-transcriptional silencing mechanisms are intact in this genetic background. RNAi appears fully functional in cells lacking either *set2⁺* or *mit1⁺*, since these strains accumulate high levels of *dh* siRNAs (**Figure 3-20A**). Surprisingly, levels of siRNA production were maintained in the *mit1Δset2Δ* double mutant. Given this lack of effect on the RNAi pathway, but the strong accumulation of centromeric transcripts in *mit1Δset2Δ* mutant cells, we asked whether centromere function was affected. Many mutants that are deficient in chromosome segregation are sensitive to the microtubule destabilizing drug, thiabendazole (191). Plating assays of cells on media containing thiabendazole demonstrated that whilst cells lacking *clr4⁺* display sensitivity (**Figure 3-20B**), the *mit1Δset2Δ* compound mutant showed only intermediate thiabendazole sensitivity, indicative of some disruption of heterochromatin function when compared with *mit1Δ* alone.

ChIP assays also revealed that H3K9me2 levels at centromeres and subtelomeres were similar in *mit1Δ* and the *mit1Δset2Δ* compound mutant cells, and were only slightly decreased compared with wild type cells (**Figure 3-20C**). Interestingly however, we found that Chp1 association with sites of heterochromatin was more severely impacted in cells lacking both Set2 and Mit1 than in either single mutant (**Figure 3-20D**), whereas Swi6 association with centromeres was similar between *mit1Δ* and *mit1Δset2Δ* cells (**Figure 3-20E**). These results indicate that heterochromatin silencing by SHREC is partially redundant with the Set2-mediated repression of transcription that functions more generally at Pol II transcribed regions, and together these factors prevent transcription and disruption of heterochromatin.
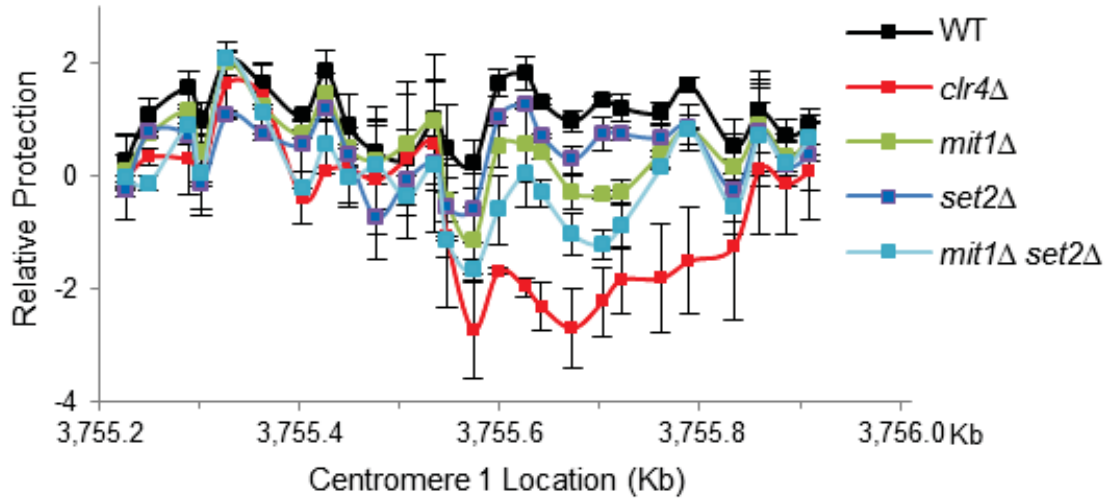
Figure 3-19. *In vivo* **nucleosome scanning of centromeric *dh* sequence.**
Nucleosome scanning experiment comparing the relative protection of part of the dh
centromeric repeat from digestion by micrococcal nuclease in wild type, *mit1Δ*, *set2Δ*,
mit1Δset2Δ and *clr4Δ* backgrounds. Mononucleosomal DNA was analyzed by Q-PCR
and normalized to amplification within *adh1⁺* and compared to wild type. Data is plotted
on a log$_2$ scale. Error bars represent SEM (n=2).
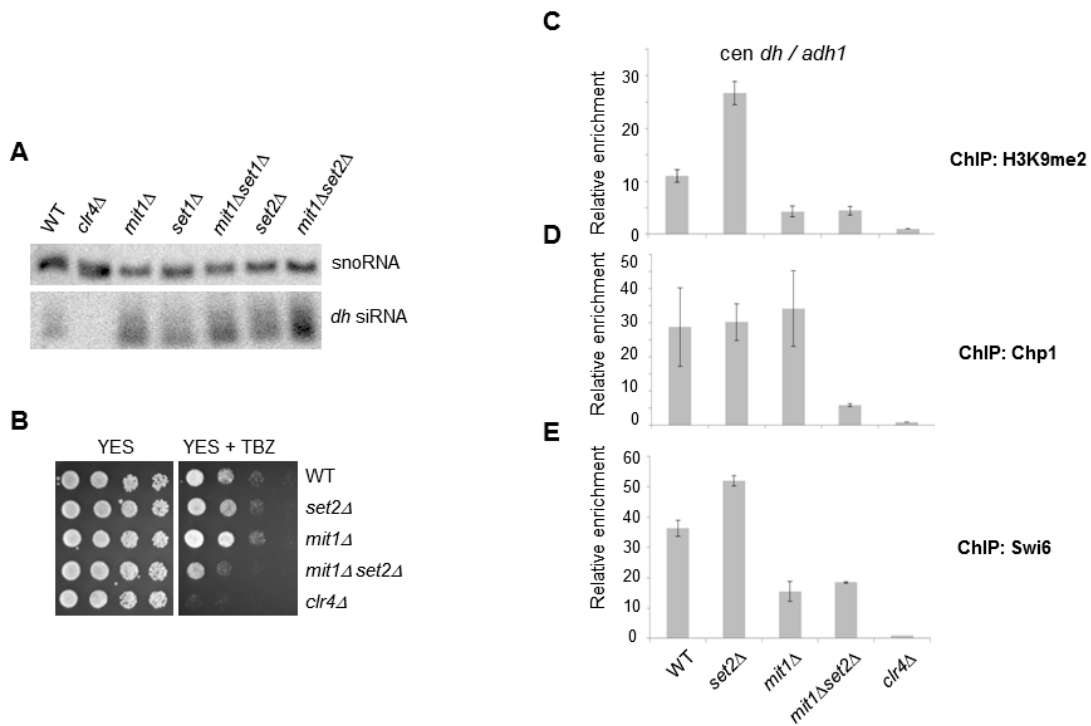
Figure 3-20. Analysis of heterochromatin in *mit1Δset2Δ* strains.
(A) Monitoring of RNAi. Northern blot of purified small RNA using probes
corresponding to cen *dh* sequences and snoR69 for loading control.
(B) Thiabendazole sensitivity plating assay. Serial dilution assay of wild type and mutant
strains plated onto YES media or YES media containing 15ug/ml TBZ. Plates were
incubated at 25°C.
(C-E) ChIP analysis in strains lacking Mit1 and Set2. Immunoprecipitated and input
chromatin was analyzed by Q-PCR for relative enrichment of centromeric dh sequence
relative to $adh1^+$ in immunoprecipitated chromatin with anti-H3K9me2 (C) anti-Chp1
(D), and anti-Swi6 (E) antibodies. ChIPs are normalized to a *clr4Δ* strain. Error bars
represent SEM (n=2).

SHREC's Role in NFR Prevention Is Not Limited to Deacetylation of H3K14

In addition to the chromatin remodeling activity of Mit1, it has been proposed that the SHREC subunit Clr3 is important for eliminating nucleosome-free regions within heterochromatin. Lysine 14 of histone H3 is thought to be the primary target of Clr3 deacetylation (118), and it has been hypothesized that an increase in acetylation of this residue may destabilize heterochromatin by recruiting the Remodels Structure of Chromatin complex (RSC) to the region (185). Recruitment of RSC chromatin remodeling activity to promoters is frequently associated with nucleosome eviction and transcriptional activation (192,193). Although we have demonstrated that Mit1 is a chromatin remodeling factor, it remained possible that rather than modifying chromatin structure and nucleosome positioning directly, the true nature of Mit1's contribution to heterochromatin silencing may be facilitation of Clr3 activity. We hypothesized that if deacetylation of H3K14 was the sole function of SHREC, preventing H3K14 acetylation may suppress the formation of nucleosome free regions observed in SHREC mutants. The fission yeast genome encodes two histone acetyltransferases responsible for H3K14 acetylation, $gcn5^+$ and $mst2^+$ (194). Importantly, while H3K14 acetylation is essentially undetectable in $gcn5\Delta mst2\Delta$ strains, they remain competent for centromeric silencing, suggesting that heterochromatin is intact (195). We combined deletion of $gcn5$ and $mst2$ with deletion of the catalytic subunits of SHREC and analyzed nucleosome occupancy at the mating-type *REII* locus. We found that combined deletion of $gcn5^+$ and $mst2^+$ only partially rescued the NFR formation seen in $clr3\Delta$ and did not suppress the $mit1\Delta$ phenotype at all (**Figure 3-21**). This experiment demonstrates that deacetylation of Lys14 on histone H3 is not the sole contribution of SHREC to nucleosome positioning.
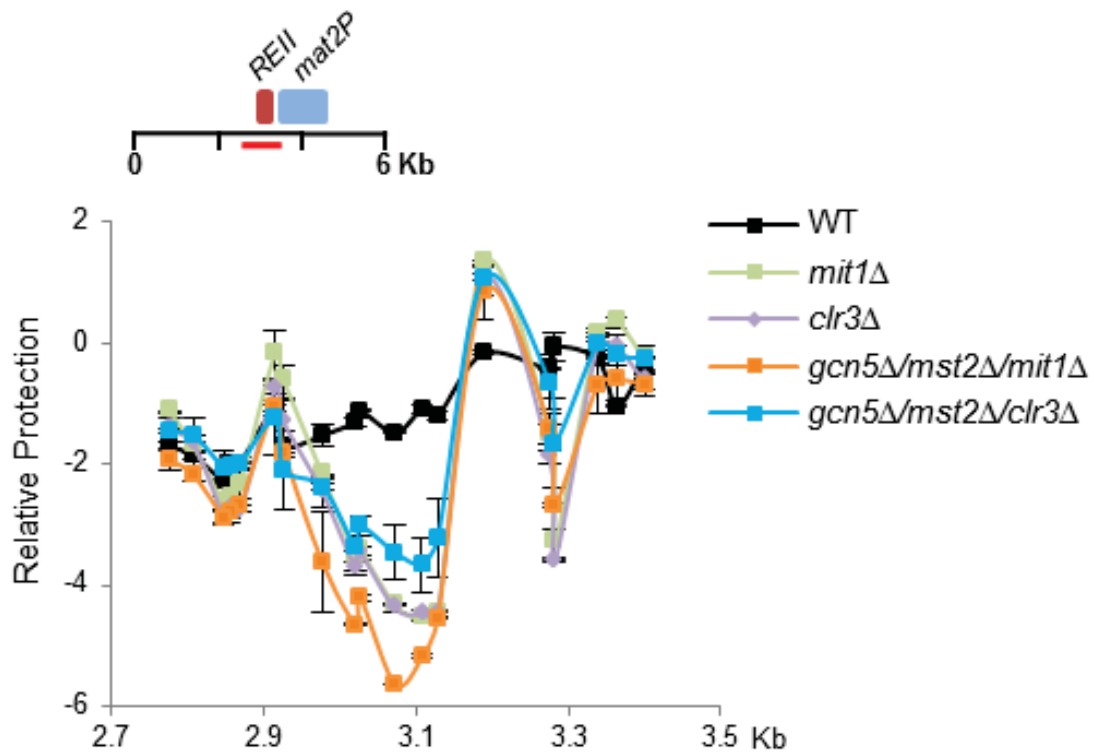
Figure 3-21. Histone acetyltransferase mutant strains cannot suppress NFR formation at *REII* in *SHRECΔ* cells.

Nucleosome scanning experiment performed as in **Figure 3-16E**, comparing wild type cells to *clr3Δ*, *mit1Δ* cells as well as triple deletion mutants *gcn5Δmst2Δclr3Δ* and *gcn5Δmst2Δmit1Δ*. Data is plotted on a $\log_2$ scale, error bars represent SEM (n=2).

CHAPTER 4.   DISCUSSION

The *S. pombe* SHREC is a known transcriptional silencing complex, but how SHREC modifies chromatin to suppress transcription is not well understood. In this study we show that Mit1 acts to silence transcription at regions of heterochromatin, but does not have a major role in regulating euchromatic gene expression. Mit1 is required to suppress access of RNA Pol II to regions of heterochromatin and does so largely downstream of H3K9 methylation in the heterochromatin assembly and silencing pathway.

This study presents for the first time evidence that Mit1 is a bona fide chromatin remodeling factor. The basic characterization of this activity has been performed, demonstrating that histone octamer mobilization by Mit1 on mononucleosomes is directional (end to center) and executed without detectable levels of octamer eviction. Efficient remodeling by Mit1 requires ATP hydrolysis and conserved PHD and chromodomain chromatin interacting motifs.

Nucleosome free regions form in the absence of Mit1 activity, most notably in the mating type regions and subtelomere on DNA sequences that are intrinsically refractory to nucleosome assembly. Mit1 requires its catalytic activity and chromatin tethering domains that are necessary for efficient mobilization of histone octamers for transcriptional silencing and to prevent the formation of these nucleosome free regions. Thus, chromatin remodeling by Mit1 seems to override the reduced intrinsic affinity of some sequences for the histone octamer in nucleosome positioning.

Interestingly, Mit1 and Set2, an enzyme associated with active transcription, act synergistically to silence heterochromatic transcripts and are partially redundant for maintaining nucleosome occupancy and function of heterochromatin at centromeres. Importantly, mutations that abrogate acetylation of Lys14 of histone H3 do not rescue loss of SHREC activity, suggesting the sole function of SHREC is not limited to deacetylation of this residue. The implications of these results are discussed below.

CHD-related Chromatin Remodeling Enzymes in *S. pombe*

The composition and activities of chromatin remodeling enzymes in fission yeast are surprisingly understudied relative to a number of other model organisms. The relationship between *in vitro* remodeling and functional changes in chromatin are perhaps best studied in the budding yeast *S. cerevisiae*. The results of these studies have been extrapolated to the study of similar complexes in other organisms, revealing overlapping themes, but also key differences likely stemming from the context-specific functions required in higher organisms (196,197). Fission yeast are an interesting example of how organisms differentially utilize chromatin remodeling enzymes to modify chromatin, as *S. pombe* curiously lack homologs of ISWI family remodeling enzymes that have been identified in most other eukaryotes (198).

ISWI-family remodelers Isw1 and Isw2 have several ascribed activities in budding yeast, including the regulation of transcriptional elongation and termination (199). Isw1 and CHD-family Chd1 are the primary remodeling enzymes responsible for spacing nucleosomes throughout the genome (200). Isw2 is well known for its role in repressing the expression of a number of genes as well as transcription of the ribosomal DNA locus (201,202). Higher organisms are known to use ISWI remodeling for heterochromatin silencing, as a murine ISWI-containing complex was found to have a role in centromeric heterochromatin maintenance (203).

Despite the lack of this family of enzymes, *S. pombe* still share many of the nucleosome positioning and regulatory characteristics observed in other organisms (204). *S. pombe* have an expanded repertoire of CHD-related remodelers, leading to speculation that fission yeast may have specialized these enzymes in order to accomplish the tasks normally performed by ISWI enzymes (198). Fission yeast have at least three CHD-related chromatin remodeling enzymes, including two Chd1 homologs (Hrp1 and Hrp3) and Mit1. Chd1-related enzymes Hrp1 and Hrp3 seem to accomplish much of the euchromatic phasing of nucleosomes performed by Isw1 and Chd1 in *S. cerevisiae*, regulating gene expression and suppressing antisense transcription in the process (205-207). Mit1 was once identified as a factor involved in the phasing of nucleosomes in euchromatic regions as well (204), however, this aspect of Mit1 function has more recently been attributed to Hrp1 and Hrp3 (207). Mit1 regulates few, if any euchromatic transcripts (this study) and is recruited primarily to regions of heterochromatin (114), suggesting that Mit1/SHREC is a specialized heterochromatin-specific CHD remodeler, though Hrp3 is known to associate with heterochromatin as well (205). The mechanistic significance of recruiting this activity, particularly in the absence of ISWI family remodeling factors is discussed below.

Mit1 and Mi-2 Share Structural, Enzymatic and Functional Properties

The co-purification of a putative chromatin remodeling enzyme with a histone deacetylase known to have a role in transcriptional silencing suggested SHREC constitution and activity might parallel that of the NuRD complexes found in higher organisms. Unlike most CHD remodeling enzymes, which utilize their chromodomains to bind histone H3 and have an additional DNA binding activity in the C-terminus, Mi-2 remodeling subunits of NuRD complexes have characteristic tandem chromodomains that bind DNA and tandem PHD fingers sensitive to methylation modification on histone H3 (127). Though a single PHD finger was identified in initial studies of the SHREC complex, it was not known how or if this domain contributed to Mit1 function. In this study, we have demonstrated that this domain mediates interactions with the histone component of chromatin. In addition, through sequence analysis we identified a region with considerable homology to chromodomains found in other proteins. Like the chromodomains of Mi-2, Mit1's chromodomain can bind DNA. Both of these domains are important for efficient octamer mobilization by Mit1. Similar observations in terms of binding specificities and requirement for efficient remodeling have been made for

Drosophila and human Mi-2 (208,209). The *in vitro* octamer mobilization activity of Mit1 is to mobilize end positioned mononucleosomes to the center of short DNA fragments, a property shared by other CHD remodeling enzymes, including Mi-2 (210).

Though we were unable to identify a specific modification that increases the binding affinity of Mit1's PHD finger to histones, murine Mi-2 is known to bind H3K9 methylation *in vitro* and can serve as a recruitment mechanism in the cell (211). Additionally, the Mbd2 subunit of NuRD recruits the complex for transcriptional gene silencing by recognition of CpG methylation in target promoters, and may aid targeting the complex to pericentric heterochromatin by recognizing DNA methylation as well (137,212). SHREC does not have an obvious Mbd2-like protein and *S. pombe* does not utilize this type of DNA methylation. Additionally, our data indicates that H3K9 methylation is likely not the motif recognized by Mit1's PHD finger. It seems likely that recruitment of SHREC to heterochromatin is functionally conserved by associating with HP1-like protein Chp2, which specifically recognizes H3K9 methylation. NuRD is not known to associate with HP1 proteins in higher organisms. The specialization of a Mi-2 PHD finger to recognize H3K9 methylation may circumvent the requirement for the association with HP1-like proteins to be recruited to this repressive mark in higher organisms.

NuRD's association with pericentric heterochromatin was first and most readily detected in rapidly dividing lymphoid cells, lending to speculation that NuRD may be particularly important for resetting repressive chromatin structures that are frequently and quickly replicated (137,138,213). Fission yeast are by nature rapidly dividing relative to metazoan cells and for this reason may require a specialized heterochromatin remodeling complex such as SHREC to ensure error-free reorganization of heterochromatic domains. Our analysis of the regions that are most dependent on SHREC activity suggests Mit1 may be particularly important for mobilization of nucleosomeomes onto refractory AT-rich sequences. It seems possible that remodeling by the related NuRD complex could perform similar functions at pericentric heterochromatin considering the repetitive, AT-rich nature of the satellite sequences (214).

Role of the PHD Finger

Our experiments demonstrate that the PHD domain of Mit1 is required for full remodeling activity of Mit1, and that it contributes to chromatin association of SHREC. Since the in *vitro* experiments were performed with unmodified nucleosomes, and loss of the PHD domain reduced mobilization activity, it is possible that the PHD finger contributes to the stabilization or activity of SHREC at heterochromatic regions through general interactions with histone H3. Our attempts to further dissect how the PHD domain associates with histones were thwarted by our inability to detect binding of the recombinant PHD domain to modified histone peptide arrays. The association of Mit1's PHD domain with signatures of transcriptionally active chromatin suggests that this domain may contribute to the recruitment or stabilization of SHREC at heterochromatic loci during a window of the cell cycle in which heterochromatin is transcriptionally

active. Transcription of centromeric repeats occurs during S phase, following the replication of centromeric DNA (215). At this time, levels of H3K9 methylation are low, and nucleosome occupancy is also reduced. It is possible that the PHD finger of Mit1 contributes to SHREC association with histone H3 following methylation on K4 and K36 as a result of transcription of these domains. Since transcription and replication of heterochromatin coincide, the recruitment of SHREC at this particular time in the cell cycle may be important for the efficiently reassembly of heterochromatin following replication.

Even if not sensitive to modification of histone tails, Mit1's PHD finger and chromodomain binding to components of chromatin seems to be important for remodeling as mobilization is decreased approximately 8-10-fold in mutants lacking this domain. Furthermore, the PHD finger may also be important for regulating more complex functions of SHREC, potentially interacting with adjacent nucleosomes to facilitate nucleosome organization or the formation of higher-order chromatin structures.

Coordination of Mit1 Remodeling and Clr3 HDAC Activity

The SHREC histone deacetylase subunit Clr3 has recently been shown to be important for suppressing histone turnover within regions of heterochromatin (122). While these subunits of SHREC may physically interact, the contributions of Clr3 to steady-state nucleosome positioning extend beyond that of Mit1, suggesting their activities may not entirely overlap.

Our experiments suggest that H3K14 deacetylation by SHREC is likely not the sole contribution of SHREC to nucleosome occupancy, since deletion of the histone acetyltransferases required for this mark does not rescue the phenotype of *mit1Δ* and only partially rescues deletion of *clr3+*. Clr3-mediated deacetylation of H3K14 is thought to act in part by limiting recruitment of the RSC chromatin remodeling complex to heterochromatin (121). Interestingly, the *in vitro* remodeling properties of RSC are opposite those we describe for Mit1, including center to end octamer mobilization and eviction under some conditions (183,216). We propose that while Clr3 acts to prevent recruitment of the RSC complex, Mit1 may antagonize its activity within heterochromatin (**Figure 4-1**). Counteracting RSC has been suggested as a possible mechanism for the suppression of aberrant transcription by the ISWI-type Isw2 chromatin remodeling complex which mobilizes histone octamers onto intrinsically refractory sequences at some budding yeast promoters (217-219). Given the lack of ISWI family remodeling enzymes in *S. pombe* and the intrinsically unfavorable sequences in which Mit1 activity is most readily detected in the cell, we propose Mit1 antagonizes the activity of factors such as RSC that contribute to nucleosome depletion. Octamer mobilization by Mit1 then prevents the formation of permanent and transient nucleosome free regions that give access to the transcriptional machinery. Future studies aimed at understanding the cooperation between nucleosome mobilization by Mit1 and histone deacetylation by Clr3 will be important for developing a comprehensive model for how SHREC acts to silence transcription.
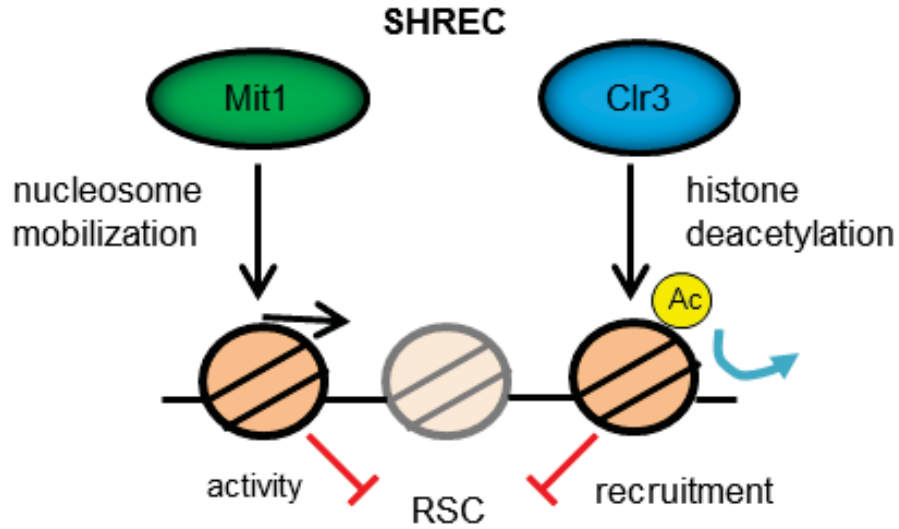
Figure 4-1.   Model for SHREC contribution to nucleosome occupancy at regions of heterochromatin.
We propose a model where SHREC, as well as other chromatin modifiers such as Set2/Clr6, Spt6, Asf1/HIRA prevent localized transient and steady-state nucleosome depletion in heterochromatic domains. SHREC performs this function using distinct but likely related activities in nucleosome mobilization and histone deacetylation in part to oppose the activity of the RSC complex. Elimination of NFRs is then important for efficient transcriptional silencing by restricting access to Pol II in regions of heterochromatin.

Redundancy Among Chromatin Modifiers

The contribution of SHREC to the assembly, silencing, and function of heterochromatin is redundant with other factors, complicating determination of the direct and specific contribution of SHREC HDAC and remodeling activities to these processes. At the level of histone deacetylation, there are at least three histone deacetylases (Sir2, Clr3, and Clr6) that contribute to heterochromatin assembly and function (115,220-222). At least two of these enzymes, Sir2 and Clr6, can remove acetylation from several lysines on histones (222,223). The results of these studies indicate that there is considerable redundancy between HDACs in *S. pombe*, and suggest the results of genetic ablation of one HDAC may be compensated by the activity of another. For example, double mutant *clr3Δ clr6-1* (a temperature sensitive mutant allele of *clr6*⁺, an essential gene) strains have considerably larger defects in heterochromatin silencing and chromosome segregation than strains with either mutant in isolation (224).

Similarly, there are a number of chromatin remodeling complexes that function in heterochromatin assembly and transcriptional silencing at these regions. Asf1/HIRA complex, known in other organisms to be involved in the reassembly of chromatin after replication, seems to be important for preventing histone turnover within regions of heterochromatin (94). CHD family remodelers Hrp1 and Hrp3, while having important functions in gene regulation, are also involved in heterochromatin silencing (205,206). The remodelers Spt6 and Fft3 (FUN30) are known to contribute to nucleosome occupancy at heterochromatic regions as well (225-227). Interestingly, Fft3 is structurally similar to a human remodeler, SMARCAD1, which seems to prevent regions of heterochromatin from acquiring euchromatic characteristics such as hyperacetylation (228). Detailed mechanistic study of the *S. pombe* counterparts have not been performed, however, budding yeast Fun30 is a known H2A/H2B exchange factor that does not efficiently slide nucleosomes *in vitro* (229), suggesting its mechanism of action is likely different then the silencing functions performed by SHREC in *S. pombe*.

Our observation that Mit1 synergizes with Set2 to silence heterochromatic transcription is interesting, given that Set2-mediated H3K36 methylation is generally thought of as a mark of active transcription. For this reason, H3K36 methylation is generally excluded from regions of heterochromatin in *S. pombe*. However, regions of heterochromatin are briefly transcribed during S phase. During this brief period in the cell cycle, the transcriptional machinery gains access (215). Set2 in particular is known to interact directly with RNA Pol II, allowing co-transcriptional methylation on Lys36 of histone H3 following DNA replication (230).

Set2 is thought to prevent aberrant transcription in euchromatin by mediating repression of intragenic cryptic transcription through recruitment of HDAC activity (231-234) and through suppression of nucleosome turnover in transcribed coding regions (235,236) without causing detectable changes in nucleosome positioning (207). Intriguingly, we have demonstrated that, in addition to synergizing to suppress centromeric transcripts, SHREC and a Set2-mediated pathway are partially redundant in preventing the formation of a nucleosome free regions in the pericentromeric repeats of

centromere 1. In contrast to the well-documented role of Set2 in co-transcriptional methylation of H3K36, Mit1 associates with and acts almost exclusively at regions of heterochromatin seemingly to oppose transcription ((114), this study). The synthetic silencing defects we observe in *mit1Δset2Δ* double deletion strains suggests that factors that prevent uncontrolled transcription in actively transcribed regions can partially compensate for the lack of specific silencing factors at constitutive heterochromatin, and that perhaps some aspects of SHREC function may be similar to the downstream pathways initiated by H3K36 methylation. This is a particularly attractive hypothesis, given that centromeric transcripts are elevated in the absence of Mit1 without dramatic changes in overall nucleosome occupancy, a phenotype that mirrors the initiation of cryptic transcription within gene bodies observed in *set2Δ* cells that similarly lack detectable changes in nucleosome occupancy. We also note that in this context Set2 activity may be stimulating the activity of the HDAC Clr6 in much the same way Set2 is known to recruit the activity of the related Rpd3 HDAC activity to gene bodies in budding yeast (232). Thus, our observation of synergistic silencing defects in *SHRECΔset2Δ* double mutant cells resembling previous defects seen in *clr3clr6*-1 cells is likely due to redundancy between SHREC and a Set2/Clr6 pathway.

Role of Chromatin Remodeling by Mit1 in Heterochromatin Assembly and Silencing

The ability of Mit1 to mobilize histone octamers and prevent the formation of nucleosome free regions may act to silence heterochromatic transcripts in several ways that are not necessarily mutually exclusive and are discussed below.

Increased Accessibility to the Transcriptional Machinery

Chromatin immunoprecipitation experiments from this study as well as others suggest that SHREC functions to antagonize access of the transcriptional machinery to regions of heterochromatin. The simple "uncovering" of the underlying DNA sequence may be the most straightforward explanation for how the formation of nucleosome depleted regions observed in mit1Δ cells can result in derepressed silencing. Histone octamers are generally a barrier to DNA binding for many proteins, including the TATA-binding protein (TBP) that promotes transcription (27). Furthermore, non-specific binding of DNA-binding proteins is known to inversely correlate with nucleosome occupancy (237). Thus, the DNA sequences no longer concealed by the presence of a histone octamer in the absence of SHREC activity have an increased potential for binding by sequence-specific or non-specific transcription factors that could promote transcription.

Curiously, the regions that experience nucleosome depletion in Mit1-deficient cells are not directly near transcription start sites and therefore do not appear to drive transcription in the way promoter NFRs stimulate transcription at actively transcribed genes (121). These regions may therefore be acting less directly as positive regulatory

elements similar to enhancers, which are also typically nucleosome-depleted (31,238). Furthermore heterochromatic domains in many organisms, including *S. pombe* tend to cluster (239,240), and it therefore seems reasonable that increased accessibility to a discrete region could allow for the accumulation of higher concentrations of the transcriptional machinery that could then compete for access to neighboring sequences. In this way mobilization of histone octamers by Mit1 at these discreet refractory sequences could conceivably impact the transcription of neighboring domains.

Formation of Specialized Structures

S. pombe heterochromatin may not be very compact in nature, as it differs little from bulk chromatin in terms of DNAse sensitivity (241), (unpublished observations), though this assay is unlikely to identify small but potentially significant changes in higher order structure. It remains possible that some amount of specialization in the chromatin structure has an impact on how heterochromatin assembles and silences transcription. How nucleosomes are positioned, in terms of spacing and presence or absence of gaps, can presumably contribute to the assembly of higher order chromatin structures (213,242). Future studies using more sophisticated assays detailing the nature of chromatin within heterochromatic domains may identify a specialized structure or level of compaction that may represent an important aspect of Mit1 function.

Disorganization of Nucleosome Patterning

Nucleosomes in fission yeast heterochromatin seem to lack the organization observed in actively transcribed regions of the genome including nucleosome depleted regions and well positioned nucleosome phasing ((121), this study). Some chromatin remodeling enzymes have been shown to disorganize regular phasing of histone octamers assembled onto nucleosomal arrays (56,243). An attractive possibility is that Mit1 may have this activity as well and actively participate in the disordering of nucleosomes within regions of heterochromatin.

The *in vitro* experiments detailed in this manuscript were performed solely on mononucleosomal substrates, largely for technical reasons with the labile nature of Mit1 making it difficult to obtain sufficient quantities of the protein for testing on polynucleosomal substrates. Thus, testing whether or not Mit1 can disorder nucleosome spacing was technically impractical. Furthermore, Mit1 may only demonstrate more complex activities such as this in the context of intact SHREC complex, as other subunits offer a number of potential contacts with chromatin that could facilitate this activity. Chp2, for instance, forms dimers that may facilitate interactions with adjacent nucleosomes (90,244). It is also possible that these subunits could alter the observed remodeling of mononucleosomes, though the activity of recombinant Mi-2 is similar to that of the purified NuRD complex (245). How the auxiliary subunits of SHREC contribute to remodeling of chromatin by Mit1, particularly in a polynucleosomal context remains a matter of speculation. It remains possible that future experiments may be able

to circumvent the labile nature of multiple SHREC subunits to test the activity of Mit1 remodeling on substrates that more closely resemble chromatin.

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APPENDIX. REAGENTS

Table A-1.   Strains used in this study.

Strain	Genotype	Figure(s)
PY42	h- ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-1, 3-2, 3-4, 3-19, 3-20B-E
PY4144	h- mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-1, 3-2, 3-4, 3-19, 3-20B-E
PY1798	h- clr4Δ::KanMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-DS/E	3-1, 3-2, 3-4, 3-19, 3-20B-E
PY743	h+/90ars1 ARS(MluI)::pREP81Xgfpswi6-LEU2+ otr1R Sph1::ura4 ade6-210 leu1-32 ura4-DS/E	3-2B
PY5251	h+/90ars1 mit1Δ::NatMX6 ARS(MluI)::pREP81Xgfpswi6-LEU2+ otr1R Sph1::ura4 ade6-210 leu1-32 ura4-DS/E	3-2B
PY5117	h- ars1 clr4Δ::KanMX6 ARS(MluI)::pREP81Xgfpswi6-LEU2+ otr1R Sph1::ura4 ade6-210 leu1-32 ura4-DS/E	3-2B
PY426	h- ade6-210 leu1-32 ura4DS/E [Ch16 ade6-216 m23::LEU2+]	3-3
PY669	swi6Δ::arg3 ade6-210 leu1-32 ura4DS/E [Ch16 ade6-216 m23::LEU2+]	3-3
PY5391	h+ mit1Δ::Natmx6 [Ch16 ade6-216 m23::LEU2+] ade6-210 leu1-32 ura4DS/E/D18	3-3
PY5169	h- pREP41-NTAP-LEU2 otr1R Sph1::ura4 ade6-210 arg3+ his3+ leu1-32 ura4-DS/E	3-5
PY5171	h- pREP41-NTAP-LEU2 otr1R Sph1::ura4 mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-5
PY5175	h- pREP41-NTAP-Nde I-Mit1-Bam HI-LEU2 otr1R Sph1::ura4 mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-5
PY5302	h- pREP41-NTAP-Nde I-Mit1K587A-Bam HI-LEU2 otr1R Sph1::ura4 mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-5

Table A-1.   (*Continued*).

Strain	Genotype	Figure(s)
PY5173	h- pREP41-NTAP-Nde I-Mit1Δ(214-269)-Bam HI-LEU2 otr1R Sph1::ura4 mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-5
PY7412	h- pREP41-NTAP-Nde I-Mit1Δ(446-502)-Bam HI-LEU2 otr1R Sph1::ura4 mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-5
PY8435	h- pREP4X-3XV5-ura4+ mit1Δ::NatMX6 chp2::3xFLAG-(x2)-chp2+ leu1-32 ura4D18	3-9A
PY8437	h- pREP4X-Mit1-3XV5-ura4+ mit1Δ::NatMX6 chp2::3xFLAG-(x2)-chp2+ leu1-32 ura4D18	3-9A
PY8441	h- pREP4X-NotI-Mit1K587A-3XV5-ura4+ mit1Δ::NatMX6 chp2::3xFLAG-(x2)-chp2+ leu1-32 ura4D18	3-9A
PY8443	h- pREP4X-Mit1Δ(214-269)-3XV5-ura4+ mit1Δ::NatMX6 chp2::3xFLAG-(x2)-chp2+ leu1-32 ura4D18	3-9A
PY8445	h- pREP4X-Mit1Δ(446-502)-3XV5-ura4+ mit1Δ::NatMX6 chp2::3xFLAG-(x2)-chp2+ leu1-32 ura4D18	3-9A
PY7259	h- pREP4X-Mit1-3XV5-ura4+ mit1Δ::NatMX6 leu1-32 ura4D18	3-9A
PY6852	h- chp2::3XFLAG(x2)-chp2+ clr4Δ::KanMX6 leu1-32 ura4-D18	3-9B
PY6854	h- chp2::3XFLAG(x2)-chp2+ mit1Δ::NatMX6 leu1-32 ura4-D18	3-9B
PY6902	h- chp2::3XFLAG(x2)-chp2+ leu1-32 ura4-D18	3-9B
PY6903	h- chp2::3XFLAG(x2)-chp2+ mit1::mit1Δ(214-269) leu1-32 ura4-D18	3-9B
PY7363	h- pREP4X-Bgl II-ATG-3XFLAG-Not I-Mit1-Not I-3XV5-ura4+ mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-10, 3-11, 3-12, 3-13, 3-14, 3-15

Table A-1.   (*Continued*).

Strain	Genotype	Figure(s)
PY7346	h- pREP4X-Bgl II-ATG-3XFLAG-Not I-3XV5-ura4+ mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-13, 3-15
PY7437	h- pREP4X-Bgl II-ATG-3XFLAG-Not I-Mit1K587A-Not I-3XV5-ura4+ mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-13, 3-15
PY7837	h- pREP4X-Bgl II-ATG-3XFLAG-Not I-Mit1(Δ214-269)-Not I-3XV5-ura4+ mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-15
PY7510	h- pREP4X-Bgl II-ATG-3XFLAG-Not I-Mit1(Δ446-502)-Not I-3XV5-ura4+ mit1Δ::NatMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-15
PY8082	mat1-msmt0 leu1-32 ura4-D18	3-16E, 3-16F, 3-21
PY8085	mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16E, 3-16F, 3-21
PY8088	clr4Δ::KanMX6 mat1-msmt0 leu1-32 ura4-D18	3-16E, 3-16F, 3-21
PY8091	pREP4X-3XV5-ura4+ mat1-msmt0 leu1-32 ura4-D18	3-16G
PY8094	pREP4X-3XV5-ura4+ clr4Δ::KanMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G
PY8097	pREP4X-3XV5-ura4+ mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G
PY8103	pREP4X-Not I-Mit1-Not I-3XV5-ura4+ mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G
PY8101	pREP4X-Not I-Mit1 K587A-Not I-3XV5-ura4+ mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G
PY8099	pREP4X-Not I-Mit1Δ(214-269)-Not I-3XV5-ura4+ mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G

Table A-1. (*Continued*).

Strain	Genotype	Figure(s)
PY8105	pREP4X-Not I-Mit1Δ(446-502)-Not I-3XV5-ura4+ mit1Δ::NatMX6 mat1-msmt0 leu1-32 ura4-D18	3-16G
PY2036	h- otr1R Sph1::ura4 ade6-210 leu1-32	3-17, 3-18, 3-20A
PY4166	h- mit1Δ::NatMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-17, 3-18, 3-20A
PY2971	h- clr4Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-17, 3-18, 3-20A
PY5472	h- set2Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-17, 3-18, 3-20A
PY5395	h- mit1Δ::NatMX6 set2Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-17, 3-18, 3-20A
PY5476	h- set1Δ::KanR otr1R Sph1::ura4 leu1-32	3-17, 3-20A
PY5400	h- mit1Δ::Natmx6 set1Δ::KanR otr1R Sph1::ura4 leu1-32	3-17, 3-20A
PY2941	h- clr3Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-18
PY4332	h- chp2Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-18
PY6429	h- clr3Δ::KanMX6 set2Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-18
PY8483	h- chp2Δ::KanMX6 set2Δ::KanMX6 otr1R Sph1::ura4 ade6-210 leu1-32	3-18
PY4405	h- set2Δ::KanMX6 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18	3-19, 3-20B-E

Table A-1. (*Continued*).

Strain	Genotype	Figure(s)
PY8515	mit1Δ::NatMX6 mst2Δ::KanMX6 gcn5Δ::KanMX6 mat1-msmt0 leu1-32	3-21
PY8516	clr3Δ::NatMX6 mst2Δ::KanMX6 gcn5Δ::KanMX6 mat1-msmt0 leu1-32	3-21

Table A-2.   **Plasmids used in this study.**

Plasmid	Genotype
JP1915	pREP4X-*BglII*-ATG-3XFLAG-*NotI*-3XV5-ura4+
JP1939	pREP4X-*BglII*-ATG-3XFLAG-*NotI*-Mit1-*NotI*-3XV5-ura4+
JP1996	pREP4X-*BglII*-ATG-3XFLAG-*NotI*-Mit1 *K587A-NotI*-3XV5-ura4+
JP2088	pREP4X-*BglII*-ATG-3XFLAG-*NotI*-Mit1(*Δ214-269)-NotI*-3XV5-ura4+
JP1994	pREP4X-*BglII*-ATG-3XFLAG-*NotI*-Mit1(*Δ446-502)-NotI*-3XV5-ura4+
pSLF972[α]	pREP4X-3XV5-ura4+
JP1889	pREP4X-*NotI*-Mit1-*NotI*-3XV5-ura4+
JP1923	pREP4X-*NotI*-Mit1 *K587A-NotI*-3XV5-ura4+
JP1929	pREP4X-*NotI*-Mit1*Δ(214-269)-NotI*-3XV5-ura4+
JP1991	pREP4X-*NotI*-Mit1*Δ(446-502)-NotI*-3XV5-ura4+
P1982[β]	pREP41-NTAP-LEU2
JP1412	pREP41-NTAP-*NdeI*-Mit1-*BamHI*-LEU2
JP1428	pREP41-NTAP-*NdeI*-Mit1 *K587A-BamHI*-LEU2
JP1414	pREP41-NTAP-*NdeI*-Mit1Δ(214-269)-*BamHI-LEU2*
JP1988	pREP41-NTAP-*NdeI*-Mit1*Δ(446-502)-BamHI*-LEU2
pGEX-KG	pGEX-KG-GST-AMP
JE102	pGEX-KG-GST-*BamHI*-GST-Mit1(205-273)-*EcoRI*-AMP
JE184	pGEX-KG-GST-*BamHI*-GST-Mit1(447-499)-*EcoRI*-AMP
JP1509[γ]	pGEX-KG-GST-Ing2 PHD-AMP

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Table A-3. Oligonucleotide primers used in this study.

Primer use	Sequence
70N0 nucleosome forward primer	5'-CGAGCTCGGTACTCGGGAGCTCGGA-3'
70N0, 0N0 nucleosome reverse primer	5'-CCGAGAGAATCCCGGTGCCGAGGGC-3'
29N59 nucleosome forward primer	5'-ACCGGCAAGGTCGCTG-3'
29N59 nucleosome reverse primer	5'-ACAGCTATGACCATGATTACGCC-3'
70N70 nucleosome reverse primer	5'-TCACACAGGAAACAGCTATGACC-3'
0N0 nucleosome forward primer	5'-CAGGATGTATATCTGACACGTGC-3'
tlh1 real-time primer	5'-CGTTTTTGATACCGGCGC-3'
tlh1 real-time primer	5'-AAAGGTGATGAATATGGTCACGC-3'
adh1 real-time primer	5'-AACGTCAAGTTCGAGGAAGTCC-3'
adh1 real-time primer	5'-AGAGCGTGTAAATCGGTGTGG-3'
cen dh real-time primer	5'-CCAGACCATTACAAGCACTACATACG-3'
cen dh real-time primer	5'-GAATCTTCTCTTGAATAAAACCGCC-3'
cen dg real-time primer	5'-GATACTGATAATATTGAGATCCACAGCAC-3'
cen dg real-time primer	5'-GCGATGCCAAACAACAATATTG-3'
end of telomere 1 real-time primer	5'-ACCGTATTTCATTTCTATTTCTTTATTCAA-3'
end of telomere 1 real-time primer	5'-GGGAATTTAGGAAGTGCGGTAA-3'
mcp4 real-time primer	5'-GTCGAAGGCATGTCACAAGA-3'
mcp4 real-time primer	5'-TGGCTCTCTCTCGGATTCAC-3'
slx1 real-time primer	5'-TGGGGAAATAGTTGGTGGTG-3'
slx1 real-time primer	5'-TCGAGATTCCCAGATTTTGC-3'
coq10 real-time primer	5'-CCGCGCTTCTAGACTTATGC-3'
coq10 real-time primer	5'-AAGGTCGGCCTTTGTAGGAT-3'
SPAC18G6.09c real-time primer	5'-CAGCGATGTCGAGTTCAGTT-3'
SPAC18G6.09c real-time primer	5'-CCATTTCGCTTACCATTGCT-3'
Mat2Pc real-time primer	5'-GCTTCAGCCAAATGCTCAAT-3'
Mat2Pc real-time primer	5'-ATGCGCTCTAACTTGGCAAT-3'

Table A-3.   (*Continued*).

Primer use	Sequence
MTR cenH homology real-time primer	5'-TTCTTATCAAACACATGCAAACG-3'
MTR cenH homology real-time primer	5'-TTCCCAAGAACTGCTGAGGT-3'
MTR Reconstitution forward	5'-AAATATAGTAATATGCTGGTATGGACATAGC-3'
MTR Reconstitution reverse	5'-CTTCCAATATCTGAACAATGATCATC-3'

Table A-4. **List of primers used for nucleosome scanning experiments at *REII*.**

Sequence	Position	Amplicon	
		Center	Length
5'-AAAAATCAACCAGTAGGACATTTTTA-3'	2687	2735	96
5'-TTTTTGCTGAGTTTAGGTTGGAA-3'	2783		
5'-CTCCACTTAAAAGAATAAAAGGAATTT-3'	2731	2777.5	93
5'-GGTGATTGTTTTTGTTTTTATGTTT-3'	2824		
5'-ATTCCAACCTAAACTCAGCAAAA-3'	2760	2808.5	97
5'-GCAATCATAGATTTGGTTTTCTTTAT-3'	2857		
5'-ACATAAAAACAAAAACAATCACCAAA-3'	2802	2848.5	93
5'-GTTCTTTTTATTGCGATTTATGTCT-3'	2895		
5'-CAAAAACAATCACCAAAAATTATAAAG-3'	2811	2857	92
5'-ATTAGTTTGTTCTTTTTATTGCGATTT-3'	2903		
5'-AAAGAAAACCAAATCTATGATTGC-3'	2834	2869	70
5'-AATTAGTTTGTTCTTTTTATTGCGATT-3'	2904		
5'-AAAAAGACATAAATCGCAATAAAAAGA-3'	2867	2915	96
5'-TTGAAAATGTTGTTAGTTCTTCCTT-3'	2963		
5'-CGCAATAAAAAGAACAAACTAATTAAC-3'	2881	2927	92
5'-TGTTGAGGAATTGAAAATGTTGTT-3'	2973		

Table A-4. (*Continued*).

Sequence	Position	Amplicon	
		Center	Length
5'-AAGAACTAACAACATTTTCAATTCCTCA-3'	2943	2977.5	69
5'-TATTTATGAAATAATTGTGGTTTAAATGG-3'	3012		
5'-AATGAATTCCATTTAAACCACAA-3'	2976	3021	90
5'-TGTTATTTGTTTTCCTACTTGTTAATG	3066		
5'-TCCATTTAAACCACAATTATTTCATA-3'	2983	3027	88
5'-AAGGAACATGTTATTTGTTTTCCTA-3'	3071		
5'-TATGAATACCACATTAACAAGTAGGAA-3'	3029	3072.5	87
5'-TTTAGTTTATAATTTTGTCCGGTTTG-3'	3116		
5'-AATAACATGTTCCTTCGCCTACG-3'	3060	3108	96
5'-TGGGTCCTTATAATTGTGTACTTTT-3'	3156		
5'-TAAGTTTACAAACCGGACAAAATTA-3'	3083	3128.5	91
5'-TAGTAATCCGTTGTTGTATGGGTCCT-3'	3174		
5'-CAATTATAAGGACCCATACAACAACG-3'	3141	3188.5	95
5'-CCAAGCAAGCAACTGTTTAAGG-3'	3236		
5'-CTTGCTTGGCAGCCTCGTA-3'	3228	3275	94
5'-ATGGCTATTAAATTAAAGGTTGTATGC-3'	3322		

Table A-4. (*Continued*).

Sequence	Position	Amplicon Center	Length
5'-GCAGCCTCGTAGGCTTTTA-3'	3236	3281.5	91
5'-GTTGTATGGCTATTAAATTAAAGGTTG-3'	3327		
5'-TAGCATACAACCTTTAATTTAATAGCC-3'	3294	3338	88
5'-ATAAACTACATATAAGCAGGGTTCAGT-3'	3382		
5'-GCCATACAACCTAATAGCCAGAA-3'	3318	3363.5	91
5'-CAAAAAGATTAAATGGACACAACA-3'	3409		
5'-ACTGAACCCTGCTTATATGTAGTTTAT-3'	3356	3400	88
5'-GAGACAAATTGCCAAGTTAGAGC-3'	3444		

Table A-5. **List of primers used for nucleosome scanning experiments at Cen1.**

Sequence	Position	Amplicon	
		Center	Length
5'-ATGGAACATGGGAAATTGATATG-3'	3755180	3755227	93
5'-TGTGTCGATGTAGTTCTCTATACCA-3'	3755273		
5'-ATTGATATGGACCTTGATATGGAC-3'	3755206	3755250	87
5'-GCAACTTTTGTTTTCTTTTCTGTG-3'	3755293		
5'-ATGGTATAGAGAACTACATCGACACA-3'	3755248	3755290	83
5'-GTACTATGGCCTGTTGATTCG-3'	3755331		
5'-AGAACTACATCGACACAGAAAAGA-3'	3755257	3755303	91
5'-GTATTTGGATTCCATCGGTACT-3'	3755348		
5'-AAAAGTTGCTAAAATGACAAAGGT-3'	3755285	3755328	86
5'-GATGTTTGAATGATTCTTGGATTG-3'	3755371		
5'-AGGCCATAGTACCGATGGAA-3'	3755320	3755364	87
5'-TTGTTCAAAACAACAATGTCGAG-3'	3755407		
5'-TCATTCAAACATCGCAAACATC-3'	3755359	3755403	88
5'-CGTTGAATGTTGTTGCTTTCA-3'	3755447		
5'-GCTCGACATTGTTGTTTTGAAC-3'	3755384	3755427	85
5'-AACAGCGAGAAATTTGTAGATACG-3'	3755469		

Table A-5. (*Continued*).

Sequence	Position	Amplicon	
		Center	Length
5'-TACTAAGAGTTGCAAATGTGAAAGCA-3' 5'-AAATAGAAATGTATATGCAAAACAGC-3'	3755409 3755489	3755449	80
5'-GCAACAACATTCAACGTATCTACA-3' 5'-GCTCCATTTCTTTCTTATACGC-3'	3755432 3755523	3755478	91
5'-CTCGCTGTTTTGCATATACATTTCT-3' 5'-CACCTACTCTTATCACTTGTAATCCA-3'	3755461 3755554	3755508	93
5'-GTGAATTAGCGTATAAGAAAGAAATG-3' 5'-TCAATAAATGAGTCCTACTCCTACAC-3'	3755494 3755577	3755536	83
5'-GCGTATAAGAAAGAAATGGAGCTT-3' 5'-ACGCAAATCACCAGATTCAA-3'	3755502 3755593	3755548	91
5'-TGGATTACAAGTGATAAGAGTAGGTG-3' 5'-CGCTCTTTATATTCTCAACCTTCC-3'	3755529 3755618	3755574	89
5'-AGTAGGACTCATTTATTGAATCTGG-3' 5'-ATTACATGGCTTAGTTTCACACG-3'	3755559 3755639	3755599	80
5'-GGTGATTTGCGTCGGAAG-3' 5'-CGAGATCTGAAAGTTTCGGTAA-3'	3755582 3755669	3755626	87

Table A-5. (*Continued*).

Sequence	Position	Amplicon	
		Center	Length
5'-GTTGAGAATATAAAGAGCGTGTGA-3'	3755600	3755642	84
5'-GGATCGGTAAATAGGCGAGA-3'	3755684		
5'-AAGCCATGTAATCTAAAACTTTACCG-3'	3755628	3755673	90
5'-GAAAATTCAAGCAACTATTACTGCAC-3'	3755718		
5'-CTTTCAGATCTCGCCTATTTACC-3'	3755657	3755703	91
5'-CGGTTTTATTCAAGAGAAGATTCA-3'	3755748		
5'-CGATCCAATTGCTAGTGCAG-3'	3755679	3755722	85
5'-GACTAGCTCTTATTGGCGGTTT-3'	3755764		
5'-TTCACCGGATGAATCTTCTCTT-3'	3755716	3755761	90
5'-CATACGCCATCTTCAATATCGT-3'	3755806		
5'-CCGCCAATAAGAGCTAGTCAA-3'	3755746	3755790	87
5'-CCATCCACCAGACCATTACA-3'	3755833		
5'-ATATTGAAGATGGCGTATGTAGTG-3'	3755788	3755834	91
5'-TCCATTTGTGTAATACTGAATGCT-3'	3755879		
5'-TAATGGTCTGGTGGATGGTG-3'	3755816	3755860	88
5'-AAAATCGTTTACCGCTTCTCC-3'	3755904		
5'-TTTCCACTCTTACTTAGCATTCAG-3'	3755841	3755886	90
5'-CAACCCATTATAATAAACGAACCT-3'	3755931		

Table A-5.   (*Continued*).

Sequence	Position	Amplicon	
		Center	Length
5'-TACACAAATGGATTAAGGAGAAGC-3'	3755868	3755910	84
5'-CTCAATTTGAATCGTGTCACTC-3'	3755952		

VITA

The son of parents Geoffrey and Ramona Creamer, Kevin Michael Creamer was born in Oxford, Ohio in 1984. After completion of his Bachelor of Science degree in chemistry with an emphasis on biochemistry from Tennessee Technological University (Cookeville, TN) in the spring of 2007, he enrolled in the Integrated Program of Biomedical Sciences at the University of Tennessee Health Science Center. His dissertation work was performed under the supervision of Janet Partridge, Ph.D. at St. Jude Children's Research Hospital, during which time he was awarded a National Research Service Award (F31) fellowship by the National Institutes of Health.

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3. Chen JS, Lu LX, Ohi MD, **Creamer KM**, English C, Partridge JF, Ohi R, Gould KL. Cdk1 phosphorylation of the kinetochore protein Nsk1 prevents error-prone chromosome segregation. J Cell Biol. 2011 Nov 14;195(4):583-93.

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5. **Creamer KM,** Job G, Shanker S, Neale G, Lin Y, Bartholomew B, Partridge, JF. The Mi-2 homolog Mit1 actively positions nucleosomes within heterochromatin to suppress transcription. Submitted article.