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Integrated Multimodal Genomic Analyses Reveal Novel Mechanisms of Glucocorticoid Resistance in Acute Lymphoblastic Leukemia

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Integrated Multimodal Genomic Analyses Reveal Novel Mechanisms of Glucocorticoid Resistance in Acute Lymphoblastic Leukemia

Abstract

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer. Much has been discovered in recent decades regarding ALL biology, and the outcome of patients with ALL has vastly improved, especially in pediatric ALL patients. Despite very promising overall cure rates, patients who relapse have a greatly decreased prognosis with survival rates ranging from 30-60%. These numbers stand to improve even further with new targeted therapies that seek to improve or maintain cure rates while reducing treatment related toxicities which affect patients both acutely and chronically. Glucocorticoids (GCs) are essential components of modern chemotherapeutic intervention for ALL. Resistance to glucocorticoids is an important factor in determining early treatment response and overall patient survival. Reduction of glucocorticoid induced toxicities, such as osteonecrosis, can significantly affect patient quality of life and are associated with high dose glucocorticoid treatment in pediatric patients. Both endogenous and exogenous glucocorticoids exert their mechanism of action through various pleiotropic effects that regulate numerous cellular functions and can cause selective cytotoxicity in lymphoid malignancies. The complex mechanism of action of glucocorticoids is evident in the number of diverse clinically relevant molecular pathways that have been previously associated with resistance to glucocorticoids in ALL. The identification of genomic and epigenomic mechanisms of glucocorticoid resistance are important for improving ALL treatment outcomes. We used an agnostic genome-wide method to interrogate multiple types of genomic information (mRNA and miRNA expression, DNA methylation, SNPs, CNAs and SNVs/Indels) in primary human acute lymphoblastic leuke

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DOCTOR OF PHILOSOPHY DISSERTATION

**Integrated Multimodal Genomic Analyses Reveal
Novel Mechanisms of Glucocorticoid Resistance in
Acute Lymphoblastic Leukemia**

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in

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DEDICATION

This dissertation is dedicated first and foremost to my wonderful wife, Lindsay. You have supported my path every step of the way, inspired me to work harder and challenged me to reach higher, without you this would have been impossible. To my amazing children Fletcher and Sloane, for providing me with endless joy to distract from the hard days. Never give up on your dreams no matter what stands in your way, and may you learn from failure so that someday you can make a difference. To my parents Don and Lorie, who have pushed me and supported me to achieve my potential from childhood. To my Nana and Papa, who taught me to work hard, to take chances and to always remember importance of family. Finally, to my grandmother Janette who was the best example of hard work, the value of education and speaking truth for its own sake.

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer. Much has been discovered in recent decades regarding ALL biology, and the outcome of patients with ALL has vastly improved, especially in pediatric ALL patients. Despite very promising overall cure rates, patients who relapse have a greatly decreased prognosis with survival rates ranging from 30-60%. These numbers stand to improve even further with new targeted therapies that seek to improve or maintain cure rates while reducing treatment related toxicities which affect patients both acutely and chronically.

Glucocorticoids (GCs) are essential components of modern chemotherapeutic intervention for ALL. Resistance to glucocorticoids is an important factor in determining early treatment response and overall patient survival. Reduction of glucocorticoid induced toxicities, such as osteonecrosis, can significantly affect patient quality of life and are associated with high dose glucocorticoid treatment in pediatric patients. Both endogenous and exogenous glucocorticoids exert their mechanism of action through various pleiotropic effects that regulate numerous cellular functions and can cause selective cytotoxicity in lymphoid malignancies. The complex mechanism of action of glucocorticoids is evident in the number of diverse clinically relevant molecular pathways that have been previously associated with resistance to glucocorticoids in ALL.

The identification of genomic and epigenomic mechanisms of glucocorticoid resistance are important for improving ALL treatment outcomes. We used an agnostic genome-wide method to interrogate multiple types of genomic information (mRNA and miRNA expression, DNA methylation, SNPs, CNAs and SNVs/Indels) in primary human acute lymphoblastic leukemia cells. We identified 463 genomic features associated with glucocorticoid resistance. Gene-level aggregation by a novel statistical method (TAP) identified 118 overlapping genes, 15 of which were confirmed by genome-wide CRISPR screening. Upon review of known glucocorticoid resistance mechanisms, we directly identified 30 of 38 (79%) genes/miRNAs and all 38 known resistance pathways, revealing 14 of 15 of our top candidate genes were not previously associated with glucocorticoid-resistance. *CELSR2*, the top novel gene downregulated in glucocorticoid resistant ALL was corroborated by single cell RNAseq and network-based transcriptomic modeling (NetBID). shRNA knockdown of *CELSR2* recapitulated glucocorticoid resistance in human leukemia cell lines and revealed a synergistic drug combination (prednisolone and venetoclax), based on high BCL-2 expression, that was able to mitigate glucocorticoid resistance in mouse xenografts.

In summation, we illustrated the power of a multi-dimensional integrative genomic strategy for elucidating genes and pathways conferring glucocorticoid resistance in patients with ALL. These findings will provide important new targets for treating glucocorticoid resistant ALL.

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LIST OF ABBREVIATIONS

AA	Homozygous Allele A
AB	Heterozygous Allele A and B
ABL	Abelson Tyrosine Protein Kinase
ADME	Absorption/Administration, Distribution, Metabolism, Excretion
ADR	Adverse Drug Response
AKT	AKT Serine/Threonine Kinase
ALAS2	5'-Aminolevulinate Synthase 2
ALL	Acute Lymphoblastic Leukemia
AMPK	5' AMP-activated Protein Kinase
ANXA2	Annexin A2
AP-1	Activator Protein 1
APAF1	Apoptotic Peptidase Activating Factor 1
ARACNE	Algorithm for the Reconstruction of Accurate Cellular Networks
ARID1A	AT-Rich Interaction Domain 1A
ARID5B	AT-Rich Interactive Domain-Containing Protein 5B
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
ATP	Adenosine Triphosphate
BAD	BCL2 Associated Agonist of Cell Death
Bag-1	BCL2 Associated Athanogene 1
BAK1	BCL2 Antagonist/Killer 1
BALLi	B-ALL Interactome
BAX	BCL2 Associated X, Apoptosis Regulator
BB	Homozygous Allele B
BCL2	B-Cell CLL/Lymphoma 2
BCL2L11	BCL2 Like 11
BCL6	B Cell CLL/Lymphoma 6 Transcriptional Repressor
BCL-XL	B-cell Lymphoma Extra Large
BCR	Breakpoint Cluster Region
BIM	Bcl-2-interacting mediator of cell death
BMI1	B Lymphoma Mo-MLV Insertion Region 1 Homolog
Ca ²⁺	Calcium
CALN1	Calneuron 1
cAMP	Cyclic Adenosine Monophosphate
CAPN3/10	Calpain 3/10
CARD11/17	Caspase Recruitment Domain Family Member 11/17
CASP1	Caspase 1
CBP	CREB Binding Protein
CD14	Monocyte Differentiation Antigen CD14
CD19	B-Lymphocyte Antigen CD19
CD3E	T-Cell Surface Glycoprotein CD3 Epsilon Chain
CD45	CD45 Antigen
CDF	Cumulative Distribution Function
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B

CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CEBPE	CCAAT Enhancer Binding Protein Epsilon
CELSR2	Cadherin EGF LAG Seven-Pass G-Type Receptor 2
cGMP	Cyclic Guanosine Monophosphate
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CLL	Chronic lymphocytic leukemia
CN	Copy Number
CNA	Copy Number Alteration
CNR2	Cannabinoid Receptor 2
CNS	Central Nervous System
CNV	Copy Number Variant
Comb	Combined
CpG	Phosphorylated Cytosine Guanosine
CR	CRISPR
CREB1	cAMP Responsive Element Binding Protein 1
CREBBP	CREB Binding Protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRLF2	Cytokine Receptor Like Factor 2
CTCF	CCCTC-Binding Factor
CYP11B1	Cytochrome P450 Family 11 Subfamily B Member 1
DAP	Death Associated Protein
DCLRE1A	DNA Cross-Link Repair 1A
DCOG	Dutch Childhood Oncology Group
COALL	Co-operative Study Group for Treatment of Childhood ALL
Del	Deleterious
DEX	Dexamethasone
DNA	Deoxyribonucleic Acid
EFS	Event-Free Survival
EMP1	Epithelial Membrane Protein 1
ENCODE	Encyclopedia of DNA Elements
ERK	Extracellular Signal-Regulated Kinase
ETP	Early T-cell Precursor
ETV6	ETS Variant Transcription Factor 6
FAM13A	Family with Sequence Similarity 13 Member A
FBS	Fetal Bovine Serum
FBXO9	F-Box Protein 9
FBXW7	F-Box and WD Repeat Domain Containing 7
FDR	False Discovery Rate
FET	Fisher's Exact Test
FGF10	Fibroblast Growth Factor 10
FGFR2	Fibroblast Growth Factor Receptor 2
FKBP52	FK506-binding protein 4
FLT3	Fms Related Tyrosine Kinase 3
FOS	FBJ Murine Osteosarcoma Viral Oncogene Homolog
GALNT1	Polypeptide N-Acetylgalactosaminyltransferase 1

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA3	GATA Binding Protein 3
GC	Glucocorticoid
GeCKOv2	Genome Wide CRISPR/Cas9 Knockout Version 2
GEO	Gene Expression Omnibus
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
gRNA	Guide RNA
GSK3B	Glycogen Synthase Kinase 3 Beta
GSTM1	Glutathione S-Transferase Mu 1
GWAS	Genome Wide Association Study
H3K27	Histone3 Lysine 27
H3K27Ac	Histone3 Lysine 27 Acetylation
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
hg19	Human Genome Build 19
HRP	Horse Radish Peroxidase
HSD11B2	Hydroxysteroid 11-Beta Dehydrogenase 2
HSD3B1/7	3-Beta-Hydroxy-Delta(5)-Steroid Dehydrogenase 1/7
HSP70/90	Heat Shock Protein 70 or 90
IDI1	Isopentenyl-Diphosphate Delta Isomerase 1
IFN γ	Interferon Gamma
IGR	Intronic Glucocorticoid Receptor Binding Region
IKBKB	Inhibitor of Nuclear Factor Kappa B Kinase Subunit Beta
IKBKG	Inhibitor of Nuclear Factor Kappa B Kinase Subunit Gamma
IKZF1	IKAROS Family Zinc Finger 1
IL	Interleukin
IL1B	Interleukin 1 Beta
IL1RAPL2	Interleukin 1 Receptor Accessory Protein Like 2
IL4R	Interleukin 4 Receptor
IL7R	Interleukin 7 Receptor
Indel	Insertion/Deletion
IQR	Interquartile Range
IRAK3	Interleukin 1 Receptor Associated Kinase 3
ITGA4/5/B1	Integrin Subunit Alpha 4/5 and Beta 1
ITPR3	Inositol 1,4,5-Trisphosphate Receptor Type 3
JAK	Janus Kinase
JNK	C-Jun N-Terminal Kinase
JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit
kb	Kilobase(s)
KLF13	Kruppel Like Factor 13
KMT2A/2D	Lysine Methyltransferase 2A/2D
KRAS	Kirsten Rat Sarcoma Viral Proto-Oncogene
LC ₅₀	Lethal Concentration for 50% of population
LCK	Leukocyte C-Terminal Src Kinase

LD	Linkage Disequilibrium
MAD	Median Absolute Deviation
MaGeCK	Model-Based Analysis of Genome Wide CRISPR/Cas9 Knockout
MAPK	Mitogen-Activated Protein Kinase
MAPK13	Mitogen Activated Protein Kinase 13
MAPK9	Mitogen-Activated Protein Kinase 9
MCL1	Myeloid Cell Leukemia Sequence 1 (BCL2-Related)
MEK	Mitogen-Activated Protein Kinase Kinase
miR	microRNA
miRDIP	microRNA Data Integration Portal
mirTarbase	database of MicroRNA-Target Interactions.
MLL	Mixed Lineage Leukemia
mM	Millimolar
MRD	Minimal Residual Disease
mRNA	Messenger RNA
MTOR	Mechanistic Target of Rapamycin Kinase
mTORC1	Mechanistic Target of Rapamycin Kinase Complex1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYB	V-Myb Avian Myeloblastosis Viral Oncogene Homolog
NALP3	NACHT, LRR and PYD domains-containing protein 3
NCOR	Nuclear Receptor Corepressor
NetBID	Network-based Bayesian Inference of Drivers
NF1	Neurofibromin 1
NFAT	Nuclear Factor of Activated T Cells
NFKB2	Nuclear Factor Kappa B Subunit 2
NFKBIB	NFKB Inhibitor Beta
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
nGRE	Negative Glucocorticoid Response Element
NLRC3	NLR Family CARD Domain Containing 3
NLRP3	NLR Family Pyrin Domain Containing 3
nM	Nanomolar
NOXA	aka. PMAIP Latin for "Damage"
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
NRAS	Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog
NRIP1	Nuclear Receptor Interacting Protein 1
NSG	NOD Scid Gamma
NTC	Non Targeting Control
P2RY8	Purinergic Receptor P2Y, G-Protein Coupled, 8
p38	Mitogen-Activated Protein Kinase P38
PARD3	Par-3 Family Cell Polarity Regulator
PARP1	Poly(ADP-Ribose) Polymerase 1
PAX5	Paired Box Gene 5 (B-Cell Lineage Specific Activator Protein)
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE4B	Phosphodiesterase 4B

PDX	Patient Derived Xenograft
PG	Polygenomic
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PI3KR1/3	Phosphoinositide-3-Kinase Regulatory Subunit 1/3
PI3K δ	Phosphatidylinositol 3-Kinase, Catalytic, Delta Polypeptide
PIK3C2B	Phosphatidylinositol 3-Kinase Class 2 Beta
PIK3CD	Phosphatidylinositol 3-Kinase, Catalytic, Delta Polypeptide
PIP4K2A	Phosphatidylinositol-5-Phosphate 4-Kinase Type 2 Alpha
pJNK	Phosphorylated c-Jun N-Terminal Kinase
PMAIP	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
PP2	Src Kinase Inhibitor PP2
Pr	Probability
PRED	Prednisolone
PRKCB	Protein Kinase C Beta
PTEN	Phosphatase and Tensin Homolog
PTPN11	Protein Tyrosine Phosphatase Non-Receptor Type 11
PTPRF	Protein Tyrosine Phosphatase Receptor Type F
PTTG1	PTTG1 Regulator of Sister Chromatid Separation, Securin
PTTG1IP	PTTG1 Interacting Protein
PVDF	Polyvinylidene Difluoride
RAC2	Rac Family Small GTPase 2
RASGRF2	Ras Protein Specific Guanine Nucleotide Releasing Factor 2
RBMS2	RNA Binding Motif Single Stranded Interacting Protein 2
RIPK1	Receptor Interacting Serine/Threonine Kinase 1
RNA	Ribonucleic Acid
Rsq	R-Squared Value
RUNX1	RUNX Family Transcription Factor 1
S.D.	Standard Deviation
S100A8/9/10	S100 Calcium Binding Protein A8/A9/A10
S6K1	Ribosomal Protein S6 Kinase B1
SAPK	Stress-Activated Protein Kinase
shCELSR2	shRNA for CELSR2
shRNA	Short Hairpin Ribonucleic Acid
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4
SMARCB1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily B, Member 1
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
SR	Standard Risk
STAT	Signal Transducer and Activator of Transcription
SWI/SNF	SWItch/Sucrose Non-Fermentable
TAOK3	TAO Kinase 3
TAP	Truncated Aggregation of P-values
TARGET	Therapeutically Applicable Research to Generate Effective Treatments

TBL1XR1	Transducin Beta Like 1 X-Linked Receptor 1
TBS	Tris Buffered Saline
TEAD4	TEA Domain Transcription Factor 4
TF	Transcription Factor
TLR	Toll Like Receptor
TLX	T Cell Leukemia Homeobox 1
TMEM126A	Transmembrane Protein 126A
TNF	Tumor Necrosis Factor
Tol	Tolerated
TOTXV	Total Therapy 15 Protocol
TOT-XV	Total Therapy 15 Protocol
TOTXVI	Total Therapy 16 Protocol
TOT-XVI	Total Therapy 16 Protocol
TP53	Tumor Protein 53
TP53INP1	Tumor Protein P53 Inducible Nuclear Protein 1
TSC22D3	TSC22 Domain Family Member 3
TXNIP	Thioredoxin Interacting Protein
UMI	Unique Molecular Identifier
USP9X	Ubiquitin Specific Peptidase 9 X-Linked
WES	Whole Exome Sequencing
WNK1	Protein Kinase with No Lysine 1
WNT	Wingless-Type MMTV Integration Site Family
XIAP	X-Linked Inhibitor of Apoptosis
μM	Micromolar
3' UTR	3 Prime Untranslated Region
5' UTR	5 Prime Untranslated Region

CHAPTER 1. INTRODUCTION

Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm composed of immature white blood cells known as lymphoblasts which can accumulate in the peripheral blood, bone marrow and other organs causing tissue damage and inhibiting normal hematopoietic cell development. The most common presenting features of childhood ALL (occurring in more than 50% of cases) at or prior to diagnosis are hepatomegaly, splenomegaly, pallor, fever and bruising.¹ Immunophenotype lineage determination by flow cytometric analysis of extracellular surface markers can classify the leukemia as either the more common B-lineage ALL (85% of cases) or T-lineage ALL.²

Prevalence

ALL is the most prevalent type of cancer in children accounting for 25% of all childhood cancer diagnoses with an estimated 3,000 cases per year in children from ages 1-19 (peaking in incidence from ages 2-4 in industrialized countries). Notably, there has been an upward trend in pediatric ALL incidence over the past 40 years.^{3,4} Recent studies have proposed that early exposure to infections (i.e. infant day care attendance) may provide protection from childhood ALL.^{5,6}

Molecular Subtypes of ALL

The majority of B-ALL (approximately 75%) exhibit aneuploidy or have recurring large chromosomal rearrangements that have been shown to be important in leukemogenesis,^{7,8} but these are unable to fully explain the complex biology of the disease.⁹ Until the last decade, ALL was described by either aneuploidy resulting in large chromosomal gains (hyperdiploidy) or losses (hypodiploidy) or by four more commonly observed translocations including 25% of patients with t(12;21) [*ETV6-RUNX1*], 5% of patients with t(1;19) [*TCF3-PBX1*], 3% of patients having t(9;11) [*BCR-ABL1*] and 5% of patients who have MLL gene translocations.

These genetic changes have important prognostic significance. For example, *ETV6-RUNX1* ALL has a favorable prognosis (97.6% vs 83% 5-year EFS).¹⁰ Conversely, *BCR-ABL1*-translocation is a high-risk feature but has been more successfully treated recently since the inclusion of targeted tyrosine kinase inhibitors into clinical protocols.¹¹ Genome-wide DNA and RNA sequencing has identified many submicroscopic alterations that may help define new molecular subtypes of ALL. Also, there are alterations seen in many protooncogenes (Ras pathway), tumor-suppressors (e.g. *CDKN2A/B*) and B-cell developmental genes (*PAX5*¹² and *IKZF1*¹³) which in the case of *PAX5* have led to the discovery of new distinct molecular subtypes of ALL (*PAX5 P80R* and *PAX5alt*) that

bring the current total to 23 unique molecular subtypes of B-ALL.¹⁴ Even with the exceptional advances in understanding the genomic landscape of ALL, the precise mechanisms underlying the pathogenesis of ALL are not fully understood, and further study is required to derive the initiating genetic lesions in many cases.

Germline Variation Involved in ALL Development

In contrast to studies looking at acquired somatic variants, the host genome and inherited germline polymorphisms have arisen as important factors susceptibility of individuals to develop childhood ALL and have been found to also affect the severity of the resulting disease. It was discovered that there is a link between germline mutations in the tumor suppressor *TP53* that are known to cause Li-Fraumeni Syndrome and an increased incidence of hypodiploid ALL in affected patients.¹⁵ Also, recurrent inherited heterozygous germline mutations within the octapeptide domain of the *PAX5* gene were discovered by studying two unrelated families and were highly associated with ALL occurrence in these patients.¹⁶ Genome-wide association studies (GWAS) identified germline variants in genes associated with increased risk of ALL occurrence *ARID5B*, *IKZF1*, *CEBPE*, *ETV6*, *CDKN2A* and *PIP4K2A-BMII*.¹⁷⁻¹⁹ Also, *GATA3* SNPs were associated with increased incidence of Ph-like ALL and increased risk of relapse which served as an example that inherited variants can cause development of specific subtypes of ALL.²⁰

Refinement of ALL Therapy and Improved Outcomes

Over the last 50 years, there have been exceptional gains in the prognostic outlook for pediatric ALL. From the inception of St. Jude Children's Research Hospital in 1962, the cure rate for ALL was less than 10% and it has now increased to greater than 90%.²¹ Recently, similar cure rates have been achieved in nationwide multi-center protocols with the most recently completed Children's Oncology Group study AALL0331 reporting an 89% 6 year event-free survival (EFS) and 95.5% overall survival rate in children with newly diagnosed standard risk (SR) ALL.²² These improvements can be attributed to a number of important advances. Initially, multi-agent chemotherapy regimens with longer duration, methods to reduce secondary infection and reduction in CNS involvement by targeted therapy provided the first wave of improvement in patient outcomes. More recently, refinements in treatment protocols have been derived from an increased understanding of the biological heterogeneity of ALL and the ability to monitor minimal residual disease (MRD)^{23,24} which has allowed for refinement of protocols to stratify patients based on risk of relapse to greatly maximize cure rates while reducing toxicity. Despite the vast improvements in treatment outcome, ALL remains a leading cause of childhood cancer-related death. Relapsed ALL though occurring at a much lower rate has a very poor prognosis with an overall survival rate of 30-60%.²⁵⁻²⁸ Assessment of drug resistance shows that leukemia blasts are more resistant to a variety of agents at relapse than at diagnosis and both *de novo* and acquired resistance are thought to be key determinants of relapse in ALL.^{29,30} Great efforts have been made to discover ways to

prevent drug resistance in ALL in hopes of decreasing the occurrence of toxicity caused by conventional chemotherapy while maintaining or improving treatment outcomes.

Glucocorticoid Mechanism of Action

The glucocorticoid receptor (GR) is a member of the steroid hormone receptor family of nuclear receptors. It functions as the receptor for the endogenous glucocorticoid cortisol. The function of endogenous glucocorticoids is to minimize inflammatory response through control of the Hypothalamic-Pituitary-Adrenal Axis.³¹ Synthetic glucocorticoids (e.g. dexamethasone and prednisone/prednisolone) can also bind the GR with high affinity and have been used therapeutically to treat inflammatory conditions such as rheumatoid arthritis.³² The inactive glucocorticoid receptor is bound in the cytosol by chaperones heat shock proteins *HSP70* and *HSP90* and co-chaperones (e.g. *FKBP52*). Glucocorticoids enter the cell and bind to the inactive glucocorticoid receptor and cause it to dissociate from its chaperone proteins.³³ The unbound glucocorticoid receptor is then translocated to the nucleus and can homodimerize with other glucocorticoid receptor monomers while binding to DNA at glucocorticoid response elements (GREs) to induce gene transcription (transactivation) or repress gene expression either directly or indirectly (transrepression). Binding site availability may vary based on tissue-specific chromatin accessibility.

Positive Glucocorticoid Receptor Functions

The most commonly accepted mechanism of transactivation suggests that the GR dimerization causes a conformational change in the GR and then it recruits co-factors like histone acetyl transferases (HATs) leading to proximal opening of the chromatin to facilitate gene expression. It has recently been observed that monomeric GR binding at “half-sites” can occur and drive transcription through transactivation and at negative GRE sites (nGREs)³⁴ to repress gene expression in the same manner. It has been shown that exogenous GCs favor homodimeric GR mechanism and disrupt these “half-sites”^{35,36}.

Negative Glucocorticoid Receptor Functions

The GR can exert negative effects on gene expression by a number of mechanisms. One important mechanism of direct transrepression is through protein-protein interactions known as “tethering” where monomeric GR cross-talks with another transcription factor (TF). This can also be seen in the context of transactivation in some cases involving assistance from co-factors. Selection of binding partners is a precise mechanism based on the specific DNA binding sequence.^{37,38} Both monomeric and dimeric GR can also compete for overlapping binding sites to act as a repressor by blocking binding of other transcription factors. It has been recently discovered that both inverted repeat nGREs exist to which agonist bound GR can bind two monomers with

reverse polarity to repress expression,³⁴ in some cases the GR β isoform can compete for active GR α binding sites and repress expression of target genes.³⁹

Non-genomic Glucocorticoid Receptor Functions

There are also other factors that can affect the mechanisms of action of the glucocorticoid receptor such as post-translational modifications (phosphorylation, acetylation, ubiquitination, methylation, nitrosylation and SUMOylation) that can regulate a wide array of GR functions. It has been observed previously that there are glucocorticoid induced effects that occur very rapidly (seconds to minutes) which belies the fact that there must be non-genomic GR mechanisms, most of which are thought to be initiated in the cytoplasm.⁴⁰ These mechanisms also include membrane bound GR which has distinct signaling functions^{41,42} and the ability of the GR to regulate transcription of mitochondrially expressed genes the trafficking of which may be mediated by a Bag-1 mediated BCL2/GR protein complex that can translocate into the mitochondria and effect function.⁴³⁻⁴⁶

In summary, the glucocorticoid receptor has a number of complex and diverse functions and regulatory mechanisms which can be highly tissue specific and provide a variety of signals to the cell.

Glucocorticoid Treatment in ALL

Glucocorticoids have been essential components of curative chemotherapy regimens for many decades, and response to this class of drugs remains pivotal in determining early treatment response and treatment outcome.⁴⁷ Glucocorticoids exhibit cell type specific cytotoxicity in lymphoid malignancies and cause cells to inhibit cytokine production, undergo cell cycle arrest and apoptosis. Initially, prednisolone was the most commonly used glucocorticoid in ALL therapy, but dexamethasone use has increased in recent years because of reports that it is better able to prevent CNS infiltration, has a longer half-life and ability penetrate the CNS. However, prednisolone has shown in a number of studies to be associated with lower incidence of an important dose limiting toxicity of glucocorticoid treatment which is osteonecrosis.³³ *Ex vivo* prednisolone response profiling of primary leukemia cells to glucocorticoids was shown to be highly correlated with patient response to therapy and treatment outcome.^{48,49}

Introduction to Pharmacogenomics

Pharmacogenomics is the study of the role of genetics as it relates to drug response essentially combining the two fields of genomics and pharmacology. Response to drug therapy can vary greatly in a population and adverse drug responses (ADRs) are a leading cause of patient mortality in hospitals.⁵⁰ Some patients may have genetic characteristics that make them highly susceptible (sensitive) to drug therapy where they

may need a lower dose of a drug than an average patient and could be at risk to develop adverse drug reactions within a normal dose range. Other patients may be poorly responsive to the drug (resistant), meaning it takes a larger dose to achieve desired therapeutic outcome. Depending on the drug being administered and its therapeutic window there may be dose limiting toxicities at higher concentrations of the drug.^{51,52}

Pharmacogenomic mechanisms can be pharmacokinetic in nature involving difference in drug absorption, distribution, metabolism or excretion (ADME) or pharmacodynamic in nature involving the effects of the drug on the body. Application of pharmacogenomic techniques in pediatric ALL and other diseases provides new avenues to discover therapeutic targets to reduce resistance and toxicity and have shown promising results in prior studies.⁵³ Anticancer agents provide particularly challenging pharmacogenomic questions because of their narrow therapeutic index which factors in determining the clinical actionability of a variant when implementing pharmacogenomics in the clinical setting.⁵⁴

Hypothesis and Specific Aims

Previous research by our group and other groups has shown that glucocorticoid resistance in ALL is defined by a diverse array of genetic changes that can be defined by alterations in important cellular processes that are in many cases involved in the downstream pleiotropic effects of glucocorticoid treatment. Though many studies have looked at glucocorticoid resistance in childhood ALL, relevant clinical strategies have not been developed or implemented to combat this very important challenge. Key mechanisms and actionable targets that are significant in large scale genome-wide clinical genomic studies remain widely undiscovered due in part to the lack of prioritization by large-scale *in vitro* functional candidate gene validation.

We hypothesize that an integrated polygenomic interrogation of primary ALL cells coupled with agnostic genome-wide CRISPR/Cas9 screening of glucocorticoids in leukemia cell lines will identify novel genomic/epigenomic alterations involved in previously undiscovered regulatory mechanisms of glucocorticoid resistance in ALL. To validate our hypothesis we propose the following specific aims.

Aim 1: Genome-Wide CRISPR/Cas9 Knockout Screening

To use genome wide CRISPR/Cas9 knockout screens to identify genes in human acute lymphoblastic leukemia (ALL) cell lines that increase resistance to glucocorticoids.

Aim 2: Integrated Polygenomic Analyses of Glucocorticoid Resistance in Patient ALL

To determine whether variants in genes identified in genome wide CRISPR/Cas9 screens of ALL cell lines are also associated with glucocorticoid resistance genes in primary leukemia cells from newly diagnosed ALL patients (*de novo* resistance).

Aim 3: Functional Mechanisms of Candidate Resistance Genes

To recapitulate drug resistant phenotypes in human ALL cell lines and/or patient derived xenografts by manipulating the expression of candidate genes, as a strategy to better understand biological pathways that are perturbed by genomic variants associated with glucocorticoid resistance.

CHAPTER 2. THE HALLMARKS OF GLUCOCORTICOID RESISTANT ALL

De Novo versus Acquired Resistance

Currently, there are two main proposed mechanisms for how drug resistance occurs in cancer cells. *De novo* (intrinsic) resistance which postulates that mutations or other genomic changes are harbored at initial diagnosis either through somatic change, inherited variance or other phenomenon that may not be directly involved in the acquisition of the cancer phenotype or the malignancy of disease. This theory relies on the heterogeneity of cancer at diagnosis and the fact that the genetic alteration must provide selective advantage for the cells to resist death from drug treatment even if only a small subset of the initial population harbors a selective advantage and persist. Acquired resistance mechanisms are described as new genetic alterations leading to drug resistance in cells that are not present at initial diagnosis but acquired either by new mutations that arise by DNA damage from conventional cytotoxic therapies or other activation of secondary lesions not found at diagnosis.^{55,56} Proper resolution to determine whether these are truly acquired lesions caused by drug-induced DNA damage and that they are not present at a low frequency initially (undetectable in bulk sequencing) is lacking. Recent studies in single cell sequencing have shown that low frequency mutations not seen in bulk sequencing may account for some of the unobserved mutations that appear at relapse and that these methods may allow for determination of mutation acquisition order.⁵⁷

Multidimensional Modalities of Drug Resistance

There are many potential ways that drug resistance can arise in ALL. There are three main categories that we will use to describe the landscape of glucocorticoid resistance in ALL in this chapter **Figure 2-1**.

Genomic Determinants

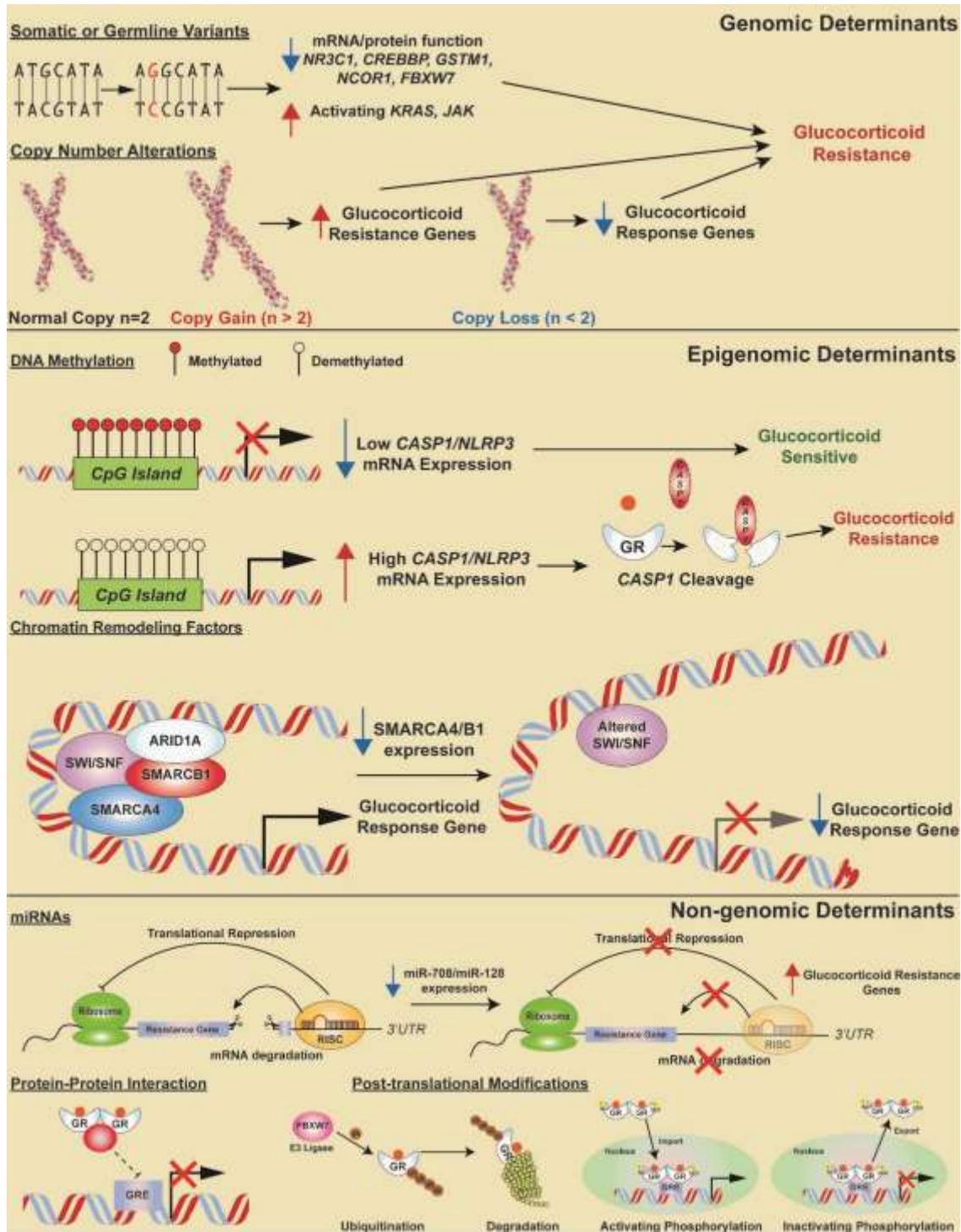
Genomic determinants of drug resistance could be somatic coding or non-coding mutations, copy number variants, germline polymorphisms or other direct changes to the DNA sequence of either the cancer cells or the patient's germline that cause the patient to be resistant to drug therapy. Another potential genomic determinant could be changes in the mRNA expression that cause the cancer cells to be resistant which could be regulated by a variety of external factors.

Epigenomic Determinants

Resistance can also arise from changes in epigenetic factors such as DNA methylation which can directly affect gene expression. Also, changes in chromatin

Figure 2-1. Modalities of drug resistance

Drug resistance can be caused by a diverse array of mechanisms. At the genomic level, there can be mutations or copy number alterations. In some cases, epigenomic changes such as DNA methylation or chromatin structural changes can cause resistance. Also, non-genomic factors such as miRNAs, protein-protein interactions post-translational modifications or other external mechanisms can lead to the drug resistant phenotype.



accessibility due to altered expression or function of SWI/SNF complex components or the histone marks that determine the heterochromatin state of the DNA are known to have important functional roles in the regulation of gene expression and could provide global phenotypic changes when altered especially in the context of resistance.

Non-genomic Mechanisms

Non-genomic mechanisms also are likely to play a role in drug resistance. miRNAs can act both post-transcriptionally and post-translationally to affect the expression of genes and functions of proteins and can cause glucocorticoid resistance if they are dysregulated. Changes in cell metabolism are another avenue of resistance especially to glucocorticoids which exert some of their cytotoxic effect by altering glucose metabolism. There could also be changes in drug metabolizing enzymes or transporters that alter the amount of available drug able to reach its target and lead to resistance. Also, changes that affect proteins directly such as posttranslational modifications that lead to increased turnover or decreased stability may be another path to resistance.

Hallmark Pathways of Glucocorticoid Resistant ALL

In this section we will discuss the many diverse pathways that have been identified previously to be associated with glucocorticoid resistance in ALL.

Glucocorticoid Receptor and Co-Factors

GR Expression

For over 35 years, it has been known that decreased glucocorticoid receptor expression impacts the prognosis for childhood acute lymphoblastic leukemia. Pui, et. al described that across a number of pediatric ALL cohorts low GR expression was associated with both induction failure and more frequent relapse, but when high-risk and standard risk were evaluated separately this was no longer related to outcome suggesting that it is directly related to the treatment efficacy and was not an independent factor.⁵⁸

GR Mutation

There has been some evidence linking glucocorticoid resistance in ALL to somatic glucocorticoid receptor mutations. Initial studies reported that alternative first exons of the GR could affect the response of leukemic cell lines to glucocorticoids.⁵⁹ There have been subsequent reports that somatic mutations were not detected in glucocorticoid resistant patients,⁶⁰ but in larger cohorts a number of deletions and

inactivating mutations have been identified that can lead to loss of GR function and increased relapse risk.^{12,61} In relapsed *ETV6-RUNX1* ALL, mutations in *NR3C1* were acquired through treatment, but it was not explored whether these may have existed at an undetectable frequency at diagnosis.⁶² Since GR mutations are rare, they cannot adequately account for the observed frequency of glucocorticoid resistance. Thus, it is likely that genes upstream or downstream of the GR must be involved in mediating glucocorticoid resistance.

GR Degradation

Altering the degradation rate of the glucocorticoid receptor has significant effects on the ability of cells to respond to glucocorticoid receptor activation by the addition of exogenous glucocorticoids in ALL and other models. Loss of function of the E3 ubiquitin ligase *FBXW7* was associated with good prognosis and early glucocorticoid treatment response in childhood T-ALL.⁶³ This specific degradation event is mediated by glycogen synthase kinase 3 β (*GSK3B*) phosphorylation of GR α at serine 404 (S404) leading to its subsequent ubiquitination and proteasomal degradation.⁶⁴

Co-Factors

NCOR1 (nuclear receptor corepressor 1) mutations were previously associated with relapse in ALL.⁶⁵ Also, higher expression of multiple HDAC family members (*HDAC3*, *HDAC4*, *HDAC7* and *HDAC9*) which are also NCOR complex components were associated with poor prognosis. NCOR complex plays a role in disease outcome in ALL,^{66,67} likely through its relationship to glucocorticoid response. This is further evidenced by recurrent deletions, mutations and decreased expression in relapsed ALL of the transcription factor *TBLXRI* which is a member of the NCOR complex.⁶⁸ *TBLXRI* is involved in the degradation of NCOR and this is essential for gene activation by a number of nuclear receptors.^{69,70}

Another transcriptional co-activator that has been implicated in glucocorticoid resistance is the cAMP responsive element binding protein (CREB) binding protein (*CREBBP*). Initially, it was observed that 18.3% of relapsed ALL cases had sequence or deletion mutations in *CREBBP*.⁶⁵ *CREBBP* (CBP) functions as a histone acetyltransferase and can act as a scaffold for other proteins in the transcriptional complex.⁷¹ CBP can directly affect glucocorticoid responsive elements and that in some cases *CREBBP* is altered in resistant leukemia cell lines.^{65,72,73} In one study, it was found that *CREBBP* knockdown by shRNA was insufficient to cause glucocorticoid resistance in the 697 leukemia cell line.⁷⁴

Inflammasome Activation

NALP3 inflammasome pathway components *NLRP3* and *CASP1* were overexpressed in glucocorticoid resistant B and T-lineage ALL. This was observed at the mRNA level, and often coincided with decreased promoter methylation at *NLRP3* or *CASP1*. It was further observed that overexpressing *CASP1* in inflammasome activated leukemia cell lines resulted in significantly increased cleavage of the GR. This caused functional loss GR activity and decreased sensitivity to glucocorticoid induced cell death providing a functional mechanism derived from the initial clinical findings.⁷⁵

TXNIP (thioredoxin interacting protein) is required for *NLRP3* inflammasome activation in presence of high glucose induced oxidative stress.⁷⁶ It also functions as a glucose feedback sensor which was confirmed via CRISPR/Cas9 knockout of *TXNIP* that showed a similar change in glucose transport to *NR3C1* CRISPR knockout in the same patient derived cells. It was associated with glucocorticoid resistance in PDX models when knocked down and agonists for this protein synergized with glucocorticoids. *TXNIP* is regulated by *PAX5* and *IKZF1* which are critical B-cell developmental factors,⁷⁷ and is known to be upregulated in leukemia cells when cells are treated with glucocorticoids.⁷⁸ *TXNIP* potentially provides a link between the inflammatory and the metabolic changes observed from glucocorticoid treatment and may lead to potential therapeutic targets in combatting glucocorticoid resistant ALL.

Glycolytic Pathway

Regulation of glucose homeostasis is important for modulating prednisolone resistance. It was shown that prednisolone resistant ALL relies on increased glucose consumption and that inhibition of glycolysis sensitized prednisolone-resistant ALL cell lines to glucocorticoids.⁷⁹ This suggests that at least some of the cytotoxicity of glucocorticoids in ALL can be attributed to their ability to negatively affect glucose uptake. Cells that can evade this glucose repressive effect may rely heavily on glycolysis which is an inefficient process for energy production and is achieved by upregulating genes such as *GAPDH* or other glycolytic components.⁸⁰ Cells that can switch to glycolysis readily are able to resist treatment with glucocorticoids.

Cytokine Signaling

Another mechanism of glucocorticoid resistance occurs when leukemia cells can alter expression and signaling in response to cytokines or other exogenous signals which can lead to large scale cellular changes both within the cell and externally. Interaction with the bone marrow microenvironment via stromal cells or normal T-cells can greatly influence the response of leukemia cells to drugs. It has been previously illustrated that combined treatment with IL-2 and IL-4 caused glucocorticoid resistance in T-cells^{81,82} and PBMCs⁸³ which was able to be reversed with IFN- γ treatment. In these T-cells, p38/MAPK activation was observed and was also abrogated by IFN- γ treatment or direct

p38/MAPK inhibition. In T-ALL, the IL-4 overexpression was attributed to glucocorticoid resistance because of hyperactivated lymphocyte cell specific kinase (LCK). IL-4 stimulation alone was sufficient to confer resistance in these T-ALL cells and PDXs and could be reversed with LCK gene silencing or inhibitors such as dasatinib.⁸⁴ Aberrant LCK activation in prednisolone resistant patients was associated with upregulation of calcineurin/NFAT signaling and triggered IL-4 overexpression. Also, IL-2 or TLR7/8 agonist stimulation caused glucocorticoid resistance in CLL cells and could be reversed by treatment with the JAK inhibitor ruxolitinib.⁸⁵

IL-7 Receptor/JAK-STAT Signaling

In T-lineage ALL, mutations in the IL-7 receptor signaling pathway genes (*IL7R*, *JAK1*, *JAK3*), Ras pathway genes (*KRAS*, *NRAS*, *NFI*) and AKT were associated with steroid resistance and poor outcome. These alterations were observed mostly in the early thymic progenitor (ETP) or T Cell Leukemia Homeobox (TLX) ALL subtypes. Expression of mutant *IL7R*, *JAK1*, mutant or wild type *NRAS*, or AKT induced steroid resistance in T-ALL cell lines P12 Ichikawa or SUPT1), with no apparent change in L-asparaginase or vincristine response. Strong activation of both MAP Kinase (MEK and ERK) and AKT signaling were implicated as causative mechanisms, and MEK, AKT and mTOR inhibitors all enhanced steroid sensitivity.⁸⁶ In another study of non-ETP T-ALL JAK/STAT inhibition or IL7 removal was able to overcome glucocorticoid resistance.⁸⁷

Avoiding Apoptosis

As noted in the initial description of the “Hallmarks of Cancer”, resisting cell death and avoiding normal apoptosis is a key factor in defining the cancer phenotype.^{88,89} Thus, it stands to reason that avoiding apoptosis would also be a “hallmark” of glucocorticoid resistance in cancer. Arguably, because chemotherapeutics push cells towards death through normal processes this pressure may act as a selection event for cells that have advantageous changes in the apoptotic regulatory pathways. Apoptotic pathways can be disturbed in glucocorticoid resistant ALL in two main ways either there is decreased pro-apoptotic protein expression or function that normally promote apoptosis or there is higher expression of anti-apoptotic proteins either of which will disrupt the balance of the cell’s ability to carry out its cell death program in response to glucocorticoid treatment.

Pro-apoptotic Mechanisms

***BCL2L11* (Bim)**

The most well described pro-apoptotic protein involved in both the mechanism of action of glucocorticoids and resistance to them in ALL is BIM (*BCL2L11*). It has been

well described that glucocorticoid treatment induces BIM in leukemia cells.⁹⁰⁻⁹² In a study of thirty matched patient bone marrow samples at either day 0 or day 8 following prednisolone monotherapy 25/30 patients (83%) had good response to PRED. Differential *BCL2* family expression showed that the pro-apoptotic protein BIM showed significantly higher induction by prednisolone treatment in prednisolone responsive patients when compared to poor responders. Furthermore, BIM expression was highly predictive of response to prednisolone independent of molecular subtype, and BIM knockdown in leukemia cell lines caused glucocorticoid resistance.⁹³ Recently, a study of genome-wide lymphocyte specific open chromatin identified an intronic GR binding region (IGR) at the BIM locus in a glucocorticoid sensitive cell line ALL-54S that was not observed in resistant cell line ALL-50R. Dexamethasone treatment induced much greater *CTCF* binding at the BIM IGR in the ALL-54S than ALL-54R. The BIM IGR was determined to be necessary mediator in the process of glucocorticoid induced cell death and resistance.⁹⁴

PMAIP1

PMAIP/Noxa which is a pro-apoptotic protein involved in the degradation of anti-apoptotic MCL-1, and it has been shown that unlike BIM which is induced by activation of the glucocorticoid receptor to be directly repressed by treatment with glucocorticoids which may be a secondary effect of glucocorticoid treatment not related to its effect on resistance.⁹⁵ PMAIP has also or been proposed to be important for the regulation of glucocorticoid sensitivity in leukemia cells⁹⁶ and may be regulated by phosphorylation state of the glucocorticoid receptor at either S211 which denotes active GR being imported to the nucleus or S226 which is a marker for GR nuclear export which is associated with GR inactivity.⁹⁷

Anti-apoptotic Mechanisms

BCL2

The opposing mechanism to loss of pro-apoptotic protein induction (e.g. BIM) leading to resistance is defective repression of or higher expression of anti-apoptotic protein expression. The namesake of this class of proteins is known as *BCL2* (B-cell lymphoma 2). Opposing regulation of BIM and *BCL2* modulates the resistance of acute lymphoblastic leukemia to glucocorticoid induced apoptosis in both cell lines and patient derived xenografts (PDXs).⁹⁵ In prednisolone resistant PDXs, GR was not able to bind at the *KLF13* promoter and subsequent *KLF13* expression was not increased resulting in sustained high expression of *MYB* and its target *BCL2*.⁹⁸

MCL1

Another anti-apoptotic member of the *BCL2* family *MCL1* was highly expressed in glucocorticoid resistant pediatric ALL⁸⁰ and MLL rearranged infant ALL.⁹⁹ Treatment with rapamycin (*mTOR* inhibitor) was able to induce sensitivity to glucocorticoids through an unknown mechanism of MCL-1 repression after being discovered as a potential glucocorticoid sensitizing agent in a chemical genomics screen¹⁰⁰ Obatoclax, a pan inhibitor of BCL-2 family proteins, could also overcome MCL-1 associated glucocorticoid resistance in ALL cell lines. Recently, a specific MCL-1 inhibitor was been developed which may provide new ways to target this in glucocorticoid and other *BCL2* inhibitor resistant cancers.¹⁰¹

Smac/Diablo

BV6,a Smac/Diablo mimetic compound synergized with glucocorticoids in patient derived xenografts of leukemia cells and in leukemia cell lines. This represents a distinctly different pathway from traditional *BCL2* family in resistance to glucocorticoid treatment mediated through the ripoptosome. This may function independently of the glucocorticoid receptor because some of the most significant effects were observed in the Reh leukemia cell line which harbors a homozygous nonsense mutation in the glucocorticoid receptor.¹⁰²

Kinase Signaling

Ras/MAPK Pathway

Ras pathway mutations (*KRAS*, *NRAS*, *NF1*, *FLT3* and *PTPN11*) are common at diagnosis in childhood ALL (ranging from 35-44%), but it has been shown that clones are retained at relapse in many cases, and that the incidence of relapse is higher in cases with Ras pathway mutations.^{103,104} Some Ras pathway mutations were found to be acquired (38.9%), but in many cases the mutations were present at initial diagnosis in bulk sequencing. This does not preclude the possibility of low frequency mutations that evade detection at diagnosis which was addressed in this study with some success and may be further understood as single cell sequencing methods improve.¹⁰⁵ Ras mutations have also been shown to have a direct impact on the prognosis of MLL-rearranged infant ALL they had higher white blood cell counts at diagnosis and also were more resistant to glucocorticoids *in vitro*.¹⁰⁶ A study in T-ALL patients with *JAK1* or *KRAS* mutations were more steroid resistant and had poorer prognosis than non-mutated patients. Ectopic expression of mutant or wildtype *NRAS* confirmed the clinical finding and induced steroid resistance in T-ALL cell lines.⁸⁶ Furthermore, *KRAS* G13V mutations were associated with *ex vivo* prednisolone resistance in patients. Consequently, Ras mutations have been proposed as a predictive biomarker for treatment with MAPK inhibitors. Downstream inhibition of the MAP kinase pathway via trametinib was able to synergize

with glucocorticoids reducing resistance in ALL cell lines and primary patient leukemia cells. Knock down of MAPK family members MEK2 and MEK4 was also able to sensitize ALL cell lines.¹⁰⁷

PI3K/AKT/mTOR Signaling

mTOR (mechanistic Target of Rapamycin) kinase signaling is an important signaling pathway for a number of cellular functions (e.g. growth, survival and autophagy) and has arisen as a central regulator of cellular homeostasis in response to nutrient deprivation and other external insults.¹⁰⁸ Genes both upstream and downstream of mTOR have been identified as important in resistance of leukemia to glucocorticoid induced apoptosis. AKT phosphorylation of mTOR in B and T lineage ALL cell lines was able to impair glucocorticoid induction of apoptosis by increasing the expression of MCL-1 and as stated previously it was seen that sirolimus (rapamycin) was able to sensitize cells and reverse MCL-1 mediated glucocorticoid resistance.¹⁰⁰ Subsequent investigation showed that the glucocorticoid sensitization induced by mTOR inhibition was facilitated by autophagy dependent necroptosis mediated through *RIPK1* kinase.¹⁰⁹

Another study showed that AKT phosphorylates GR at S134 and decreases its nuclear localization blocking downstream transcriptional targets of GR, and that AKT inhibitors can sensitize cells to glucocorticoids.¹¹⁰ Downstream mechanisms of this pathway have also been associated with glucocorticoid resistance. AKT phosphorylation inhibited BAD (a pro-apoptotic *BCL2* member) and direct AKT mediated phosphorylation of *XIAP* (an anti-apoptotic factor) prevents its ubiquitination and degradation.¹¹¹ Upregulation of metabolic pathway genes by AKT was associated with glucocorticoid resistance in T-ALL by acting in direct opposition to the metabolic inhibition caused by glucocorticoids treatment.¹¹² A genome-wide shRNA screen in the NALM-6 B-precursor ALL cell line identified PI3K pathway genes (*PIK3CB2*, *PIK3CD* and *IL7R*) were associated with resistance to glucocorticoids through their interaction with the B-cell receptor. Idelalisib (PI3K δ inhibitor) treatment or knockdown of endogenous PI3K inhibitory phosphatase *PTEN* sensitized cells to glucocorticoids in B-ALL.¹¹³

Furthermore, inhibition of the de-ubiquitinating enzyme *USP9X* (Ubiquitin Specific Peptidase 9 X-Linked) which is highly expressed in B-ALL sensitized cells to prednisolone induced apoptosis, and when knocked down in the RS4;11 leukemia cell line downregulated MCL-1, BCL-2/BCL-XL and increased *BAX* levels. This was attributed to reduced mTORC1 phosphorylation of its substrate S6K1.¹¹⁴

Src/Fyn/Lck Pathway

The Src-family kinases Lck and Fyn are critical in T-cell receptor (TCR) transduction.¹¹⁵ It was shown that in MLL-rearranged infant ALL that overexpression of

SI00A8 and *SI00A9* was associated with prednisolone resistance because of failure of cells induce free cytosolic calcium Ca (2+), and this was mitigated by treatment with the Src kinase inhibitor PP2.¹¹⁶ Also, high *ANXA2* expression and activation via Src kinase phosphorylation requiring its adapter protein p11 (*SI00A10*) caused prednisolone resistance in MLL-rearranged infant leukemia. shRNA knockdown of *ANXA2*, *FYN*, *LCK* or *SI00A10* all were individually sufficient to inhibit *ANXA2* phosphorylation and cause sensitization to prednisolone.¹¹⁷ *EMPI* (a gene involved in adhesion to stromal cells) was associated with poor prognosis in B and T-ALL due to its association with prednisolone resistance. Pathway analysis confirmed that *EMPI* signals through the Src kinase family and that this is a possible mechanism for its normal function in maintaining interactions with the stromal microenvironment.¹¹⁸ As described above the association with *LCK* and the IL-7 receptor pathway has been described in great detail in T-ALL. Collectively, these data suggest that Src kinase family inhibitors are a promising option for therapeutic intervention to mitigate glucocorticoid resistance in both B and T lineage ALL.

cAMP/AMPK Pathway

cAMP signaling is a well described signaling pathway and has implications in a variety of phenotypes. One important component of cAMP signaling are phosphodiesterases (PDEs) which degrade the phosphodiester bond in second messengers cAMP and cGMP. They regulate the localization, duration and amplitude of cAMP signaling within subcellular domains.¹¹⁹ It was first discovered that germline *PDE4B* mutations were associated with relapse in pediatric ALL.¹⁹ It was further observed that PDE4 inhibitors could alter the levels of the glucocorticoid receptor in CLL cells and sensitize them but not in circulating hematopoietic cells.¹²⁰ AMPK, an inhibitory kinase for the conversion of ATP to cAMP which is a critical step in cAMP signaling sensitized cells to glucocorticoids when inhibited which is in concordance with previous findings.⁷⁷

Epigenomic Mechanisms of Glucocorticoid Resistance in ALL

Chromatin Modifiers (SWI/SNF)

The glucocorticoid receptor acts on many of its targets through direct transcriptional activity. To bind directly to DNA and effectively modulate transcription dynamic structural changes in chromatin are required. The SWI/SNF chromatin remodeling complex is essential in this efficient restructuring of chromatin, especially in the case of nuclear receptors.^{121,122} SWI/SNF components *SMARCA4*, *SMARCB1* and *ARID1A* all exhibited decreased expression in glucocorticoid resistant primary acute lymphoblastic leukemia.¹²³ SNPs in the promoter of *SMARCB1* were also associated with glucocorticoid sensitivity in lymphoblastoid cell lines which was attributed in part to the alteration of a *PARP1* binding site.¹²⁴

Histone Modifiers

Another way the chromatin landscape can be regulated is through post-translational modification of histones which are proteins that the DNA is “wound” around in its heterochromatin (closed) state. One major amino acid residue that is known to be modified with important functional consequences in signaling for chromatin remodeling complexes to act on DNA and either open or close it are lysine residues which are most commonly either acetylated in the cases of open chromatin¹²⁵⁻¹²⁷ or mono-, di- or trimethylated which can signal the chromatin to be in a closed state.¹²⁸ These marks are commonly found at promoter or enhancer regions in the DNA where the regulation of gene expression is most highly affected.

One family of enzymes that catalyzes the methylation of histones are the lysine methyltransferases (*KMT2A* or *KMT2D*) these were originally referred to as MLL (mixed lineage leukemia) genes. Commonly altered in ALL, MLL-rearranged leukemias have been designated as a unique molecular subtype. MLL Patients exhibit poor response to therapy and are more commonly younger individuals, especially infants.^{9,129} Nearly 80% of infant leukemias have MLL rearrangement, and it has been shown in several studies that in both B-lineage leukemia and T-lineage leukemia MLL rearranged leukemias were more resistant to glucocorticoids especially those with the t(4;11) translocation.^{112,130} In a study in T-ALL, it was shown that changes in expression in wild-type (non-rearranged) MLL also can contribute to glucocorticoid resistance.¹³⁰

Other epigenomic factors may be potential targets to combat glucocorticoid resistant leukemia. In GC poor responsive patient leukemias where the BIM gene is silenced the HDAC inhibitor vorinostat was able to recover antileukemic efficacy in leukemia xenograft models of ALL.¹³¹ Overall, epigenomics is still a burgeoning field of research, and there is great effort to understand the epigenomic landscape of leukemia especially in the case of glucocorticoid resistance. With advances in the ability to interrogate and deconvolute the 3D interactions of chromatin and the epigenetic “code” the future of epigenomics in the context of drug resistance is very promising.

B-Cell Development Factors

ALL development can often be attributed to specific genetic lesions or developmental blocks in normal development of B-cells leading to aberrant growth of leukemic cells that can ignore normal differentiation signals leaving cells in an immature state. Normal B-cell development is controlled by a number of transcription factors known to regulate lineage specific development.¹³²

PAX5

PAX5 is a transcription factor that is critical in mediating the maturation of B-cells by repressing the expression of non-lineage specific genes and upregulating the

expression of genes involved in B-lymphoid signaling.¹³²⁻¹³⁴ Alterations in *PAX5* increase susceptibility to and are common in acute lymphoblastic leukemia occurring in over 40% of all cases. They exert their leukemogenic effect by altering the normal B-cell transcriptional program leading to aberrant growth and malignant phenotype.^{12,135} *PAX5* was differentially expressed between glucocorticoid sensitive and resistant leukemias, and this resistance was associated with the altered maturation state of the cells. In patient derived pre-B ALL cells, expression of a dominant negative mutant of *PAX5* was able to cause glucocorticoid resistance. Also, cells expressing a *PAX5* haploinsufficient mutant were glucocorticoid resistant which could be recovered by addition of wild-type *PAX5*.⁷⁷

IKZF1

IKZF1 is another important B-cell developmental factor that is frequently mutated in ALL. It has been associated with poor prognosis likely due to its high frequency in the high-risk ALL subtypes *BCR-ABL1*+ and *BCR-ABL1*-like ALL.^{12,13} Similar to *PAX5*, patient derived B-ALL expressing haploinsufficient *IKZF1* exhibited glucocorticoid resistance and this could be recovered by addition of wild-type *IKZF1*. Dominant negative *IKZF1* was able to cause resistance when transduced into wild-type patient derived B-ALL cells. It was postulated that *PAX5* and *IKZF1* act as metabolic gatekeepers in which loss of function increased glucose uptake and ATP levels which was likely causative in the ability to resist the effects of glucocorticoid induced apoptosis. This was proposed as an explanation for why glucocorticoids are effective against lymphoid but not myeloid malignancies.⁷⁷ CRISPR/Cas9 screening was performed to identify transcriptional targets of *PAX5* and *IKZF1* and three genes were identified *NR3C1* (GR), *TXNIP* (involved in inflammation, reactive oxygen sensing and glucose-feedback) and *CNR2* (cannabinoid receptor 2). They confirmed these initial findings by showing that *TXNIP* and *CNR2* agonists were able to strongly synergize with glucocorticoids.⁷⁷

Drug Metabolism and Transport

GSTM1 is an enzyme involved in detoxification, metabolism of xenobiotics and the negative regulation of apoptosis signaling cascades. Germline genetic polymorphisms in *GSTM1* were associated with a greater risk of relapse in pediatric high risk ALL with corresponding decreased gene expression in *GSTM1*.¹³⁶ *GSTM1* inhibited glucocorticoid induced apoptosis by suppressing Bim expression in two T-ALL cell lines, presumably via down-regulation of both p38-MAPK and upregulation of NF-kappaB p50.¹³⁷

There is evidence that enzymes involved in the metabolism of steroids such as 11 β -hydroxysteroid dehydrogenase 2 (*HSD11B2*) which converts active glucocorticoids into inactive glucocorticoids which cannot bind to GR. *HSD11B2* inhibition by carbenoxolone made T-ALL cell lines more sensitive to glucocorticoid treatment.¹³⁸

Non-genomic Mechanisms of Glucocorticoid Resistant ALL

miRNAs have been shown to be important in the post-transcriptional and post-translational regulation of gene expression. Recently, a set of miRNAs mir-27a, mir-223 and mir-708 were found to be associated with clinical outcome in childhood ALL, but in this study only miR-708 was found to be associated with *in vivo* response to glucocorticoids.¹³⁹ miR-128b was differentially expressed in relapsed vs. non-relapsed childhood ALL¹³⁹ and was shown to be associated with poor prognosis in MLL-AF4 ALL along with miR-221 and downregulation of these miRNAs led to glucocorticoid resistance. Re-expression of both miR-128 and miR-221 (which down-regulates CDKN1B) sensitized two ALL (MLL-AF4) cell lines to glucocorticoids.¹⁴⁰ However, miR-221 has conflicting reports as a study in multiple myeloma showed that inhibition of miR-221 sensitized cells to glucocorticoids.¹⁴¹ These findings illustrate one example of how non-genomic mechanisms may provide promising discoveries for the future of drug resistance in ALL.

CHAPTER 3. METHODOLOGY*

Patients

The sensitivity of primary leukemia cells to prednisolone was determined *ex vivo* for a total of 444 patients aged 18 years or younger with newly diagnosed ALL. Of these, 298 patients were enrolled in the St. Jude Total Therapy XV (TOTXV, NCT00137111) or XVI (TOTXVI, NCT00549848) protocol, the initial 225 were the “discovery” cohort and the subsequent 73 constituted a validation cohort. We also used publicly available mRNA expression data and prednisolone LC₅₀ values for 145 European pediatric ALL patients previously described in detail 62 as a second validation cohort. Also, 45 T-lineage leukemia patients from St. Jude Total Therapy XV or XVI protocols were included for investigating ALL subtype differences in prednisolone sensitivity. Leukemia cells from an additional cohort of 335 patients with ALL were studied: 226 pediatric patients (14 St. Jude Total Therapy XV, 182 St. Jude Total Therapy XVI [73 from validation cohort] and 30 from St. Jude Total Therapy XVII) and 109 adult patients (66 from the Eastern Cooperative Oncology Group, 33 from M.D. Anderson Cancer Center, 8 from the University of Chicago and 2 from the Alliance for Clinical Trials in Oncology) were included to further assess the expression of *CELSR2*; in a subset of these patients (n=96) the sensitivity to venetoclax was measured. The level of minimal residual disease (MRD) in bone marrow was determined by flow cytometry and/or polymerase chain reaction at day 15-19 and after completion of induction, as previously described.²⁴ Written informed consent was obtained from all patients or their parents or guardians. The use of these samples was approved by the institutional review board at St. Jude Children’s Research Hospital.

Mice

Unconditioned mice were seven to nine weeks old when injected with leukemia. Daily observations were carried out on the mice and they were sacrificed when leukemia cells reached 50% in the peripheral blood, or the veterinarian determined they showed clinical symptoms (ruffled fur, respiratory stress, hindlimb paralysis, or significantly decreased mobility). This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were maintained in an American Association of Laboratory Animal Care accredited facility and were treated using a protocol approved by the St. Jude Animal Care and Use Committee (Protocol Number: 580-100498) in accordance with NIH guidelines. NOD. Cg-Prkdc scid Il2rg tm1Wjl/SzJ (NSG) mice were obtained from the St. Jude colony for all experiments and were kept under pathogen free conditions.

*Portions of chapter from previously published article; final submission modified with permission. Autry, R. J. et al. Integrative genomic analyses reveal mechanisms of glucocorticoid resistance in acute lymphoblastic leukemia. *Nature Cancer*, doi:[10.1038/s43018-020-0037-3](https://doi.org/10.1038/s43018-020-0037-3) (2020).¹⁴²

Animals were sacrificed by carbon dioxide asphyxiation using the gradual displacement method, consistent with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition. Great efforts were made to minimize suffering.

Human Leukemia Cell Lines

Human B-lineage acute lymphoblastic leukemia cell lines (NALM-6 and 697) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine at 37° C with 5% CO₂. *CELSR2* knockdown cell lines were generated in NALM-6 and 697 cells transduced with lentivirus containing short-hairpin RNA targeting *CELSR2* (MISSION pLKO.1-puro shRNA TRCN0000011243; sequence : 5'-CCGGGCCACTGAAGACACTGACATACTCGAGTATGTCAGTGTCTTCAGTGGC TTTT or TRCN0000011240 sequence: 5'-CCGGCGCTTGGACAAAGGGAAGTTTCTCGAGAAAGTTCCTTTGTCCAAGCG TTTT; Sigma-Aldrich) or a non-targeting control (MISSION pLKO.1-puro Non-mammalian shRNA control (SHC002); Sigma-Aldrich), and selected in media containing 5µg/mL puromycin. *PAX5* knockdown NALM-6 cell lines were generated by transducing with lentivirus short-hairpin RNA targeting *PAX5* (MISSION pLKO.1-puro shRNA TRCN0000016059; sequence: 5'-CCGGCCCTCAGTATTCCCTCGTACAACACTCGAGTTGTACGAGGAATACTGAGGG TTTT; Sigma-Aldrich) or a non-targeting control (MISSION pLKO.1-puro Non-mammalian shRNA control (SHC002); Sigma-Aldrich), and selected in media containing 5µg/mL puromycin.

GR rescue experiments were performed by stable lentiviral transduction of non-target control or shRNA targeting *CELSR2* transduced NALM-6 cells with plx304 vector (Addgene) with cDNA of GR (Origene; RC220189) tagged with V5 or GFP control. These cells were then selected with 15µg/mL Blasticidin and 5µg/mL Puromycin and assessed for in vitro prednisolone sensitivity at 72hr.

To constitutively express Cas9 in the NALM-6 cell line, we transduced cells with lentivirus containing the Cas9 expression vector (Addgene: 52962) and selected cells in media containing 15µg/ml blasticidin (Sigma-Aldrich).

Prednisolone *Ex Vivo* Resistance Assay

Primary leukemia cells were isolated from the bone marrow or peripheral blood of newly diagnosed ALL patients, and tested for prednisolone sensitivity by MTT assay, as previously described.^{75,80} In brief, cells were seeded in a 96-well plate at a concentration of 2x10⁶ cells/mL for primary cells, in phenol red-free RPMI 1640 medium supplemented with 20% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B, and 1X insulin-transferrin-selenium supplement or 2.0x10⁵ cells/mL for ALL cell lines, in phenol red-free RPMI 1640 with

10% FBS and 2mM L-glutamine. In wells of round-bottom 96-well plates, 80 μ L of each cell suspension was combined with 20 μ L of methylprednisolone at varying concentrations (Solu-Medrol®, Pfizer) diluted serially. Plates were incubated at 37° C in 5% CO₂ for a total of 96 hours (primary ALL) or 72 hours (ALL cell lines). 10 μ L of 5 mg/mL MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide) was added to each well for the final 6 hours of incubation. Acidified isopropanol was used to solubilize formazan crystals and absorbance was measured at 562 nm with a background correction of 720 nm, (uQuant, BioTek Instruments). For ALL cell lines, the CellTiterGlo® Assay (Promega) was used to measure prednisolone LC₅₀. For patients enrolled on St. Jude TOTXV or TOTXVI protocols LC₅₀ was determined at St. Jude Children’s Research Hospital. Publicly available data from European patients had been previously assayed in The Netherlands by MTT as previously described.⁸⁰ Patients from all cohorts were classified as resistant ($\geq 64\mu$ M) or sensitive ($<0.1 \mu$ M), according to previously described criteria.⁷⁵ For a subset of patients included in the whole exome sequencing mutation analysis who did not have prednisolone LC₅₀ determined, their dexamethasone LC₅₀ values were used after multiplying by a factor of eight to adjust for the difference in potency.

Gene Expression by Microarray

Total RNA was harvested from primary leukemia cells obtained from 203 patients at diagnosis using TRI Reagent (Molecular Research Center, Inc.). Gene expression was assessed in the Hartwell Center for Bioinformatics & Biotechnology at St. Jude Children’s Research Hospital using either HG-U133A (GPL96) or HG-U133 Plus 2.0 (GPL570) microarray platforms (Affymetrix), according to the manufacturer’s protocol. The “affy” Bioconductor R-project package or Affymetrix Microarray Suite version 5.0^{80,144,145} was used to implement the MAS5 algorithm for processing the gene expression data.

Gene Expression by RNA Sequencing

Total RNA was harvested from 217 patients enrolled on St. Jude protocols (13 from St. Jude TOTXV, 176 from St. Jude TOTXVI and 26 St. Jude TOTXVII) and 103 adult patients (62 from the Eastern Cooperative Oncology Group, 32 from M.D. Anderson Cancer Center, 7 from the University of Chicago and 2 from the Alliance for Clinical Trials in Oncology), and total stranded RNA sequencing was performed. Total RNA was harvested from primary ALL samples using TRI reagent. In ALL cell lines, total RNA was isolated using the RNAeasy Mini kit (Qiagen), and stranded mRNA sequencing was performed. All RNA sequencing was carried out via the Illumina HiSeq platform by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital.

DNA Methylation Analysis

DNA was isolated from the bone marrow or peripheral blood of 178 newly diagnosed leukemia patients using the Blood and Cell Culture DNA kit (Qiagen). DNA methylation status was interrogated genome-wide using either the Infinium HumanMethylation27 BeadChip kit or the Infinium HumanMethylation450 BeadChip kit in accordance with the manufacturer's protocol (Illumina). HumanMethylation27 BeadChip experiments were performed at either the Emory Integrated Genomics Core (EIGC; Atlanta) or the Wellcome Trust Centre for Human Genetics Genomics Lab (Oxford). HumanMethylation450 BeadChip experiments were performed at the Heflin Center for Genomic Science at the University of Alabama at Birmingham (Birmingham, Alabama, USA). Beta-values (β)¹⁴⁶ were derived from the raw output for each CpG site. Individual loci were grouped according to ENCODE criteria¹⁴⁷; DNA methylation status was classified as: hypomethylated ($\beta \leq 0.2$), hemimethylated ($\beta > 0.2 < 0.6$) or hypermethylated ($\beta \geq 0.6$).

SNP Analysis

DNA was extracted from ALL cells from bone marrow or peripheral blood samples from 184 newly diagnosed patients using the Blood and Cell Culture DNA kit (Qiagen). DNA samples were genotyped using the Affymetrix GeneChip Human Mapping 500K set or the SNP 6.0 array (Affymetrix). The arrays were scanned, and genotype calls were made using the BRLMM algorithm as implemented in the GTYPE software (<http://www.affymetrix.com/products/software/specific/gtype.affx>) as previously described.¹⁴⁸ SNPs were excluded for call rates of less than 95% amongst all patients or a minor allele frequency less than 1%. SNPs were annotated to genes for further comparative analysis between methods only if they were an expression quantitative trait locus (eQTL) as defined by Haploreg v 4.1.

miRNA Expression Analysis

Total RNA was extracted from ALL cells isolated from bone marrow or peripheral blood samples from 163 newly diagnosed patients. All microRNA expression microarrays were analyzed in the Hartwell Center for Bioinformatics & Biotechnology at St. Jude Children's Research Hospital, as previously described.¹⁴⁹ High-quality RNA was hybridized to miRCURY LNA 10.0 (GPL7722) generated from ready to spot probe sets or preprinted 6th generation miRCURY LNA microRNA microarrays (GPL11434) in accordance with the manufacturer's protocol (Exiqon, Woburn, MA). Upon removal of background signal data were log₂ transformed and quantile normalized prior to analysis.

Copy Number Alteration Analysis

DNA was extracted from ALL cells isolated from 184 newly diagnosed patient bone marrow or peripheral blood samples, as described above to obtain data with the Affymetrix Genome-wide Human SNP Array 6.0. To identify copy number abnormalities for each sample, the SNP arrays were processed as follows. For each probe-set, we computed the raw signal as the mean of the log-transformed CEL file intensities across all probes annotated to that probe-set. Noting that the distribution of raw signals differed according to probe type (copy number or genotyping), we then transformed raw signals to have the same mean and variability across probe types. Probes were categorized by quintiles of the GC content according to Affymetrix annotations. For each probe category, the raw signals were median centered. After median centering, a pooled standard deviation estimate was computed as the square root of the average of first differences¹⁵⁰ within each probe category. Then, for each probe category, the median-centered signals were divided by the Rice (1984)¹⁵⁰ standard deviation estimate based on first differences. After this re-centering and scaling, the distribution of signals for each probe category had median 0 and Rice standard deviation of 1. These signals were then multiplied by the pooled standard deviation so that the relationship of the processed signals to actual copy number more closely approximated that of the original signals. After this re-scaling, all probe categories had signal distributions centered at zero with scale comparable to that of the original raw signals. These processed signals were provided as input to the circular binary segmentation algorithm.^{151,152} The endpoints of all segments were used to empirically partition the genome into a series of non-overlapping regions to represent results in a matrix form with the segment mean of each subject (matrix column) for each region (matrix row). These segmentation results were post-processed by first inferring copy numbers (CN=0, 1, 2, 3, 4) based on segment means. Histogram of segment means for all inferred segments were generated and a clear trimodal distribution was shown with center mode at 0 corresponding to CN=2. The nadirs (+/-0.2) between the center mode and two adjacent modes were chosen to be used as cutoff for copy number gain or loss. Among copy number loss segments, we further assigned those with mean less than 3 median absolute deviation(MAD) from the median of all CN loss segments as CN=0. Similarly, we assigned those segments with mean above 3 MAD from the median of all CN loss segments as CN=4. After copy number has been assigned, adjacent segments with same CNs across all patients in the study cohort were further collapsed for downstream analysis. The detection, prevalence estimates, and association results for CNAs should be considered preliminary in that they are limited by the resolution of the microarray platforms used in this study. These analyses were performed using the DNACopy package developed for R software.

Whole Exome Sequencing Coding Variants

DNA was extracted from ALL cells isolated from bone marrow or peripheral blood from 201 newly diagnosed pediatric B-lineage leukemia patients using Blood and Cell Culture DNA kit (Qiagen). Alignment was performed to the reference human genome assembly GRCh37-lite with BWA and analyzed as previously described using

matched germline sample as reference for somatic mutations.¹⁵³⁻¹⁵⁵ Mutations were filtered for non-synonymous variants only in the coding region (excluding 3' and 5'UTR variants). They were then aggregated to individual genes and gene-level clustering via the Ward method was performed using Euclidean distance.

Hierarchical Clustering of Genomic Features

Each individual genomic feature type (mRNA, miRNA, DNA methylation, SNP, CNA and SNV/Indels) was rank ordered based by their linear regression p-values for association with prednisolone LC₅₀. Instanced hierarchical clustering was performed in a stepwise fashion beginning with the two most statistically significant probes. For each instance, Fisher's Exact Test (FET) was utilized to assess how well the clustered data could segregate resistant and sensitive leukemias when the highest clade of the dendrogram was split in two. Probes were added individually at each instance and hierarchical clustering and Fisher's Test calculations were repeated for up to 500 probes. Fisher p-values for the different patient cohorts were combined using meta-analysis (Stouffer's method) and the combination of probe sets that generated the lowest meta-analysis p-value was used as the signature for each individual feature type. 110,000 rounds of permutation were performed on the data to determine the likelihood that the observed meta clustering p-values for each feature was due to chance. Analyses were carried out in R using packages gtools, gdata, bitops, caTools, gplots, and amap. All data were clustered using the hcluster function in R using the "ward" method and distance used was "correlation" for all features except WES ("Euclidean") and SNPs ("binary"). All FDR corrected values were calculated using the Benjamini-Hochberg correction method for cutoffs of selected feature lists and can be found in **Supplementary Tables 1-9** for each respective feature type.

Connectivity Among Genomic Features

To determine connectivity between mRNA expression and each of the other genomic feature types, we compared expression levels with each genomic feature type for all samples interrogated for both features.

Associations between DNA methylation and gene expression were deemed significant if there was a negative and statistically significant ($p < 0.05$) association according to linear regression (patient cohort included as a covariate), and the CpG site was within 100 kb of the gene's transcription start site. Connections between DNA methylation probes found to be inversely associated with expression of genes in the prednisolone resistance mRNA signature were included only if the methylation probe was also significantly associated with prednisolone LC₅₀ ($p < 0.05$).

Connections between miRNA expression and gene expression were required to meet the following conditions: a negative association by linear regression (p -value < 0.05) and the gene's transcript contains a miRNA binding site based in either of two in

silico prediction databases (miRaNDA or miRDIP) or a database of experimental evidence of biological connection from public databases (mirTarbase). Connections between SNPs, CNAs, SNVs/Indels and gene expression were required to have a linear regression p-value < 0.001 in both patient cohorts.

Genome-Wide CRISPR Knockout Screens

We transduced 12×10^6 NALM-6 human leukemia cells that were constitutively expressing Cas9 protein with either GeCKOv2 library A or B by spinfection for 2hr at 568g at a MOI between 0.3-0.5 to ensure only one gRNA per cell, as previously described.¹⁵⁶ Both libraries contained 6 gRNAs per gene and 1000 non-targeting controls and library A contained 4 gRNAs per miRNA. Cells were selected with 15ug/ml Blasticidin (Cas9) and 5ug/ml Puromycin (gRNA) to ensure that cells contained both gRNA and Cas9 protein. Representation of gRNAs was verified by sequencing the gRNA region using a two-step nested PCR reaction to amplify the region, as previously described.^{156,157} For each library, 2×10^7 cells were treated with 100 μ M prednisolone for 72hr. After 72hr, viability of these cells ranged from 10-20% for all treatments. Cells were grown out until they reached >90% viability, and DNA was extracted using the Blood and Cell Culture Maxi kit (Qiagen). The gRNA region was sequenced via Illumina HiSeq 2500 using single reads in rapid run mode and gRNA enrichment/depletion analysis performed using the MaGeCK algorithm.^{158,159} Genes that were not expressed based on RNA-seq of the NALM-6 cell line were removed from the analysis, and at least 4 significant (p<0.005) gRNAs were required to be included in the gene-level knockout reduced analysis. Gene level aggregation of gRNAs was performed using logit p-value transformation method in the R package metap. Effect sizes were calculated as Cohen's D.

Integrated Gene Level (TAP) Analysis

To assess a known gene's potential involvement in leukemia cell sensitivity/resistance to prednisolone, we combined evidence from all six genomic features within or in proximity to (50kb) every known human gene. Each genomic feature was evaluated individually for its association with prednisolone LC₅₀, features that were significant (linear regression p < 0.05) were included in the overall gene level model. A hybrid permutation approach was used along with a non-parametric smooth CDF (cumulative distribution function) with a variation diminishing spline to obtain a TAP (Truncated Aggregation of P-values) statistic for every gene.^{160,161} Adaptive thresholding was used, as previously described to define the threshold of significance.¹⁶² Genes meeting this threshold were used to select top candidate genes for further analysis. Annotation of genomic features to gene regions: We downloaded from the UCSC <<https://genome.ucsc.edu/index.html>> the genomic locations of all 19,725 mapped human genes. A genomic feature with genomic location information, (i.e. mRNA expression probeset, CpG methylation marker, SNP, SNV/in-del from WES and CNV segment) is annotated to a gene region if the feature is either inside the gene (between the

beginning of the first exon and the end of the last exon), or within or overlaps with (in case of CNV segment) the region 50 kb up and down stream of the gene.

Truncated Aggregation of P values (TAP) statistic: Each genomic feature annotated to a gene region was tested individually for its association with the prednisolone LC₅₀ by linear regression, with treatment protocol (TOT-XV and TOT-XVI) as a covariate, the P value of the t test of the regression coefficient on the genomic feature is obtained, representing the level of statistical evidence on the genomic feature's association with LC₅₀ (a single piece of evidence). The P values are then combined to form the TAP statistic in a modified form of Fisher transformation **Equation 3-1**.

$$T = - \sum_{i=1}^M \log(p_i) I(p_i \leq \delta) \quad (\text{Eq. 3-1})$$

where M is the number of genomic features interrogated on the various assay platforms and annotated to the gene region; I(A) is the indicator of condition A, I(A)=1 (0) if A is true (false); and δ is a truncation threshold which was set to 0.05 in our analysis following.¹⁶³ The truncation is included here to better contrast small P values out of a possibly large set of P values (a gene region can contain dozens or hundreds of genomic features).

Hybrid Permutation Test

Assessment of a gene region's potential involvement in the biological process underlying leukemic cells' sensitivity/resistance to prednisolone was formulated by testing the null hypothesis that none of the interrogated genomic features annotated to gene region is associated with LC₅₀, vs. the general alternative hypothesis which states that the null hypothesis is false. The test can be carried out using the statistic T defined above. To compute the statistical significance (P value) one needs to know (or adequately approximate) the probability distribution of T under the null hypothesis. Notably this statistic is not a sum of independent log-transformed P values because the genomic features are generally related (e.g., SNPs in LD, reduced mRNA expression due to CpG methylation, etc.), and the truncation adds more complexity. In principle a permutation test can be performed, where in each round the LC₅₀ and covariate data points are randomly permuted together, and the P value of each individual genomic feature, and then the test statistic T are recomputed based on the permuted data. In each permutation round genome-wide association tests have to be conducted on several platforms, and to reflect high significance level at least 100,000 permutations is necessary; this can be extremely time consuming.

To introduce computational and statistical efficiency, we took a hybrid permutation test approach with the following steps: (1) Perform a few hundred permutation rounds to obtain a set of observations of the test statistic under the (simulated) null hypothesis: T_b , $b=1, \dots, B$. The number of permutations was set to $B=200$ in our analysis. (2) Transform the observations onto the unit interval [0,1] by a probability integral transformation, $W_b = F_0(T_b)$, $b=1, \dots, B$, where F_0 is the

null cumulative distribution function (cdf) of the test statistic under the null hypothesis, derived under the condition that the P values are independent. Clearly naively applying this cdf to compute the statistical significance for a gene region will inflate the type-I error rate because the P values are not independent. Our approach then is to properly correct this cdf by combining permutation (resampling), probability integral transformation, and non-parametric smoothing as described in the next two steps. (3) Construct a nonparametric smooth cdf F^*_W on $[0,1]$ using the approach described in,¹⁶¹ where variation diminishing spline¹⁶⁰ was chosen as the smoothing kernel for its good numerical and analytical properties. (4) Construct an estimator of the cdf of T under the null hypothesis by back-transformation $F^*_T(x) = F^*_W(F_0(x))$. Here F^*_W is a non-parametric correction to F_0 . The P value is computed as $P = 1 - F^*_T(T_{obs})$. It is shown in¹⁶⁰ that proper non-parametric smoothing can introduce substantial efficiency. Results from the simulation study (see below) show that this procedure works quite well even with as few as 200 permutations.

Significance Threshold Adjusting for Massive Multiple Tests

The TAP test was applied to 19,725 gene regions, generating 19,725 gene-level p-values. The significance threshold was determined by applying the adaptive threshold criteria developed in,¹⁶² which has been implemented in R and applied to the 19,725 p-values.

A Simulation Study

To gauge the operating performance of the TAP method, we conducted a simulation study with 10,000 simulation rounds. We simulated the data of a gene region mimicking our observed data on the *NLRP3* gene region (chr1: 247529458-247662406 plus 50kb up and down stream), assuming 47 SNPs, 2 CpG methylation loci, 1 CNV segment and 1 gene expression probe are measured. To maintain the LD structure of the SNPs, we downloaded the genotypes of the 47 SNPs from the One-thousand Genomes Project (<http://www.internationalgenome.org/data>), totally 2,504 samples. Then nucleotides of the 47 SNPs in one copy of a chromosome were treated as a copy of a pseudo-chromosome. Thus, we had 5,008 copies of pseudo-chromosomes from 2,504 samples. Those pseudo-chromosomes were randomly paired to form simulated genotypes for the 47 SNPs. Methylation levels were considered as ordinal variables. For one methylation loci, we assumed the probabilities of high, medium and low methylation to be 0.4, 0.4 and 0.2 respectively, and for the other 0.3, 0.1 and 0.6 respectively. The copy number status probabilities (loss, no change and gain) were set as 0.7, 0.2 and 0.1. The logarithm of gene expression ($\log(\text{expr})$) was modeled by a linear relationship with the genotypes of 2 selected SNPs (SNP1, SNP2), the 2 methylation loci (meth1, meth 2) and the copy number locus (cn) **Equation 3-2:**

$$\log(\text{expr}) = 0.15 * \text{SNP1} - 0.07 * \text{SNP2} - 0.03 * \text{meth1} - 0.02 * \text{meth2} + 0.08\text{cn} + e \quad (\text{Eq. 3-2})$$

The continuous phenotype y was generated by a simple linear regression mode **Equation 3-3**.

$$y = \beta * expr + \epsilon \quad (\text{Eq. 3-3})$$

Here e and ϵ are independent random errors set to follow the standard $N(0,1)$ distribution. Different coefficient (beta) values were used to depict effect sizes (see tables below); $\beta=0$ corresponds to the null hypothesis. We simulated several scenarios defined by two truncation thresholds $\delta=0.05, 0.2$, several different values of β , and a few nominal significance levels. The sample size was set to $n=300$, and the number of permutations was set to $B=200$ in all scenarios. Estimated power (sensitivity, rows for $\beta \neq 0$) and level (1 minus specificity, row for $\beta=0$) are compiled in **Table 3-1** and **Table 3-2**.

The TAP test by the hybrid permutation procedure can hold the nominal significance levels well (good control on false positive probabilities); while provides good statistical power (sensitivity) to detect meaningful effect sizes ($|\beta| > 0.3$). As expected, power/sensitivity decreased as the truncation threshold δ was relaxed from 0.05 to 0.2.

Statistical Significance of Top Candidate Genes

We estimated the probability that finding 15 genes by all three of methods of genomic interrogation is due to chance by considering the two aspects in genome-wide tests: the probability of capturing one non-associated gene by chance (producing a false positive), and the multiplicity. First, for any given gene, let PG, TAP and CR be respectively the event that the gene is captured by chance in the polygenomic, TAP and CRISPR analyses. The CRISPR experiment is completely separate from the other two analyses, and thus is fully independent. By conditional probability, the event of “being captured in all three analyses” has the probability **Equation 3-4**:

$$\pi^* = \Pr(PG \cap TAP \cap CR) = \Pr(PG \cap TAP)\Pr(CR) = \Pr(PG|TAP) \Pr(TAP) \Pr(CR) \quad (\text{Eq. 3-4})$$

Because PG and TAP were done on the same cohort, the results of the two analyses can be highly associated; which raises the concern of whether a false positive captured in one analysis is also captured in the other analysis. Quantitatively this is reflected by the conditional probability $\Pr(PG|TAP)$. We here conservatively assume that this probability is very high, $\Pr(PG|TAP)=0.9$. $\Pr(TAP)$ and $\Pr(CR)$ are the respective P value thresholds in the TAP and CRISPR analyses, each was determined with accounting for massive multiple tests: $\Pr(TAP)=0.00053838$ and $\Pr(CR)=4.1707 \times 10^{-8}$. Thus $\pi^*=2.0209 \times 10^{-11}$. Next consider multiplicity. Even if our results were presented at the gene level, significance tests were performed on individual genomic features of mRNA and microRNA expressions, methylation probes, SNPs, SNVs/in-dels from WES, CNV

Table 3-1. Estimated power TAP ($\delta = 0.05$)

β	Nominal significance levels α			
	0.05	0.01	0.005	0.001
-0.5	1	0.9989	0.9998	0.8899
-0.4	0.9979	0.97	0.8625	0.5973
-0.3	0.9401	0.7512	0.4749	0.1944
-0.2	0.5456	0.2725	0.106	0.03
-0.1	0.1205	0.0405	0.0141	0.0021
0	0.0475	0.0162	0.0047	0.0009
0.1	0.1237	0.0409	0.0131	0.0026
0.2	0.549	0.2638	0.1016	0.0235
0.3	0.94	0.753	0.4796	0.1909
0.4	0.9978	0.9697	0.8607	0.5883
0.5	1	0.9979	0.9819	0.8907

Table 3-2. Estimated power TAP ($\delta = 0.2$)

β	Nominal significance levels α			
	0.05	0.01	0.005	0.001
-0.5	0.9997	0.9919	0.9434	0.7333
-0.4	0.9801	0.9192	0.7537	0.3952
-0.3	0.8196	0.6077	0.3551	0.1037
-0.2	0.404	0.2022	0.079	0.0162
-0.1	0.1056	0.0359	0.0088	0.001
0	0.0493	0.0156	0.0038	0.0003
0.1	0.1084	0.0375	0.0091	0.0009
0.2	0.4027	0.1995	0.0729	0.0098
0.3	0.833	0.6189	0.3549	0.107
0.4	0.9795	0.9191	0.7405	0.3861
0.5	0.999	0.9927	0.9479	0.7344

segments and CRISPR probes. The mRNA and microRNA expressions, methylation probes, SNPs, SNVs/in-dels from WES and CNV segments were tested twice (once in PG and once in TAP), resulting in 1,861,210 tests; and there were 13,267 tests in CRISPR. So, we regard the study-wide multiplicity as $m=1,874,477$. The number of genes captured in all three analyses purely by chance, N , can be modeled by a random variable following the Binomial(m, π^*) distribution. Thus $\Pr(\text{capturing } \geq 15 \text{ genes in all three analyses purely by chance}) = \Pr(N \geq 15)$ which is described in **Equation 3-5**.

$$\begin{aligned} \Pr(\text{capturing } \geq 15 \text{ genes in all three analyses purely by chance}) &= \Pr(N \geq 15) \\ &= \sum_{i=15}^m \binom{m}{i} (\pi^*)^i (1 - \pi^*)^{m-i} \approx 8.1655 \times 10^{-79} \end{aligned}$$

(Eq. 3-5)

Therefore, the probability of capturing 15 genes in all three analyses purely by chance is extremely small (8.2×10^{-79}).

Single Cell RNA Sequencing

To interrogate the expression of genes at the single cell level in patients who were either sensitive or resistant to prednisolone in vitro, primary patient ALL cells were collected at diagnosis and incubated for 96 hours with or without prednisolone. Patient cells were re-suspended at a concentration of 2×10^6 cells/mL in culture media, as described above. On day zero, 80 μ L of cells were plated in round bottom 96-well plates. Cells were incubated for 4 days with either 63 μ M prednisolone or no drug, collected and washed with 50 μ L of PBS and 150 μ L total volume was collected. Both control and treated samples were processed and subjected to single cell RNA-sequencing on the 10x Genomics platform. The Cellranger software from 10x Genomics was used to demultiplex each of the samples, align the demultiplexed reads to the hg19 human genome, collapse PCR duplicate reads into UMIs, and generate a matrix of UMI counts for each cell and Ensembl ID combination.

All UMIs aligning to ribosomal protein-coding or mitochondrial genes were removed from the count matrices. Cells were removed if less than 500 genes were detected from the remaining UMIs. UMI counts within a cell were normalized by dividing each UMI count by the total UMI count across the cell, scaling by the median total UMI count across all cells from the four samples, adding a pseudocount of one, and taking the natural logarithm.

PCA was performed jointly on control and treatment samples from each patient. The first 40 principal components of overdispersed genes were used to generate a two-dimensional embedding of cells using the Barnes-Hut implementation of tSNE with a perplexity of 30.¹⁶⁴ Dispersion was calculated as described elsewhere¹⁶⁵ and as implemented in Seurat (<https://github.com/satijalab/seurat>), with overdispersed genes being genes in each bin that have z-scores at or above 1.4 ($n = 922$ genes for sensitive and $n=663$ for resistant).

Cell-cell Euclidean distance matrices were computed jointly for control and post-treatment samples of each patient using the over-dispersed genes. Hierarchical clustering of the cells using the Euclidean distance matrix was performed using the Ward method.¹⁶⁶ Clusters were then identified using DynamicTreeCut, an iterative cluster partitioning and agglomeration method.

Clusters with at least 20% of the component cells expressing *CD19* were classified as B cells. The null hypothesis that the proportion of CD19+ cells between control and treatment samples for each patient was tested using the prop.test function in R, and differential expression of genes was tested using the Wilcoxon rank sum test as implemented in the Seurat R package. This resulted in seven clusters for the resistant patient and eleven clusters from the sensitive patient (**Figure 3-1a,d**)

Clusters of the B-lineage leukemia cells were identified based on the expression of *CD19* in the component cells. Other cell types were identified by identifying the most highly differentially expressed genes in each cluster that are known markers of hematopoietic lineage (e.g. T-cells, red blood cells; **Figure 3-1b,e**). The sensitive patient in our single cell analysis had a B-lineage ALL with *P2RY8-CRLF2* fusion. The resistant patient in this analysis had a *BCR-ABL* positive B-lineage ALL.

Immunoblot

Cells were pelleted at 500xg. Lysates were prepared in RIPA buffer (Sigma-Aldrich) containing cComplete Protease Inhibitor Cocktail (Roche Life Science) and PhosStop (Sigma-Aldrich). Equivalent amounts of extract (20 µg) were separated on 3-8% Novex Tris-Acetate polyacrylamide gels (Thermo Fisher Scientific) and transferred to polyvinylidene fluoride (PVDF) membranes (0.2 µm). Non-specific binding was blocked with 5% Milk in TBS with 0.05% Tween-20 for at least 1hr prior to incubation of membranes with primary and secondary antibodies. Primary antibodies used were rabbit anti-CELSR2 monoclonal (Cell Signaling; D2M9H) diluted 1:1,000, mouse anti-BCL2 monoclonal (Cell Signaling;124) diluted 1: 1,000, mouse anti-BIM monoclonal(Cell Signaling;C34C5) diluted 1:1,000, mouse anti GR(BD Biosciences ; #611227) diluted 1:1,000 ,mouse anti p-JNK (Santa Cruz Biotech; sc-6254) diluted 1:500, rabbit anti cJun (Cell signaling; 9165), rabbit anti phospho-cJun Ser63 (Santa Cruz Biotechnology; sc-822) diluted 1:500, rabbit anti NFAT1 (Cell Signaling ; 4389) diluted 1:1,000, SAPK/JNK (Cell Signaling ; 9252) diluted 1:1,000 , mouse anti GAPDH (Santa Cruz Biotechnology; sc-47724), mouse anti PAX5 (Cell Signaling ;12709) diluted 1:1000mouse anti BCL2 (Cell Signaling; 15071) diluted 1:1000, mouse anti glucocorticoid receptor (BD Biosciences; 611227) diluted 1:1000 rabbit anti LaminB1 (Cell Signaling; 12586) diluted 1:1000 and anti-β-actin (Sigma-Aldrich; A5441) diluted 1:100,000. Membranes were then incubated with appropriate HRP-Conjugated IgG secondary antibodies (Jackson Immunoresearch) and developed with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific) prior to signal acquisition using an Odyssey Fc Imager (LI-COR). Image processing and signal quantification were

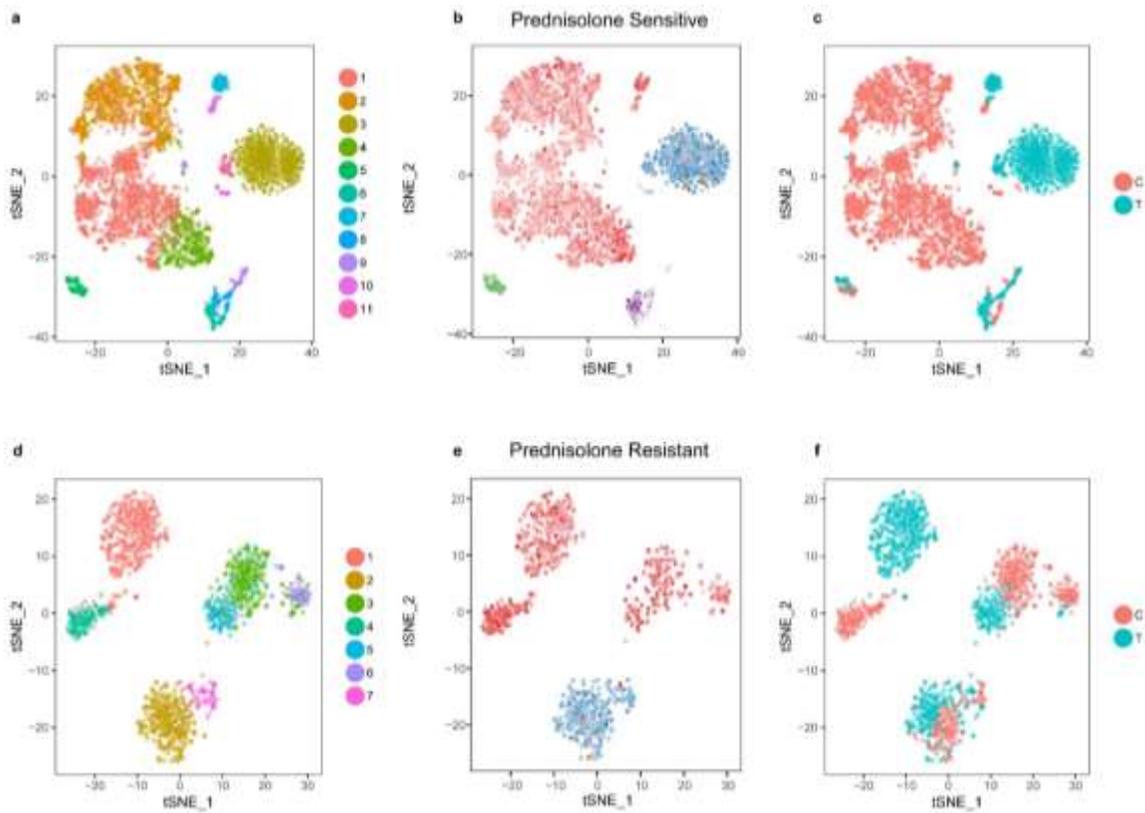


Figure 3-1. Single cell transcriptomics defines distinct expression signatures in primary B-ALL cells

(a.) Clustering of bone marrow cells from a prednisolone sensitive patient (n=2,427 control cells; n= 924 treated cells) based on top 1000 most highly expressed genes (b.) Identification of distinct cell populations in a prednisolone sensitive patient CD19+ B-cells (red), CD3E+ T-cells (blue), ALAS2+ Erythrocytes (purple) and CD14+ Macrophages (green) (c.) Control vs. treatment for all cell clusters in prednisolone sensitive patient (red = control, blue = treated) (d.) Clustering of bone marrow cells from a prednisolone resistant patient (n= 686 control cells; n=759 treated cells) based on top 1000 most highly expressed genes (e.) Identification of distinct cell populations in a prednisolone resistant patient CD19+ (red) and CD3E+ T-cells (blue) (f.) Control (C) vs. treatment (T) for all cell clusters in prednisolone resistant patient (red = control, blue = treated)

performed with Image Studio software (Version 4.0; LI-COR). Nuclear/Cytoplasmic protein extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher) using standard protocol.

ChIP-seq and ATAC-seq

ChIP-seq was performed as previously described.¹⁶⁷ Briefly, 20 million NALM-6 cells were crosslinked using 1% formaldehyde and sonicated on a Diagenode Bioruptor Plus sonicator. Chromatin immunoprecipitation was performed using 5 μ g anti-PAX5 antibody (Abcam, ab15164). ChIP-seq and input control libraries were run on an Illumina HiSeq4000 next-generation sequencing machine using single-end 50bp sequencing, reads were mapped to the hg19 reference genome using BWA and binding sites were called using MACS2 peak caller. ATAC-seq was performed using the Fast-ATAC protocol.¹⁶⁸

Briefly, 10,000 cells were transposed in a cocktail containing 1% digitonin. Following transposition, DNA was collected using the Qiagen MinElute Reaction Cleanup Kit (Qiagen #28204) and amplified for 5 cycles using barcoded Nextera PCR primers. A quantitative PCR reaction was performed on an Applied Biosystems QuantStudio 3 Real-Time PCR machine using 5 μ L of PCR product to determine the additional number of PCR cycles required. PCR products were subsequently re-amplified for the appropriate number of additional PCR cycles and DNA was size-selected using SPRIselect beads (Beckman Coulter, B23317). ATAC-seq libraries were run on an Illumina HiSeq4000 next-generation sequencing machine using paired-end 100bp sequencing, reads were mapped to the hg19 reference genome using Bowtie2 and open chromatin sites were called using the MACS2 peak caller.

Previously Reported Mechanisms of Prednisolone Resistance

To assess the performance of our agnostic polygenomic method to identify genes that confer resistance to glucocorticoids, we used Illumina BaseSpace® Literature Correlation Engine to perform a literature search to identify all genes previously reported to confer resistance to glucocorticoids (performed January 2018). The search terms “glucocorticoids, leukemia” were used for our initial search, with a secondary filter of “resistance”. This resulted in 426 total publications, which we narrowed using the “genes and proteins” tab set on the “top 1000” setting and exporting the list of top word cloud tags that were found from our search. This list of word cloud tags was entered into the HUGO database (https://www.genenames.org/cgi-bin/symbol_checker); the resulting list was trimmed by removing unmatched terms. This generated a list of 347 unique genes published through 2016. We also included genes published in papers from January 2017-January 2018 that were found by a PubMed search using search terms “glucocorticoids, resistance, leukemia” with results limited by publication date, yielding 27 additional publications.

For all genes included in our tabulation of known genes previously associated with glucocorticoid resistance, we required a published report linking the gene to poor patient response (i.e. remission induction failure, persistence of minimal residual disease [MRD], or disease relapse) or glucocorticoid resistance as measured in primary ALL cells and that the gene/pathway was confirmed through either direct manipulation or chemical inhibition in a human cell culture model or patient derived xenograft (PDX). Using the aforementioned methods and criteria, a table of “known mechanisms” was generated (**Supplementary Table 7**) and comparisons were then made to genes identified as significantly related to glucocorticoid resistance by our three methods (polygenomic, TAP, CRISPR screening), using either all genes identified by any one of these methods or using the subset of genes identified by two or more of these methods. Direct matches of genes were considered the strongest evidence. Genes that were not directly matched were considered to have an associated pathway component if they were found by searching for the gene in STRINGdb with the following criteria: 1) Only 30 first shell interactors with a correlation > 0.7 (high confidence), 2) only “Text-mining”, “Experiments”, “Databases”, “Co-expression” were used to define informative data sources. In all cases, concordance of directionality of the relationship was required between published genes and those discovered in our analyses.

NetBID Analysis to Identify Drivers of GC-Resistance in ALL Patients

One pitfall from the conventional gene expression analysis is that important signaling proteins might not change at individual mRNA expression level, thus network-based methods were used to infer master regulator activity which would help overcome this pitfall. We applied the network-based integrative NetBID¹⁶⁹ algorithm to identify “hidden” drivers in GC-resistant primary leukemia cells (ALL cells from patients) using gene expression profiles. First, NetBID used an improved version of ARACNE 88, an information theory-based algorithm to reverse-engineer a B-ALL specific interactome (BALLi) from RNA-seq profiles of B-ALL primary B-All patients (N=185) from TARGET¹⁷⁰ project against 1,673 transcription factors and 6,247 signaling proteins annotated by Gene Ontology.¹⁷¹ With parameters NB=100, e=0 and p=1e-7, the data-driven BALLi resulted in 21,655 nodes and 830,213 edges. Then we applied ‘netbid’ function (signed=TRUE) in NetBID package, which used z-normalization and z-statistic for activity inference and Bayesian linear modeling for differential activity analysis, to compare resistant (level=3) versus sensitive (level=1) as well as intermediate (level=2) vs. sensitive ALL patients from TOTXV and TOTXVI cohorts respectively and then applied ‘netbidi’ function, which used Stouffer’s method, to integrate results from TOTXV and TOTXVI cohorts that used two different microarray platforms (HG-U133A and HG-U133_Plus_2). In TOTXV cohort, we observed and removed the batch effects from sample source by using the “removeBatchEffect” function in limma.¹⁷² Finally, the 48 top drivers were selected by the following criteria: network size > 50 , $p < 5 \times 10^{-5}$ in TOTXVI and integrated (combined) analysis of highly-resistant vs. sensitive patients, and that the intermediate vs. sensitive and the resistant vs. sensitive results were concordant. The NetBID package can be found online at: <https://github.com/jyyulab/NetBID>.

Drug Synergy Experiments and Response Surface Modeling

Relationship of response was determined when prednisolone was given alone or in combination with three concentrations of venetoclax using both NALM-6 cells (10nM, 100nM, 1 μ M) and 697 cells (1nm, 10nm, 50nm). Concentration ranges of venetoclax differed because of differences in sensitivity to venetoclax between the two cell lines. Viability assays were carried out using Cell Titer Glo. In synergy experiments with primary leukemia cells from patients, 1nM, 10nM and 100nM concentrations of venetoclax were used to assess synergy with prednisolone. Response surface modeling, a well-established method calculating synergy in drug combinations^{173,174} as implemented in MATLAB version R2016a (MathWorks), was performed to evaluate changes in the response of cells to prednisolone and venetoclax alone or in combination at three concentrations (low, medium, high).¹⁷⁵⁻¹⁷⁷ A drug combination was considered synergistic if the (α) which represents the change in response relative to additive model was positive and antagonistic if this value was negative. Results were confirmed using two other established models (Loewe's Additivity and ZIP) using the synergyfinder R package (data not shown).¹⁷⁸

***In Vivo* Drug Combination Studies**

NALM-6 cells were injected into non-irradiated 8-12 week old female NSG mice (100,000 cells/mouse). Treatment was started three days post injection and continued until the endpoint was reached for each mouse. Mice were treated with either continuous dexamethasone (4mg/L) alone, venetoclax alone (50 or 100mg/kg), as previously described¹⁷⁹ or either dosage of venetoclax in combination with dexamethasone. Dexamethasone was given daily in drinking water with tetracycline (1g/L; Sigma-Aldrich, St. Louis, MO), and half of each week the water contained Sulfamethoxazole (600 mg/L) and trimethoprim (120mg/L; from Hi-Tech Pharmacal, Amityville, NY). Mice were randomized following injection. Mice were sacrificed when they became moribund for any reason, as determined by the veterinarian.

Flow Cytometry

Blood was collected from the retro-orbital sinus, facial vein, or tail vein of anesthetized mice to assess engraftment of human ALL cells. Blood was lysed with the BD FACS Lyse Wash Assistant (BD Biosciences, San Jose, CA). Cells were stained with antibodies to human CD19 (eBioscience;45-0199-42, PerCP-Cy 5.5) and mouse CD45 (Tonbo; 20-0451-U100, APC). Samples were assayed on the BD LSR II or LSR Fortessa (BD Biosciences) and analysis was performed with FlowJo version 10 (FlowJo, LLC, Ashland, OR).

Statistics and Reproducibility

All statistical analyses were performed using R software or Graphpad Prism 8 unless otherwise stated. For box plots unless stated explicitly, upper and lower values in each box depict the 75th and 25th percentiles, respectively, the solid line represents the median, and the top and bottom of each dashed vertical line depict the most extreme data points that were no more than 1.5 times the interquartile range (75th percentile–25th percentile) from the box. All bar plots are representing mean \pm standard deviation (S.D.) and each dot represents an independent experiment or sample. For all analyses comparing means of groups student's t-test was performed, Chi-Squared analysis was used for MRD group data, linear model was used as the predominant method for statistical significance in genome-wide analyses using LC₅₀ as a continuous variable and Fisher's tests were used to describe clustering of genomic data vs LC₅₀. Customized methods were also used such as NetBID which relied on Bayesian inference and used z-scores to report significance¹⁶⁹ and the TAP method which utilized a hybrid permutation and an adaptive thresholding approach to assessing statistical significance of higher order problems.

Data Availability

DNA methylation, gene expression and ChIP-seq data are available at the Gene Expression Omnibus (GEO) under accession GSE66708. miRNA data can be found at GEO under the accession number GSE76849. Cell line RNA-seq data can be found at GEO under the accession number GSE115384. Validation cohort #1 RNA-seq data from 73 of the 320 patients in the independent second cohort can be found at GEO under the accession GSE115525. Additional RNA-seq data from validation cohort #1 (n= 247) can be found at GEO under GSE124824. PAX5 CHIP-seq can be found at GEO under the accession GSE115764. Cell line ATAC-seq data can be found at GEO under the accession GSE129066. Genotype data can be found in dbGaP at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000638.v1.p1

Code Availability

Code used to generate for the polygenomic and TAP analyses can be found on GitHub at <https://github.com/evanslabSJCRC/Polygenomic-Analysis>. The NetBID code can be found at <https://github.com/jyyulab/NetBID>. Any custom code generated for our analyses not specifically listed here or in the text may be requested from Dr. William E. Evans (William.Evans@stjude.org). All R packages or other software used are listed in methods section for each relevant analysis.

CHAPTER 4. RESULTS*

Introduction

Drug resistance is a major cause of treatment failure for disseminated human cancers.¹⁸⁰ Acute lymphoblastic leukemia (ALL) has long served as a model for developing curative chemotherapy for disseminated malignancies. Long-term disease-free survival in childhood ALL has increased dramatically in recent decades, with 5-year event-free survival approaching 90%, yet drug resistance makes it less curable in adult patients and it remains a leading cause of cancer deaths in children.²¹ Much of the improvement in cure rates can be ascribed to refinement of therapy based on improved understanding of clinical and biological characteristics of the disease and the intensification of treatment when there is poor early response or persistence of minimal residual disease (MRD).^{21,47,181,182} Glucocorticoids, such as prednisone (PRED) and dexamethasone (DEX), are essential components of curative combination chemotherapy for ALL in adults and children³¹ and the intrinsic sensitivity of ALL cells to glucocorticoids, as measured *ex vivo*, is predictive of treatment outcome (event-free survival or survival) in childhood ALL.^{48,49,180,183} Although several mechanisms of leukemia cell resistance to glucocorticoids have been identified,^{33,184-186} the genomic and epigenetic determinants of *de novo* glucocorticoid resistance remain poorly understood. Whole genome sequencing offers a comprehensive approach for identifying sequence variants that confer drug resistance, but this technology does not assess the complex interaction of multiple genomic, transcriptomic and epigenetic mechanisms.¹⁸⁷ In the current study, we integrated genome-wide interrogation of multiple genomic and epigenetic features of primary leukemia cells to identify genes associated with drug resistance, using glucocorticoids as a model. This directly identified over 78% of genes and 100% of pathways previously associated with glucocorticoid resistance and further revealed 14 genes not previously known to confer glucocorticoid resistance. Collectively, this represents an agnostic, multi-dimensional genome-wide strategy for discovery of genomic mechanisms of drug resistance in primary cancer cells.

Results

Drug Sensitivity and Treatment Response

The sensitivity to prednisolone of primary leukemia cells from bone marrow aspirates of 225 newly diagnosed patients with B-lineage ALL ranged over 5 orders of

*Portions of chapter from previously published article; final submission modified with permission. Autry, R. J. et al. Integrative genomic analyses reveal mechanisms of glucocorticoid resistance in acute lymphoblastic leukemia. *Nature Cancer*, doi:[10.1038/s43018-020-0037-3](https://doi.org/10.1038/s43018-020-0037-3) (2020).¹⁴²

magnitude (LC₅₀ 0.00176 -1387.4μM) (**Figure 4-1a**).

Using previously reported criteria, patients with prednisolone LC₅₀ values <0.1 μM were classified as sensitive, those >64 μM were classified as resistant, and the remaining patients were designated as intermediate sensitivity.⁷⁵ Patients whose leukemia cells were intermediate or resistant to prednisolone were significantly more likely to have minimal residual disease (MRD) >1% at day 15-19 of remission induction therapy (p=1.3 x10⁻⁵; **Figure 4-1b**). Likewise, MRD at the end of remission induction therapy, on day 46 of St. Jude Total XV protocol (TOTXV) or day 42 of St. Jude Total XVI protocol (TOTXVI), was more likely to be >0.1% in patients whose leukemia cells were intermediate or resistant to prednisolone (p=1.1 x10⁻⁴; **Figure 4-1b**). These MRD levels have been previously associated with a significantly worse event free survival.¹⁸⁸⁻¹⁹⁰

Polygenomics of Glucocorticoid Resistance

Six distinct genomic/epigenetic features were interrogated genome-wide in primary leukemia cells and assessed for their association with prednisolone resistance (LC₅₀) in two independent patient cohorts [**Figure 4-2**]. Hierarchical clustering of each feature type was performed to identify genomic features (mRNA, miRNA, CpG methylation, single nucleotide polymorphisms [SNPs], copy number alterations [CNAs] and SNV/Indels [WES]) that best discriminated prednisolone sensitive and resistant ALL (**Supplementary Table 1**). These analyses identified 254 mRNA expression probes (permutation p-value < 8.2x10⁻⁵), 203 CpG methylation sites (permutation p-value < 1x10⁻⁵), 49 miRNA probes (permutation p-value < 1x10⁻⁵), 380 SNPs (permutation p-value < 1x10⁻⁵), 25 CNA segments (permutation p-value < 4.5x10⁻⁴) and 227 WES mutations (permutation p-value < 1x10⁻⁵) that best individually discriminated prednisolone resistant from prednisolone sensitive ALL (**Figure 4-3; Figure 4-4a-b; Supplementary Table 1**). This mRNA expression signature was verified in two independent validation cohorts (**Figure 4-4c-d**). Collectively, these features identified 192 distinct genes associated with prednisolone resistance.

Connectivity of these genomic features (miRNA, methylation, SNPs, CNAs and SNV/Indels) was initially assessed based on significant relation to mRNA expression and filtered based on biologically relevant criteria (cis CpG site or miRNA binding site as described in Methods), revealing that the expression of 94% of the significant mRNAs was significantly associated with at least one of the other genomic features and five were connected to all other features (*ID11, ITPR3, PTPRF, WNK1, and PAX5*; **Supplementary Table 2**). mRNA expression probes that were associated with LC₅₀ in the polygenomic analysis were also associated with treatment response, as assessed by the in vivo level of residual leukemia on day 15-19 and day 42-46 of remission induction treatment (**Figure 4-4e**). Prednisolone LC₅₀ across all subtypes revealed some ALL subtypes that were more resistant than others, but prednisolone resistant and sensitive cases were documented in all major subtypes (**Figure 4-4f**).

Genes identified in the polygenomic analysis (**Figure 4-3**) were interrogated for

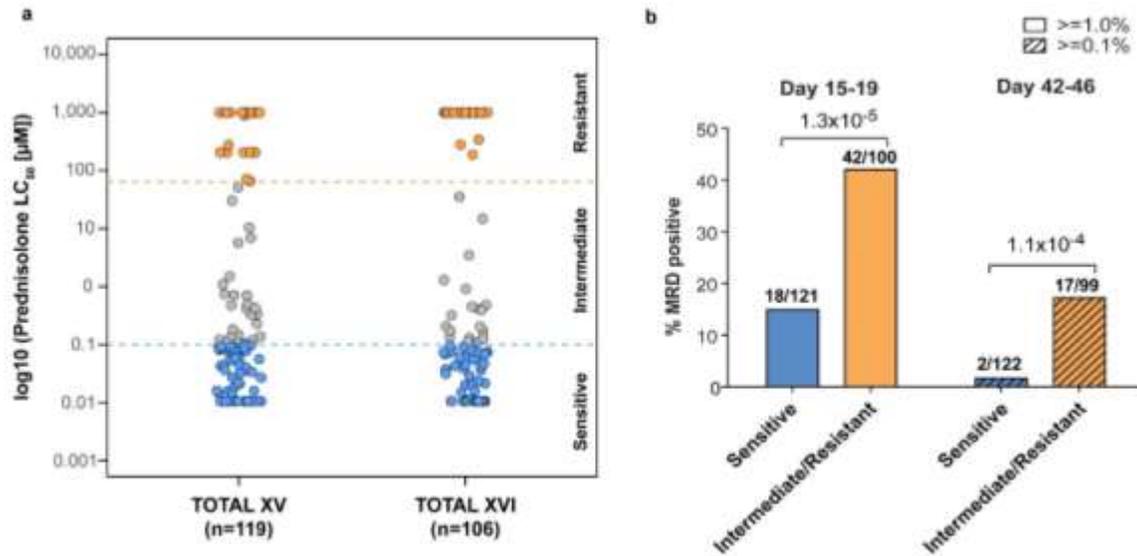


Figure 4-1. *De novo* sensitivity of primary leukemia cells to prednisolone and clinical treatment response

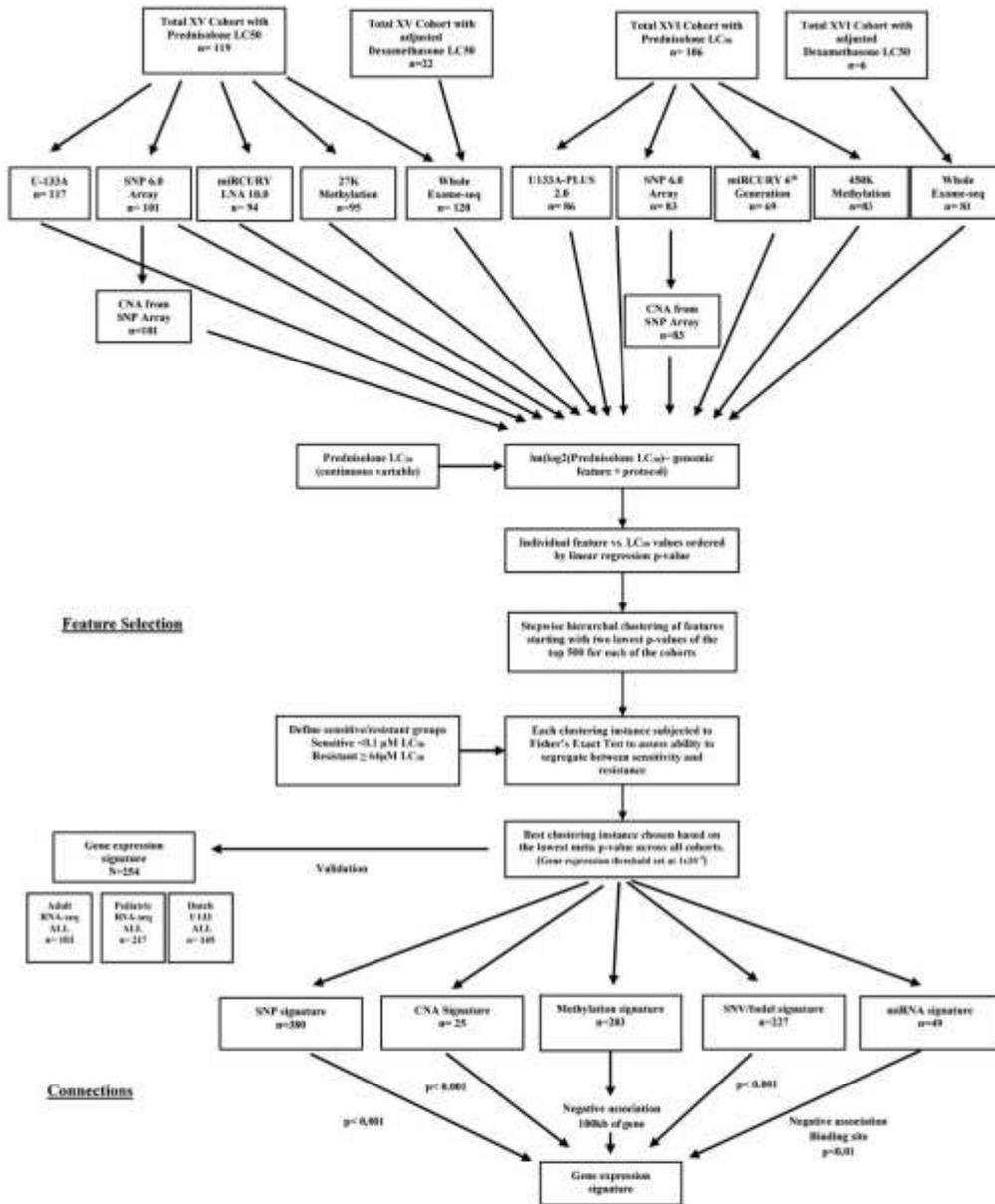
a) Distribution of prednisolone LC₅₀ values in the discovery cohort, comprising children with acute lymphoblastic leukemia enrolled on two consecutive research protocols at St. Jude (n=119 and n=106 ALL patients, respectively) depicting >10,000- fold range in *ex vivo* sensitivity. Horizontal dashed lines depict LC₅₀ values discriminating prednisolone sensitive, intermediate and resistant cases using previously reported values.⁷⁵ b) The percentage of patients who had minimal residual disease (MRD) in their bone marrow at day 15-19 of treatment (MRD ≥ 1%) or at day 42-46 of treatment (MRD ≥ 0.1%) differed significantly based on prednisolone sensitivity (Chi-Square test p-value; n=221 ALL patients).

Figure 4-2. Polygenomic analysis workflow

(a.) Flowchart depicting cohorts, genomic assays and detailed analysis pipeline for polygenomic analyses of multiple feature types (mRNA, miRNA, DNA methylation, SNVs, CNVs and WES mutations) as determinants of prednisolone sensitivity in patients diagnosed with acute lymphoblastic leukemia (“lm” = linear model). (b.) Table describing age, race, gender and molecular subtype of discovery cohort (n=225 patients) from polygenomic analysis. The P-values represent differences between the discovery cohort enrolled on the two clinical trials (Fisher’s Exact Test p-value; Total 15 and Total 16).

a

Genomic Analyses of Primary ALL Cells



b

Variable	Levels	n _{prof XV}	% _{prof XV}	Σ % _{prof XV}	n _{prof XVI}	% _{prof XVI}	Σ % _{prof XVI}	n _{all}	% _{all}	Σ % _{all}	
Age > 10	>= 10	16	16.8	18.9	20	18.9	18.9	36	18.9	18.9	
	< 10	69	81.2	100.0	86	81.1	100.0	155	81.2	100.0	
p = 1.00	all	80	100.0		106	100.0		191	100.0		
Sex	Female	64	53.8	53.8	52	49.1	49.1	116	51.6	51.6	
	Male	25	46.2	100.0	54	50.9	100.0	109	48.4	100.0	
p = 0.51	all	119	100.0		106	100.0		225	100.0		
Race	White	80	87.3	67.8	64	61.0	61.0	144	64.5	64.5	
	Black	17	14.4	82.2	16	15.2	76.2	33	14.8	79.4	
	Hispanic	17	14.4	96.5	14	13.3	99.5	31	13.9	93.3	
	Asian	1	0.8	97.5	0	0.0	89.5	1	0.5	93.7	
	Other	3	2.5	100.0	11	10.5	100.0	14	6.3	100.0	
	p = 0.51	all	118	100.0		105	100.0		223	100.0	
	Molecular Subtype	B-other	28	21.9	21.9	24	22.6	22.6	50	22.2	22.2
Hyperdiploid		27	22.7	44.5	25	23.6	46.2	52	23.1	45.3	
Hypodiploid		9	7.6	52.1	4	3.8	50.0	13	5.8	51.1	
Ph-like		4	3.4	55.5	5	4.7	54.7	9	4.0	55.1	
Ph-like-CRLF2		4	3.4	58.8	1	0.9	55.6	5	2.2	57.3	
BCR-ABL		2	1.7	60.5	2	1.9	57.5	4	1.8	59.1	
CRLF2-non-Ph-like		1	0.8	61.3	3	2.8	60.4	4	1.8	60.9	
TCF3-PBX1		8	6.7	68.1	5	4.7	65.1	13	5.8	66.7	
ETV6-RUNX1		27	22.7	90.8	30	28.3	93.4	57	25.3	92.0	
MLL-AF4		3	2.5	93.3	4	3.8	97.2	7	3.1	96.1	
ERG		8	6.7	100.0	3	2.8	100.0	11	4.9	100.0	
p = 0.70	all	119	100.0		106	100.0		225	100.0		

Figure 4-3. Polygenomic analyses identify genomic features related to prednisolone resistance

Leukemia cell mRNA, miRNA, DNA methylation, copy number alterations (CNAs), single nucleotide polymorphisms (SNPs) or coding SNVs/Indels (by WES) that significantly discriminate prednisolone sensitive and resistant ALL, by hierarchical clustering in the discovery cohorts (TOTXV and TOTXVI). Each column represents an individual patient's ALL cells, those labeled at the top with green are sensitive and those with red are resistant to prednisolone; each row indicates a different probe. (Center panel) mRNA expression vs prednisolone LC₅₀: heat map depicts high (red) or low (blue) gene expression relative to the mean signal for that probe set in the entire cohort [n=254 mRNA probes; n= 203 patients]. (Top left) heat map for miRNA expression versus LC₅₀; red and blue denote higher versus lower expression relative to mean signal for probe amongst the entire cohort [n=49 miRNAs; n=163 patients]. (Top Right) DNA methylation versus LC₅₀; red and blue denote higher versus lower methylation signal [n=203 CpG probes; n=178 patients] (Bottom left) single nucleotide polymorphisms (SNPs) associated with LC₅₀ blue = AA, orange = AB, purple = BB [n=380 SNPs; n=184 patients]. (Bottom right) copy number alterations (CNAs) associated with LC₅₀; red = copy gain, blue = copy loss, orange = copy neutral [n=25 CNAs; n= 184 patients]. (Bottom center) SNVs and Indels by WES [n=227 SNVs/Indels] associated with LC₅₀; purple = mutation, orange = non-mutated. (Lines) lines connecting probe sets are drawn where significant associations were found between mRNA expression levels and a specific peripheral genomic feature. DNA methylation and miRNA connections were required to be significantly negatively associated with mRNA expression; DNA methylation probes were also required to be within 100kb of the gene encoding the mRNA and miRNAs were required to have a predicted binding site in the gene and/or experimental evidence from literature. Connections between SNVs/Indels and mRNA are provided in **Figure 4-4a**. P-values for each heatmap (at top) indicate the results of Fisher's exact tests comparing the distribution of sensitive and resistant cases when the highest level of the dendrogram is split in two. Overall clustering P-values for each heatmap (at bottom) are the result of Stouffer's meta-analysis of corresponding individual Fisher's exact test (two-sided) p-values within each cohort.

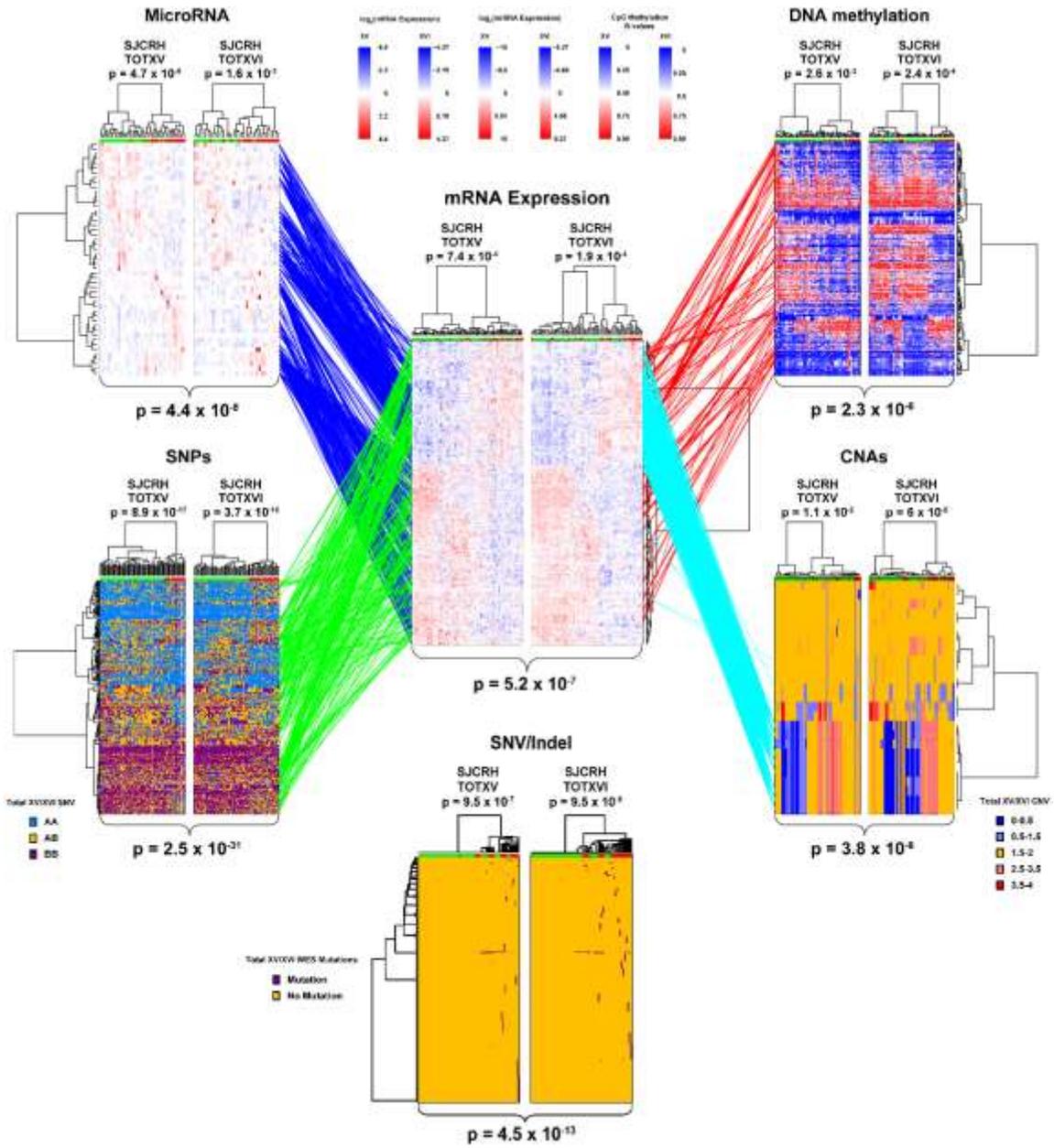
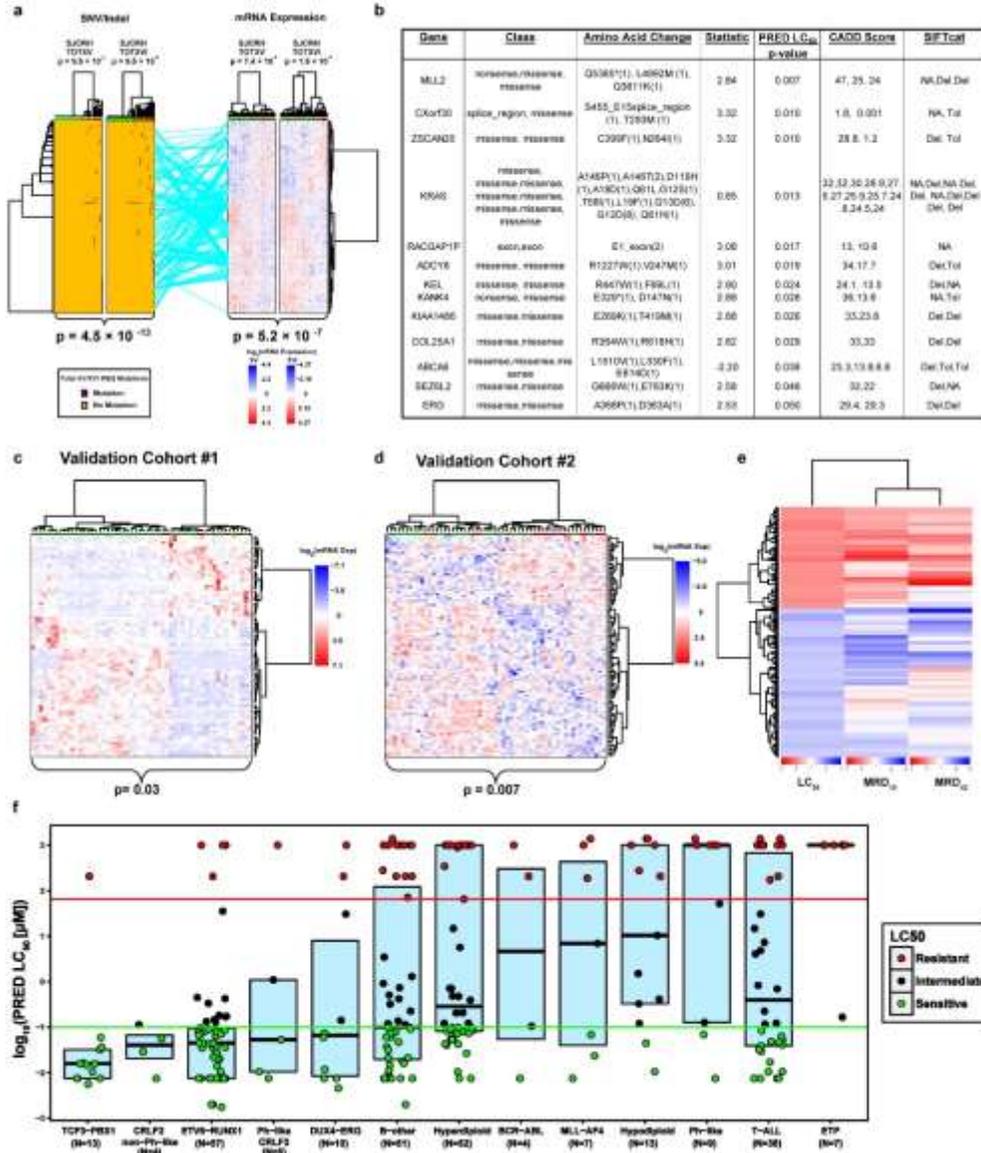


Figure 4-4. Validation of gene expression signature, relation to treatment response and WES variant connectivity

(a.) Connectivity between polygenomic signatures for mutation (n=227 mutations) and mRNA expression (n=254 mRNA probes; Fisher's Exact Test clustering p-values and linear model p-value for connectivity). (b.) Characteristics of WES mutations with linear model p-value <0.05 vs. LC₅₀. (SIFTcat Del = Deleterious and Tol = Tolerated). (c.) RNA sequencing of ALL cells from St. Jude Total XVI patients (n=73 patients; validation cohort #1; Fisher's Exact Test clustering p-value) clustered with gene expression signature from discovery cohort analysis. (d.) Publicly available DCOG/COALL patient cohort (n=145 patients; validation cohort #2; Fisher's Exact Test clustering p-value) clustering with gene expression signature from patient discovery cohort. (e.) Clustering of gene expression vs. LC₅₀. Red denotes genes correlated with LC₅₀ or minimal residual disease (MRD) in positive direction. Blue denotes genes correlated in negative direction with LC₅₀ or MRD. Clustering performed to show concordance of genes discriminating LC₅₀ or MRD. (f) Boxplot denoting Prednisolone LC₅₀ in patients from discovery cohort with the major ALL molecular subtypes. Red circles denote prednisolone resistant patients, green denotes sensitive patients, and black denotes intermediate sensitivity. Upper line is the upper quartile (75%) middle line is the median and lower line is lower quartile (25%) boundary for prednisolone LC₅₀.



relevant pathway connections (**Supplementary Table 3**), that included B-cell development (*PAX5* mRNA, *FLT3* methylation and SNV/Indel, *ITGA4* SNP eQTL), B-cell receptor signaling (e.g., *CD19* methylation, *PAX5* mRNA, *TCF3* mRNA), non-canonical Wnt signaling (*CELSR1/2* methylation and mRNA, *ROR1* mRNA), *IL7R*-signaling (e.g., *IL2RG* mRNA, *JAK3* SNP eQTL), SWI/SNF complex (*SMARCA4* mRNA), TGF- β signaling, apoptosis signaling (*BCL2* methylation, *FAS* mRNA), drug transporters (*ABCC1* methylation), and inflammatory signaling (*CASP1* methylation and mRNA, *NLRP3* methylation).

Gene Level Integration of Multiple Genomic Variants

To assess the influence of each gene on prednisolone resistance, we aggregated p-values of all genomic features within 50 kilobases upstream or downstream of the coding region for all annotated human genes ($n = 19,725$), to obtain a gene-level TAP (Truncated Aggregation of P-values) statistic with its associated p-value (see Methods). This identified 903 genes associated with prednisolone resistance ($p < 5.38 \times 10^{-4}$; **Figure 4-5a; Supplementary Table 4**). **Figure 4-6** illustrates four gene-level plots and their TAP statistic. *SMARCA4* and *NLRP3* illustrate genes previously associated with prednisolone resistance,^{75,123} whereas *CELSR2* and *PTTG1IP* illustrate the top two novel genes in current study (**Figure 4-6; Supplementary Table 5**). Many of these genes, 118/463 (25%), are common between the gene-level TAP analysis and the polygenomic analysis that assessed each feature independently (**Figure 3c**). Pathway analysis of genes significant in the TAP analysis is detailed in **Supplementary Table 3**.

Validation by Genome-wide CRISPR Knockout Screen

To validate hits by an orthogonal method, we performed genome-wide CRISPR/Cas9 knockout screening using the GeCKOv2 library.¹⁵⁷ In cells treated with 100uM prednisolone, we identified 1024 genes that were significantly “knockout enriched” in prednisolone resistant leukemia cells ($FDR < 5.2 \times 10^{-7}$; **Figure 4-5b; Supplementary Table 6**). *NR3C1*, the gene encoding the GR, was the top “knockout enriched” gene ($p = 4.6 \times 10^{-78}$). This screen identified genes affecting multiple cellular functions, including several pro-apoptotic genes (*BAK1*, *PMAIP*, *APAF1*, *CAPN3/10*) and genes involved in GR signaling, cell-cell communication genes (*ITGA5/B1*, *CELSR2*), modulators of GR transcriptional activation (*NR1P1*, *JUN*), inhibitors of NF- κ B kinase (*IKBKB*, *IKBKKG*), B-cell developmental genes (*BCL6*), glucocorticoid biosynthetic components (*HSD3B1/7*, *CYP11B1*), cytokine signaling genes (e.g., *IL4R*, *IL1RAPL2*), toll-like receptor signaling (*TLR6*, *IRAK3*, *TNF*), inhibitors of PI3K signaling genes (*PTEN*), and other genes previously associated with glucocorticoid resistance (*TBL1XR1* and *CNR2*). When the knockout enriched genes were used to perform clustering on the RNA-seq from a 320 patient validation cohort, this significantly discriminated resistant and sensitive leukemias (clustering p-value = 0.006).

We also identified 1000 genes that were significantly “knockout reduced” (i.e.

Figure 4-5. Genomewide orthogonal validation identifies *CELSR2* as a key mediator of glucocorticoid resistance

(a.) Manhattan plot of gene-level aggregated p-values (TAP statistic) for all 19,725 genes (n= 203 patients) (illustrated in regional plots for four genes in **Figure 4-6**). Circles above the blue dotted threshold line represent genes only significant in TAP analysis, squares depict genes significant in both the TAP and CRISPR screen, triangles represent genes significant in both the polygenomic analysis and the TAP analysis, and red stars depict the 15 genes significant in all three analyses (linear regression p-value cutoff [adjusted for massive multiple testing by adaptive thresholding] = 5.38×10^{-4}). (b.) $-\log_{10}$ p-values for genes (n=19,050 genes in two replicate experiments) interrogated in the CRISPR knockout screen (“knockout enriched”). The threshold for statistical significance (logit gene-level one-sided $p = 4.0 \times 10^{-8}$ [FDR adjusted $p = 5.2 \times 10^{-7}$]) of association with prednisolone resistance is depicted by the horizontal dotted line. (c.) Venn diagram showing overlap among genes significant in each analysis with 15 genes significant in all three analyses. (d-e) *CELSR2* mRNA expression in leukemia cells from newly diagnosed patients enrolled on St. Jude clinical trials, grouped based on prednisolone sensitivity (LC_{50}) in (d.) the discovery cohort (n=203; linear model p-value) and (e.) an independent validation cohort (n=320; linear model p-value). (f-g) *NR3C1* mRNA expression in leukemia cells from newly diagnosed patients enrolled on two St. Jude clinical trials, grouped based on *CELSR2* expression (low \leq lower quartile, high \geq upper quartile and intermediate falls between the upper and lower quartile of *CELSR2* expression) in (f.) the discovery cohort (n=203; linear model p-value) and (g.) an independent validation cohort (n=320; linear model p-value). (h.) Representative western blot and bar graph quantifying (n=3 biologically independent experiments) knockdown of *CELSR2* (mean \pm SD) in two human B-lineage leukemia cell lines (NALM-6 two tailed t-test p-value = 0.0026 and 697 two-tailed p-value = 0.0081; ** = <0.01 ; cropping performed uncropped image available as source data). (i.) Prednisolone LC_{50} values in human ALL cells lines (NALM-6 and 697) expressing *CELSR2* shRNA (~70% knockdown) or non-target control (results of triplicate experiments). For all boxplots, horizontal bars depict medians and boxes represent 25th and 75th percentiles, whiskers represent ± 1.5 x interquartile range (IQR); p-values are two-tailed t-test).

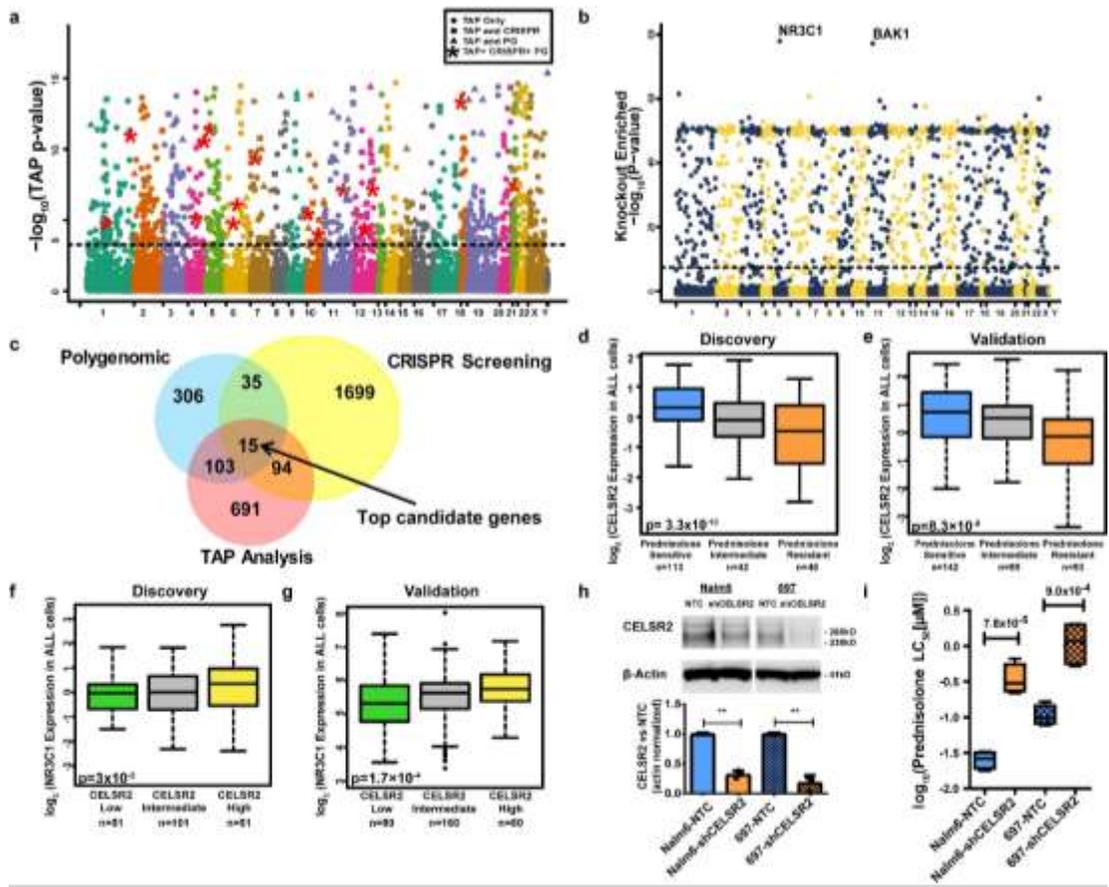
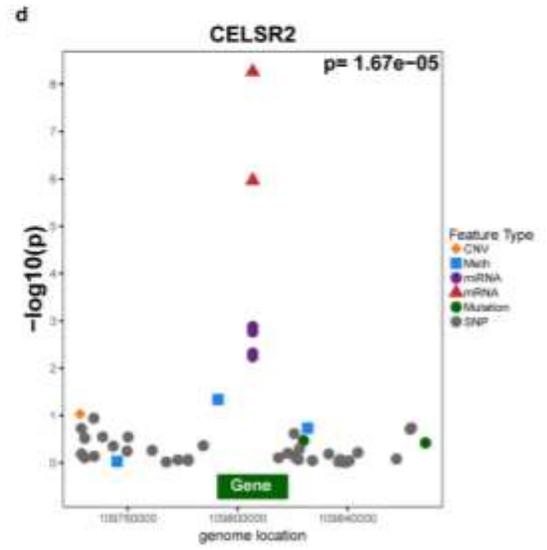
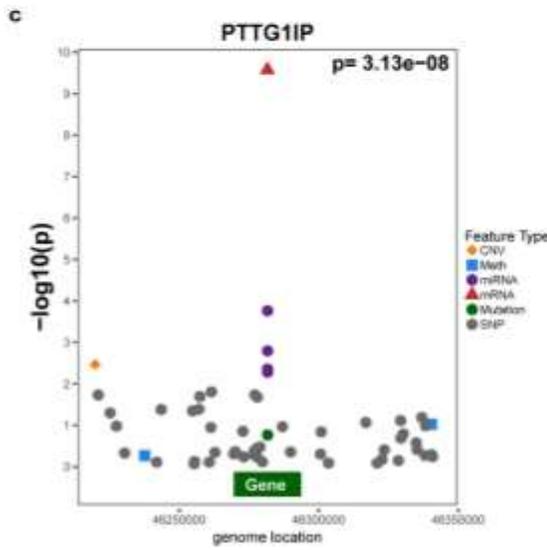
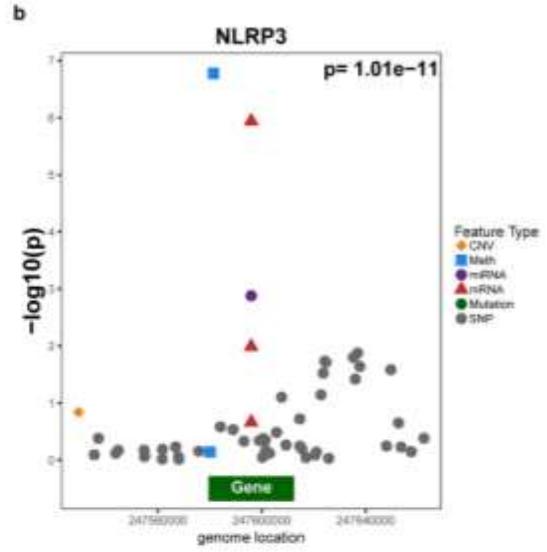
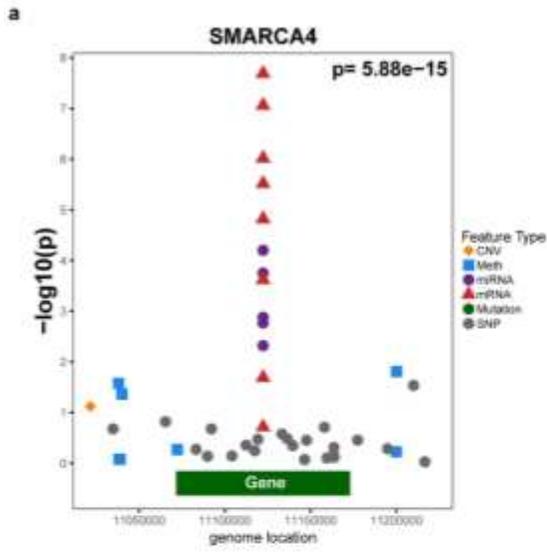


Figure 4-6. Gene level integration of genomic variants related to prednisolone resistance

Each panel depicts $-\log_{10}$ p-values for the association of the indicated genomic feature with prednisolone LC₅₀, and the aggregated gene-level linear model p-value based on all genomic features is shown for each gene at the top right. Red triangles represent mRNA probes within the gene body, orange diamonds depict copy number variants, blue squares are DNA methylation probes, grey circles SNVs, and purple circles miRNAs within 50kb upstream or downstream of gene region (n=203 patients). (a.) *SMARCA4*, a component of the SWI/SNF complex, has been previously linked to glucocorticoid resistance in pediatric ALL.¹²³ (b.) *NLRP3* encodes NALP3, an inflammasome component that activates caspase 1, and has been previously associated with ALL resistance to glucocorticoids.⁷⁵ (c.) *PTTG1IP* encodes the pituitary tumor-transforming gene 1 protein-interacting protein that interacts with the proto-oncogene *PTTGI* (also known as securin). (d.) *CELSR2* is a G-protein coupled receptor involved in non-canonical Wnt signaling. *PTTG1IP* and *CELSR2* are novel genes from the current study associated with glucocorticoid resistance.



depletion of gRNAs targeted to knockout these genes, $FDR < 1.19 \times 10^{-14}$; **Supplementary Table 6**), suggesting their absence enhanced glucocorticoid sensitivity. This revealed genes associated with various signaling pathways such as inflammasome activation (*NLRP3*, *NLRC3*, *CARD11/17*), NF- κ B signaling (*NFKB2*, *NFKBIB*), JNK/SAPK signaling (e.g., *MAPK9*[*JNK2*], *RAC2*), PI3K signaling (*PI3KR1/3*, *PI3KR3*, *PRKCB*), apoptotic proteins (*FAS*), TNF signaling and growth factor signaling (*FGFR2*, *FGF10*) [**Supplementary Table 3**]. The knockout reduced genes were also able to discriminate glucocorticoid resistant and sensitive leukemias in the 320 patient validation cohort (clustering p-value = 1.7×10^{-5}).

Genes Significant by Multiple Methods

As summarized in **Figure 4-5c**, 247 genes were significant by at least two methods and significantly discriminated resistance in the 320 patient validation cohort (clustering p-value = 0.001), as did the 118 genes significant by both TAP and polygenomic analyses (clustering p-value = 1.34×10^{-6}), and the 50 genes in both CRISPR and the polygenomic analysis (clustering p-value = 6×10^{-4}). Fifteen genes (*CELSR2*, *MAPK13*, *PARD3*, *CALN1*, *DAP*, *RBMS2*, *PTTG1IP*, *NLRP3*, *FAM13A*, *TAOK3*, *DCLRE1A*, *RASGRF2*, *FBXO9*, *GALNT1* and *TMEM126A*) were significant by all three methods, thereby constituting the top candidate genes (**Figure 4-5c; Supplementary Table 5**), and they discriminated sensitive from resistant leukemias in the validation cohort (clustering p-value = 1×10^{-4}). Only one of the 15 top candidate genes (*NLRP3*) has been previously associated with glucocorticoid resistance.⁷⁵ The statistical likelihood of a gene being significant by all three methods by chance is very low ($p = 8.2 \times 10^{-79}$).

Corroboration of Known Resistance Mechanisms

To assess the robustness of our approach, we compared genes/miRNAs that were significantly related to glucocorticoid resistance in our analyses, to genes involved in previously published mechanisms of glucocorticoid resistance (35 genes and three miRNAs, identified as described in Methods, summarized in **Supplementary Table 7**). Of these 38 previously reported genes/miRNAs, 30 (79%) were found to be significant by one or more of our three methods. This improved to 38/38 (100%) genes/miRNA when we included other members of the involved resistance pathway using StringDB (strict criteria as defined in Chapter 3).

CELSR2 Knockdown Alters Transcriptional Response and Prednisolone Resistance

CELSR2 was the top novel candidate gene by all three methods, with decreased expression associated with glucocorticoid resistance in the discovery cohort ($p = 3.3 \times 10^{-10}$; **Figure 4-5d; Supplementary Table 5**). *CELSR2* remained significant after adjusting for leukemia molecular subtype ($p < 9.5 \times 10^{-6}$). Lower *CELSR2* expression in glucocorticoid resistant ALL was subsequently validated in primary leukemia cells from

two independent validation cohorts, one comprising 320 pediatric and adult patients with newly diagnosed ALL ($p= 8.3 \times 10^{-8}$; **Figure 4-5e**), and the other comprising 145 pediatric patients with newly diagnosed ALL ($p= 0.033$). We also documented in primary leukemia cells that patients with low expression of *CELSR2* (defined as mRNA \leq the lowest quartile of expression) had significantly lower expression of the GR (*NR3C1*; **Figure 4-5f,g**; $p\text{-value}= 1.7 \times 10^{-4}$). We also observed 5 patients with copy number alterations in *CELSR2*, and 4 were resistant to glucocorticoids. Only two patients had predicted damaging missense mutations in *CELSR2*, one was prednisolone resistant and the other prednisolone intermediate sensitivity. Reduction of *CELSR2* expression in two human ALL cell lines by shRNA (**Figure 4-5h**), significantly increased prednisolone resistance (LC_{50}) compared to non-targeting control (**Figure 4-5i**). There was a 12.7-fold increase in LC_{50} in *CELSR2* knockdown NALM-6 cells ($0.026 \pm 0.033 \mu\text{M}$ vs $0.37 \pm 0.1 \mu\text{M}$ (mean \pm s.e.m), $p=7.8 \times 10^{-5}$), and a 20-fold increase in LC_{50} in 697 cells ($0.095 \pm 0.003 \mu\text{M}$ vs. $1.98 \pm 0.046 \mu\text{M}$ (mean \pm s.e.m), $p=9.0 \times 10^{-4}$).

To assess the mechanism of GC resistance, we identified differentially expressed genes and alterations in global transcriptional effects of glucocorticoids in *CELSR2* knockdown cells. Knockdown of *CELSR2* led to a significant decrease in basal expression of the GR (*NR3C1*) in both NALM-6 cells (1.8-fold decrease; $p= 1.34 \times 10^{-7}$; **Figure 4-7a**), and 697 cells (1.3 fold-decrease, $p= 4.3 \times 10^{-5}$; [**Figure 4-8a**]). Decreased expression of total cellular GR in *CELSR2* knockdown cell lines was more prominent after 24 hours of prednisolone treatment in the NALM-6 cell line (2.3-fold decrease; $p= 2.8 \times 10^{-12}$; **Figure 4-7b**), and in the 697 cell line (1.44 fold-decrease; $p= 1.45 \times 10^{-5}$; [**Figure 4-8b**]). A second shRNA was used to confirm on-target specificity and phenotype (**Figure 4-8c**). Stable *CELSR2* protein knockdown (**Figure 4-8d**) showed decreased glucocorticoid receptor protein expression (**Figure 4-8e**) in ALL cells. Stable re-expression of GR (97% of control) significantly re-sensitized leukemia cells to prednisolone (**Figure 4-7c-d**; two-tailed t-test $p\text{-value} = 0.02$).

After activation of the GR with 24 hours of glucocorticoid treatment, many genes were differentially expressed in *CELSR2* knockdown versus control ALL cells, including a robust upregulation of the antiapoptotic gene *BCL2* in *CELSR2* knockdown cells, documented at the transcriptional (2.5-fold increase; $p= 3.7 \times 10^{-12}$; **Figure 4-7b**) and protein level (1.3-fold increase; $p= 0.0128$; **Figure 4-8f**), consistent with the known repressive effect of activated GR on *BCL2*.¹⁹¹ *BCL2L11* (Bim), a pro-apoptotic gene known to be up-regulated by glucocorticoids¹⁹² did not exhibit upregulation in *CELSR2* knockdown cells after 24 hours of prednisolone treatment (1.3-fold decrease, compared to a 1.8-fold increase in non-targeting control $p= 4.6 \times 10^{-6}$; **Figure 4-8g**), yielding a lower ratio of BIM/BCL2 protein expression in the *CELSR2* knockdown NALM-6 cells after 24 hours of prednisolone treatment. (**Figure 4-8h**). Genome-wide analysis of glucocorticoid-induced gene expression changes in NALM-6 cells (3 replicate experiments) identified 415 genes that were induced at least three-fold compared to untreated cells, 72% (298/415) of which had lower induction (at least 25% less induction) by prednisolone treatment in *CELSR2* knockdown cells, consistent with lower GR (**Figure 4-7e**). Furthermore, 69 genes were repressed by at least three-fold in control cells, and 90%

Figure 4-7. CELSR2 knockdown decreases GR expression and attenuates glucocorticoid modulation of gene expression

(a.) Volcano plot (n= 3 independent experiments) for untreated *CELSR2* knockdown human leukemia cell line (NALM-6) vs. non-target control (NTC). Left side of plot depicts genes with reduced expression in *CELSR2* knockdown cells and genes to the right exhibiting increased expression in *CELSR2* knockdown cells. Orange and red symbols depict mRNAs with significant changes in expression (linear model p-value); red symbols have a fold change greater than 2. (b.) Volcano plot of gene expression after 24 hours of 10 μ M prednisolone treatment of *CELSR2* knockdown vs. non-target control ALL cells (NALM-6, n=3 independent experiments; linear model p-value). (c.) (left) *NR3C1* protein quantification (n= 2 independent experiments) or (right) Prednisolone LC₅₀ (n=3 independent experiments; mean \pm SD) in Nalm6 NTC or sh*CELSR2* cells with either GFP control or re-introduction of *NR3C1* (two tailed t-test p-value; * =p < 0.05, ** = p <0.01, *** = p <0.001). (d.) Representative western blot of GR protein expression (n= 2 independent experiments) in NTC or sh*CELSR2* NALM-6 cells with or without GR re-expression. (e.) The 75 most highly upregulated (top) or downregulated (bottom) genes in human NALM-6 ALL cells after 24 hours treatment with 10 μ M prednisolone (n= 3 independent experiments). Blue and green bars depict mRNA expression in NALM-6 cells transfected with non-target control vector and gold bars depict blunted induction or repression in cells expressing shRNA for *CELSR2* knockdown (mean \pm SD). (Inset) Representative western blot (n= 3 independent experiments) showing significantly lower GR levels in *CELSR2* knockdown cell lines compared to non-targeting controls, with or without prednisolone treatment for 24hr (cropping performed uncropped image available as source data).

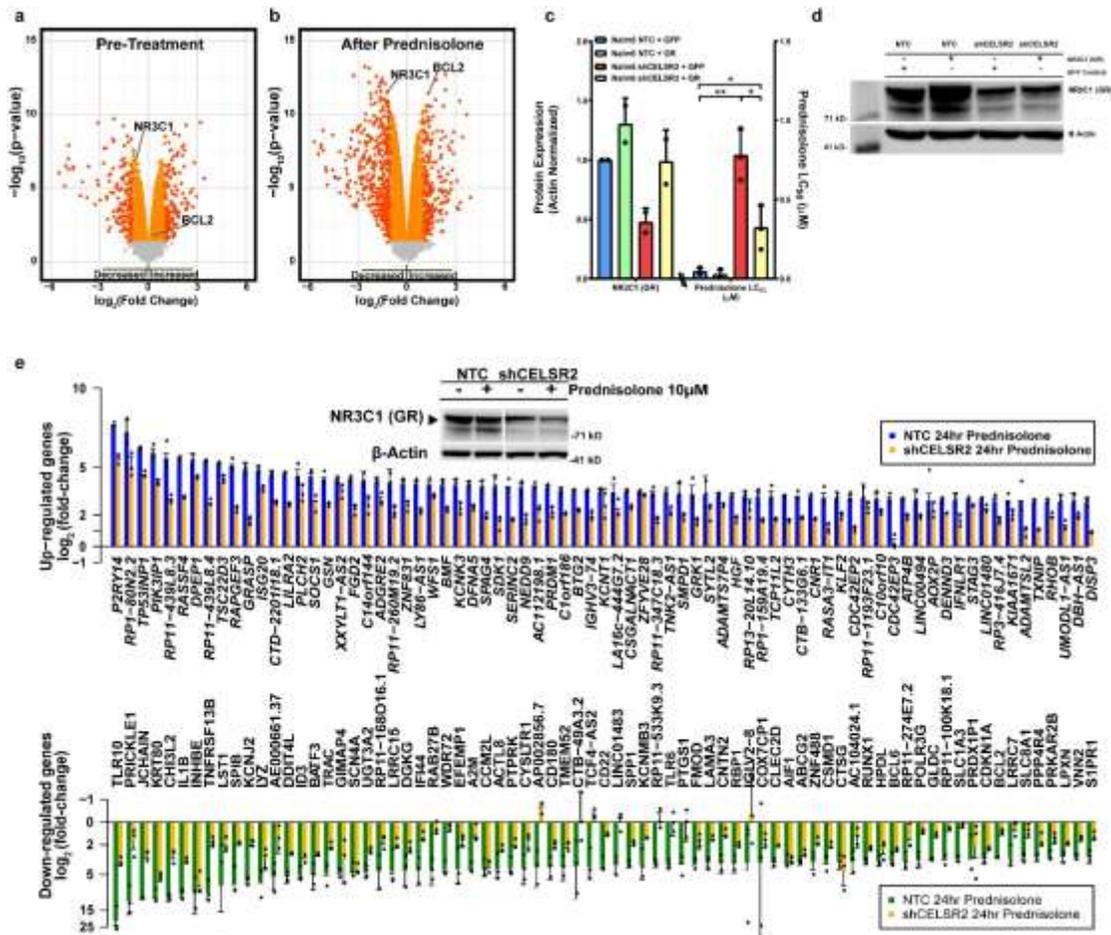
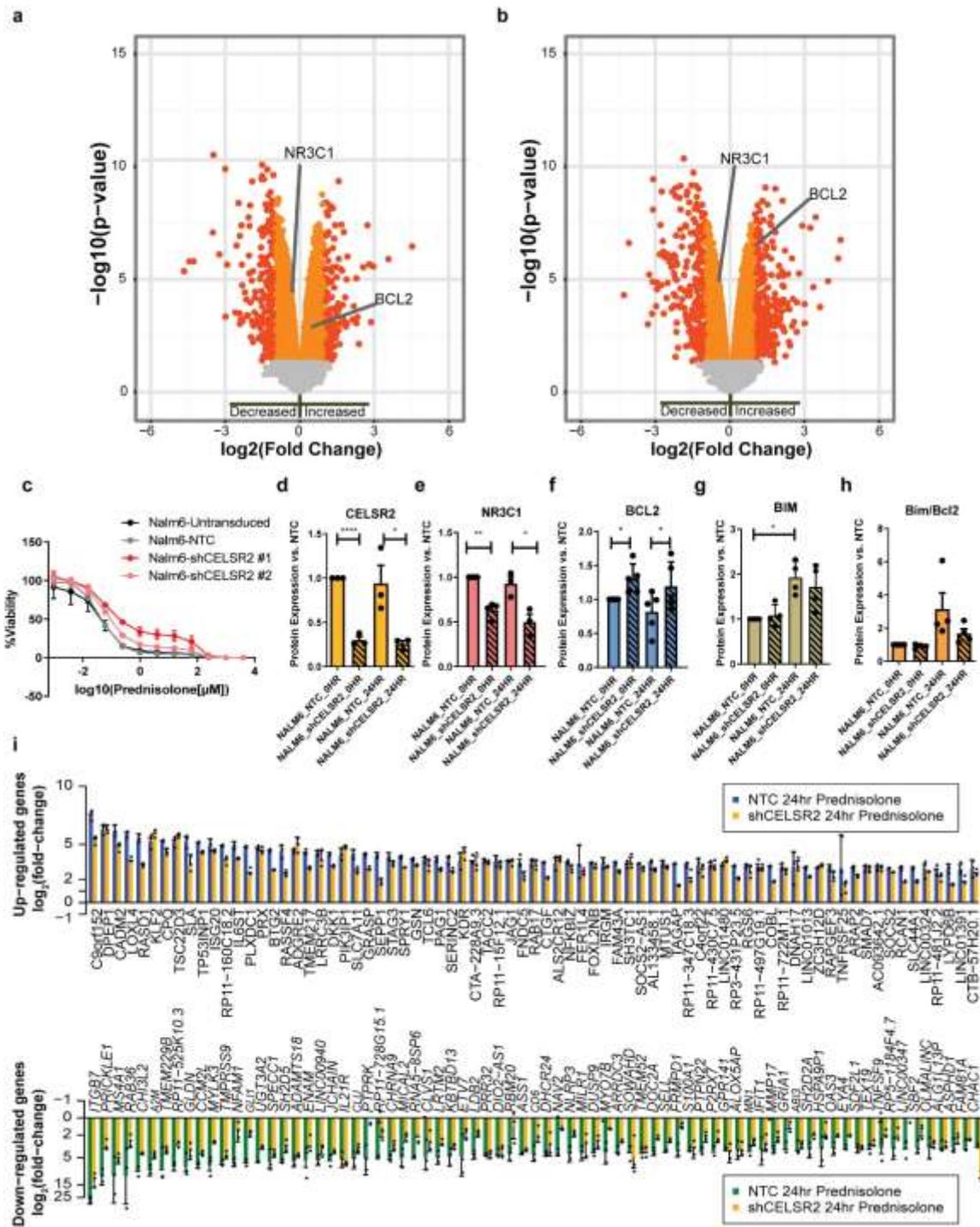


Figure 4-8. CELSR2 knockdown blunts glucocorticoid responsiveness of 697 cells and increases sensitivity to venetoclax

(a.) Volcano plot for untreated *CELSR2* knockdown ALL cell lines vs. non-target control in 697 cell line (n= 3 independent experiments; linear model p-value). Left side of plot depicts genes with reduced expression in *CELSR2* knockdown cells and genes to the right had increased in expression in *CELSR2* knockdown cells. (b.) Volcano plot of gene expression after 24 hours of prednisolone treatment of *CELSR2* knockdown vs. non-target control ALL cells (697; n= 3 independent experiments; linear model p-value). (c.) Dose-response plot (mean \pm S.D.; n= 3 independent experiments) of two shRNA constructs vs non-targeting control and un-transduced NALM6 leukemia cell line. (d.) *CELSR2* (n= 3 independent experiments) (e.) *NR3C1* (n= 3 independent experiments) (f.) *BCL2* (n=5 independent experiments) (g.) BIM (n= 4 independent experiments) and (h.) Bim/Bcl2 protein expression (mean \pm S.D; n=4 independent experiments; two-tailed t-test p-values; * =p < 0.05, ** = p <0.01, *** = p <0.001, **** = p <0.0001) in Nalm6 cells comparing controls (NTC; solid bars) to *CELSR2*-knockdown (sh*CELSR2*) either prior to prednisolone treatment (0HR) or after 24hr prednisolone treatment (24HR). (i.) The 75 most highly upregulated (top) or downregulated (bottom) genes after 24 hours treatment with 10 μ M prednisolone. Blue and green bars depict mRNA expression (mean \pm S.D.; n= 3 independent experiments) in 697 cells transfected with non-target control vector and gold bars depict cells expressing shRNA for *CELSR2* knockdown.



(62/69) had at least 25% lower repression in *CELSR2* knockdown cells (**Figure 4-7e; Supplementary Table 8**). Similar results were observed in the 697 cell line (**Figure 4-8i**).

Mitigation of Glucocorticoid Resistance Caused by Low *CELSR2* Expression via Inhibition of *BCL2*

Because the anti-apoptotic gene *BCL2* was highly induced in *CELSR2* knockdown cells after prednisolone treatment, we tested venetoclax (a BCL-2 inhibitor) for its ability to mitigate glucocorticoid resistance in ALL cells with low *CELSR2* expression. When *CELSR2* knockdown ALL cells were treated for 72 hours with prednisolone (0.954nM - 4mM) and varying concentrations of venetoclax, synergy was evident in two human leukemia cell lines (NALM6 $\alpha = 2.07$; 697 $\alpha = 2.47$; **Figure 4-9a.; Figure 4-10a,c**), but synergy was greatly increased in leukemia cells in which *CELSR2* was knocked down (NALM-6 $\alpha = 5.22$; 697 $\alpha = 4.38$; **Figure 4-9b; Figure 4-10b,d**), and confirmed using other methods for assessing drug synergy (Loewe's additivity and ZIP method; data not shown).

In mice inoculated with NALM-6 leukemia cells with shRNA targeting *CELSR2*, there was a significant prolongation of survival in mice treated with venetoclax 50 mg/kg plus dexamethasone, compared to dexamethasone alone (median survival 60 vs 69 days; $p=0.0062$; **Figure 4-9d**) or venetoclax alone (median survival 56 vs 69 days; $p=0.0046$; **Figure 4-9d**). In mice inoculated with NALM-6 leukemia cells with the non-targeting control shRNA (NTC), there was modest but significant prolongation of survival when treated with venetoclax 100 mg/kg plus dexamethasone compared to dexamethasone alone (median survival 39 vs 41 days; $p=0.02$; **Figure 4-9c**), whereas there was not significant improvement with the lower dosage of venetoclax (50 mg/kg) combined with dexamethasone ($p=0.836$; **Figure 4-9c**), consistent with greater synergy in ALL with lower *CELSR2* expression.

To verify these findings in primary leukemia cells, we documented that primary leukemia cells ($n= 96$ patients) that were resistant to prednisolone were significantly more sensitive to venetoclax ($p=0.014$; **Figure 4-10e**). As reported for other malignancies,^{193,194} higher BCL-2 expression in primary ALL cells was associated with increased sensitivity to venetoclax ($p=2.5 \times 10^{-3}$; **Figure 4-10f**).

We also measured the effects of the two drugs given separately or together in primary leukemia cells isolated from the bone marrow or peripheral blood of six patients (3 freshly isolated and 3 xenograft samples). In the two prednisolone sensitive patients, we observed low levels of additivity/synergy, whereas in the four leukemias that were intermediate or resistant to prednisolone, we documented greater synergy based on significantly higher alpha values in all cases (**Figure 4-10g**). Three of the four prednisolone resistant patients exhibited much lower mRNA expression of *CELSR2* and *NR3C1* when compared to the prednisolone sensitive patients (**Figure 4-10h**), and primary ALL cells from all four of the prednisolone resistant patients exhibited decreased

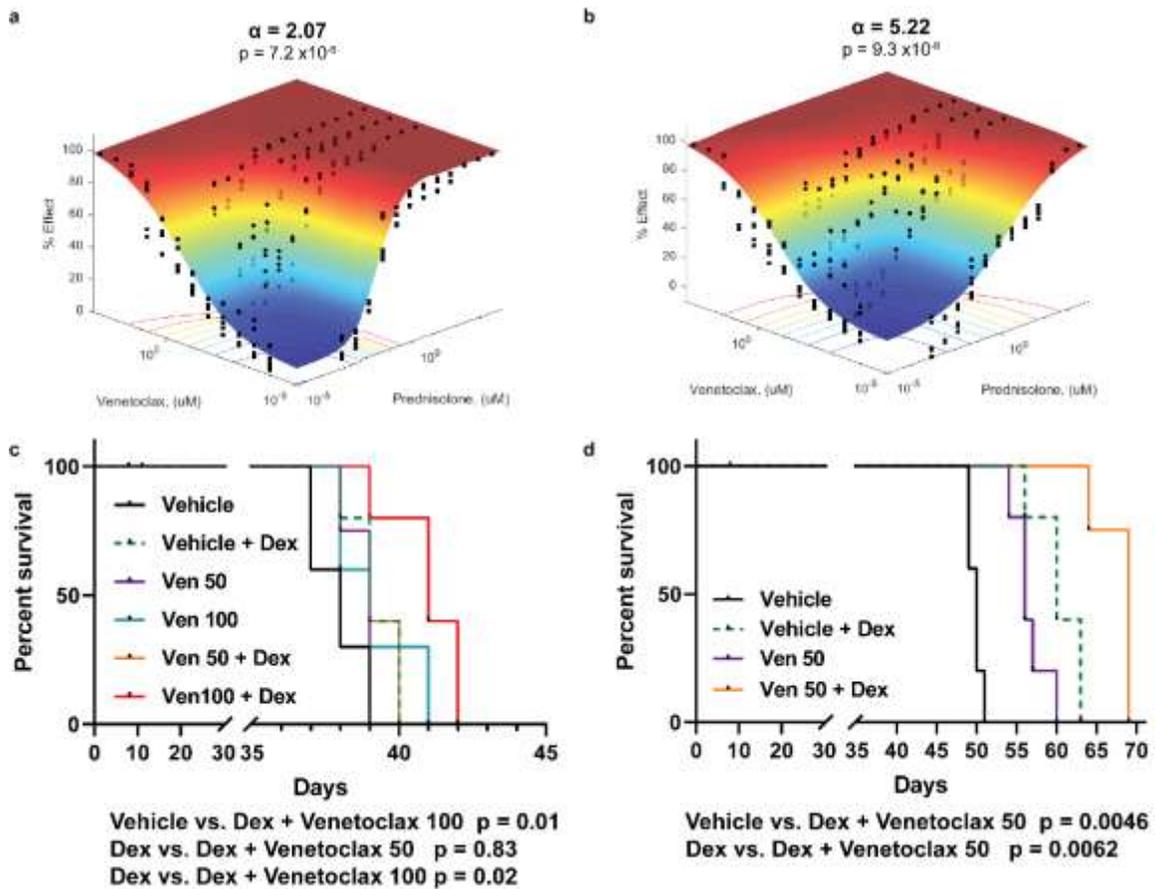
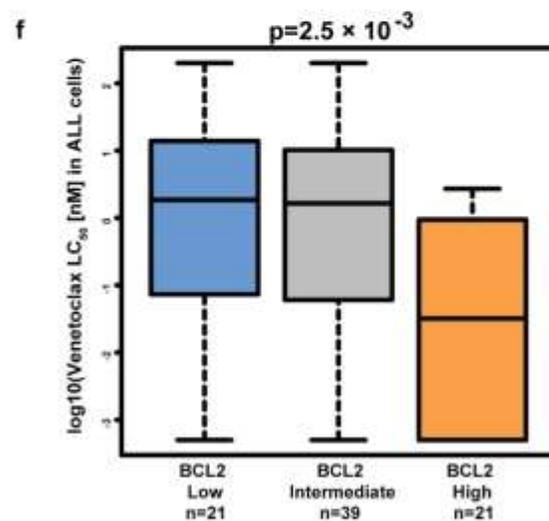
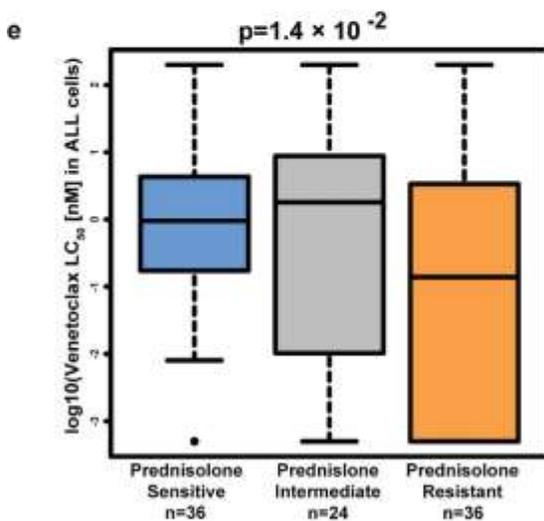
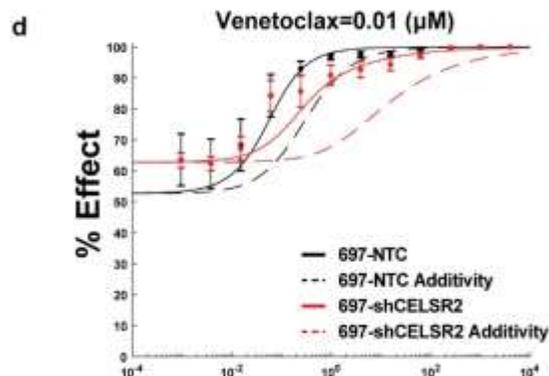
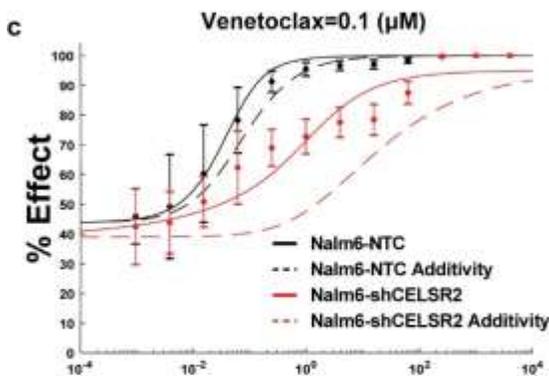
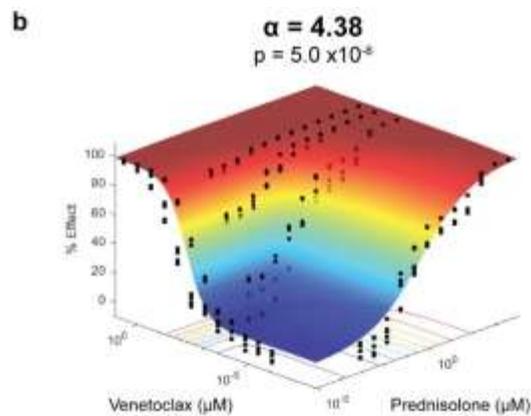
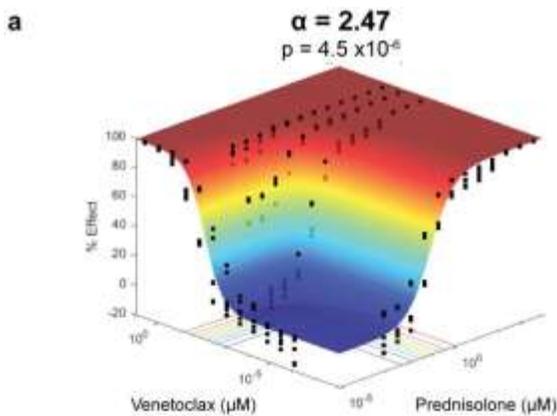


Figure 4-9. Increased synergy and mitigation of glucocorticoid resistance by inhibition of *BCL2* in ALL with low *CELSR2* expression

(a.) Response surface model (% Effect = % cell kill) depicting synergy between prednisolone and Bcl-2 inhibitor venetoclax in NALM-6 cells transduced with non-target control or (b.) *CELSR2* knockdown ALL cells (n= 3 independent experiments; response surface model two-tailed t-test p-value). The (α) value indicates antagonism < 0 or synergy > 0, with higher values representing greater synergy. P-value assesses overall model fit. (c.) Percent survival of NSG mice inoculated with 100,000 NALM-6 non-target control cells or (d.) 100,000 *CELSR2* knockdown leukemia cells treated with either vehicle, dexamethasone alone (4 mg/kg), venetoclax alone (50 or 100 mg/kg) or combination of venetoclax with dexamethasone (n= 5 mice per treatment group; Log-rank Mantel-Cox test p-values).

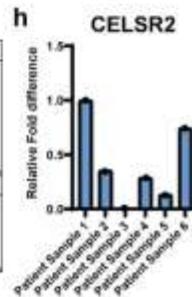
Figure 4-10. Venetoclax and prednisolone synergize in primary ALL with low *CELSR2* expression and *CELSR2* knockdown in cell lines dysregulation of Bim/Bcl2 axis

(a.) Response surface model plot of cytotoxicity from prednisolone plus venetoclax at concentrations indicated for the 697 leukemia cell line transduced with non-targeting control vector. (b.) Response surface model plot for the 697 leukemia cell line transduced with *CELSR2* shRNA knockdown vector (for a and b individual points represent n= 3 independent experiments performed in technical duplicate; response surface model two-tailed t-test p-value). The alpha (α) value indicates antagonism < 0 or synergy > 0 with greater synergy from higher value. P-value describes overall model fit. Individual plots of prednisolone effect (mean \pm S.D.; n= 3 independent experiments) (c.) NALM-6 and (d.) 697 leukemia cell lines at one concentration of venetoclax (mean \pm S.D.; n= 3 independent experiments). Black lines are non-targeting control cells and red lines are *CELSR2* knockdown cells, dashed lines indicate predicted additivity curve fit based on single drug treatments; data left of the dashed lines represent additivity/synergy. Solid lines represent fit of measured values. (e.) Venetoclax sensitivity of independent cohort of patients (n=96 ALL patients) grouped based on prednisolone sensitivity (LC_{50}) (f.) Bcl2 expression associated with sensitivity to venetoclax (n= 81 ALL patients) (g.) Primary ALL cells from patients (n=6 patient samples) and human leukemia cell lines assessed for additivity/synergy with prednisolone and venetoclax (for all box plots horizontal bars depict medians and boxes represent 25th and 75th percentiles, whiskers represent ± 1.5 IQR; linear model p-values). (h.) mRNA expression (n=1 experiment run in technical triplicate) of *CELSR2* in patient samples assessed for synergy.



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Patient Samples (Primary ALL)	PRED LC50(μM)	Venetoclax LC50(nM)	Synergy (g)	Combination Pred LC50 (Venetoclax conc.)
Patient Sample 1 (Prednisolone Sensitive)	< 0.015	0.3877	0.77	<0.015 (1nM)
Patient Sample 2 (Prednisolone Sensitive)	0.1219	>100	0.065	0.03 (100nM)
Patient Sample 3 (Prednisolone Intermediate)	4	313	16.8	<0.015 (100nM)
Patient Sample 4 (Prednisolone Resistant)	>500	0.22	10.2	<0.015 (1nM)
Patient Sample 5 (Prednisolone Resistant)	>500	>100	8x10 ²	0.66 (10nM)
Patient Sample 6 (Prednisolone Resistant)	>500	0.54	32	< 0.015 (10nM)
Human ALL Cell Lines	PRED LC50(μM)	Venetoclax LC50(μM)	Synergy (g)	Combination Pred LC50 (Venetoclax conc.)
NS-NTC	0.021	3.825	2.07	0.006 (10nM)
NS-shCELSR2	1.92	0.77	5.22	0.01328 (100nM)
697-NTC	0.09	0.018	2.47	0.0044 (10nM)
697-shCELSR2	0.4	0.015	4.38	< 9x10 ⁻⁴ (10nM)



ability to induce the pro-apoptotic protein BIM when treated with 10 μ M prednisolone for 24 hours (data not shown).

***CELSR2* Is a Negative Hub Driver of Prednisolone Resistance**

We used NetBID,¹⁶⁹ a data-driven systems biology approach, to reconstruct a B-ALL-specific interactome (B-ALLi), composed of “hubs” representing central components of larger regulatory networks, using RNA-seq profiles of 185 B-ALL patients in the TARGET¹⁷⁰ cohort (**Figure 4-11a**). B-ALLi identified hub drivers whose network activities differed significantly in prednisolone resistant versus sensitive leukemia cells from two patient cohorts (SJCRH TOTXV and TOTXVI; **Figure 4-11b**). Known glucocorticoid resistance genes including *SMARCA4*, *PAX5*, *CASPI* were significantly enriched in NetBID top predictions ($p=0.011$, **Figure 4-12a**). Network topology analysis of the top 48 NetBID drivers ($p<5 \times 10^{-5}$, **Figure 4-11b**; **Figure 4-12b**) identified *CELSR2* as a hub that modulated other top drivers (**Figure 4-11c**). NetBID-inferred activity of *CELSR2* was markedly down-regulated ($p=8.6 \times 10^{-8}$; **Figure 4-11d**) in prednisolone resistant relative to sensitive leukemias, as was the expression of *CELSR2* (**Figure 4-11b**). More strikingly, *CELSR2* regulons (**Figure 4-12c**) inferred by NetBID from baseline RNA-Seq profiles of B-ALL patients were significantly enriched among differentially expressed genes in ALL cells after *CELSR2* knockdown ($p=1 \times 10^{-4}$ in NALM-6 and $p=1 \times 10^{-3}$ in 697 cells, **Figure 4-11e**; **Figure 4-12d**). Several previously reported glucocorticoid resistance genes (e.g. *TSC22D3*, *IL1B* and *TP53INP1*), were also regulated by *CELSR2* ($p=1.8 \times 10^{-3}$ in NALM-6 and $p=0.01$ in 697; **Figure 4-12e**).

CELSR2* Expression Is Significantly Related to *PAX5

NetBID analysis also identified *CELSR2* as a top downstream target of *PAX5* (**Figure 4-13a**). For *CELSR2*, the most highly co-expressed gene in primary ALL cells was *PAX5*, which was highly positively correlated in leukemia cells from 203 patients ($p=3 \times 10^{-11}$; **Figure 4-13b**). Accordingly, lower expression of *PAX5* was observed in leukemia cells with higher prednisolone LC₅₀ ($p=7.47 \times 10^{-5}$; **Supplementary Table 1**). Recent studies have highlighted the importance of chromatin accessibility in the discovery of novel regulators of glucocorticoid resistance.^{94,98} ATAC-seq profiles of multiple human leukemia cell lines revealed open chromatin regions in proximity to the *CELSR2* coding region. When combined with ENCODE transcription factor binding site data,¹⁴⁷ *PAX5* binding sites were found within the cis-regulatory elements of *CELSR2* in the B-lymphocyte cell line GM12878 (**Figure 4-13c**). Furthermore, CHIP-seq peaks for *PAX5* in NALM-6 ALL cells confirmed that *PAX5* binds in these open chromatin regions in a leukemia cell line (**Figure 4-13c**). We constructed a multivariate model using the expression of all miRNAs associated with *CELSR2* as co-variates along with *PAX5* mRNA, revealing that *PAX5* expression accounted for about 25% of the variability in *CELSR2* expression ($p=1.6 \times 10^{-12}$), and mir-31-5p accounted for an additional 4% of the variability in *CELSR2* expression ($p=0.002$). Alone, miR-31-5p was significantly negatively associated with *CELSR2* expression ($p=0.001$), as was *PAX5* ($p=3 \times 10^{-11}$).

Figure 4-11. NetBID identifies *CELSR2* as a hub driver of prednisolone resistance (a.) Schematic workflow representing NetBID algorithm (b.) Heatmap of top 48 NetBID-predicted drivers from total of n=7,920 drivers inferred from the B-ALL interactome (n=185 patients) that were associated with prednisolone resistance (n=203 patients with gene expression and LC₅₀). Drivers (as denoted by “symbol_regulon size”, e.g. “*CELSR2_399*”) are ranked by integrated NetBID p-value. (Left) Combined NetBID results color-coded by z-score (red = positive, blue = negative) and labeled by p-value of integrated NetBID results of TOTXV and TOTXVI patient cohorts; Right: differential expression (DE) of each driver itself, color-coded by z-score and labeled by signed fold-change of integrating the two cohorts (shown separately in **Figure 4-12**). (c.) Subnetwork of the top 48 drivers versus prednisolone LC₅₀ in relation to one another (limited to top 50 interactions for each driver ranked by mutual information of each hub gene) from B-ALLi. Node size is proportional to the regulon size; nodes in green represent known resistance genes. Edges: width is proportional to mutual information, red is for positive and blue for negative Spearman correlations of the connecting nodes. (d.) *CELSR2* NetBID activities (horizontal bar depicts median and boxes represent 25th and 75th percentiles, whiskers represent $\pm 1.5 \times$ IQR) in prednisolone resistant and sensitive patients from TOTXV and TOTXVI patient cohorts (Stouffer’s combined Bayesian generalized linear model “NetBID” p-value; n=203 patients). (e.) Enrichment of NetBID-inferred *CELSR2* regulon (n= 399 genes) in differentially expressed genes (n= 222 genes) of *CELSR2* knockdown vs. control in NALM-6 (top) and 697 (bottom) cell lines without prednisolone treatment.

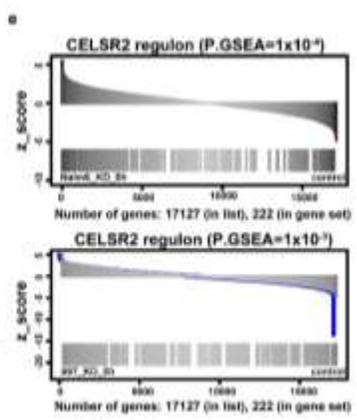
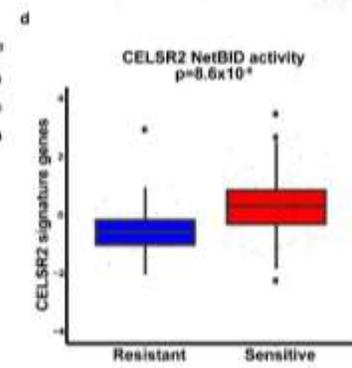
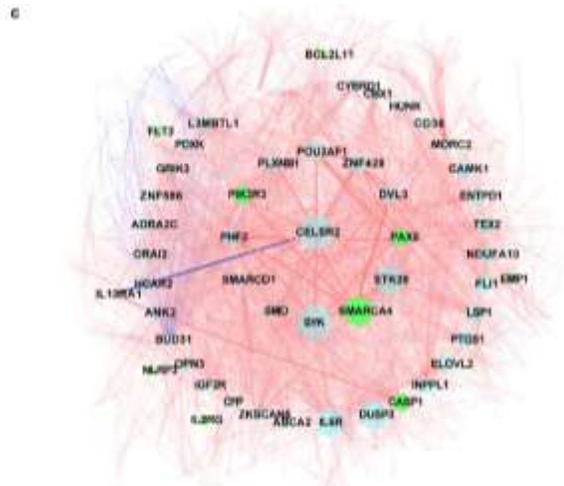
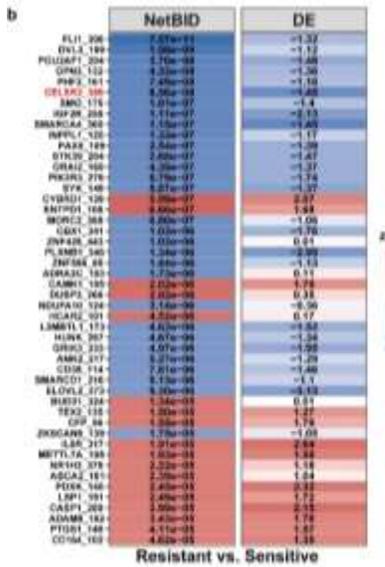
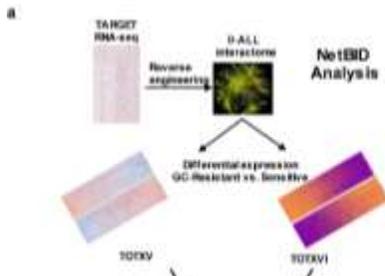


Figure 4-12. NetBID identifies regulatory nodes of prednisolone resistance
(a.) Enrichment of previously reported resistance genes (n=40 genes and miRNAs; Wilcoxon two-tailed p-value) in NetBID results. (b.) Heatmap of top 48 NetBID-predicted drivers ('symbol'_'regulon size') are ranked by integrated NetBID p-value. Left: color-coded by z-score and labeled by p-value of NetBID results in TOTXVI, TOTXV, and combination (Comb); Right: differential expression of each driver itself, color-coded by z-score and labeled by signed fold-change in TOTXVI, TOTXVI and combination (Comb; Stouffer's combined Bayesian generalized linear model "NetBID" p-value; n=203 patients). (c.) *CELSR2* regulon from B-ALLi (n=399 genes). Legends of node and edge follow **Figure 4-11c**. (d.) Enrichment of NetBID-inferred *CELSR2* regulon (n=399 genes) in differentially expressed genes of *CELSR2* knockdown vs. NTC in NALM-6 human ALL cell lines (n=222 genes; Wilcoxon two-tailed p-value) upon prednisolone treatment for 24hr (top) Blue lines inside the box indicate the down-regulation of *CELSR2* itself, labeled p-value and signed fold-change. (e.) Enrichment of previously reported resistance genes (n= 40 genes and miRNAs; Wilcoxon two-tailed p-value) in differentially expressed genes of *CELSR2* knockdown vs. NTC in NALM-6 ALL cell lines without prednisolone treatment.

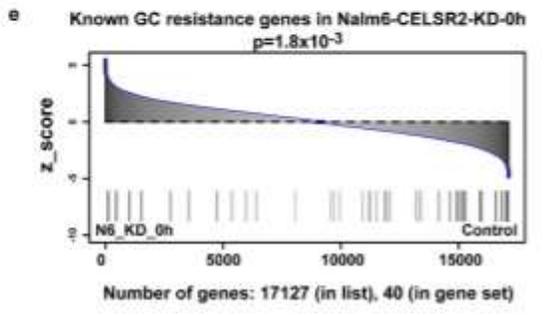
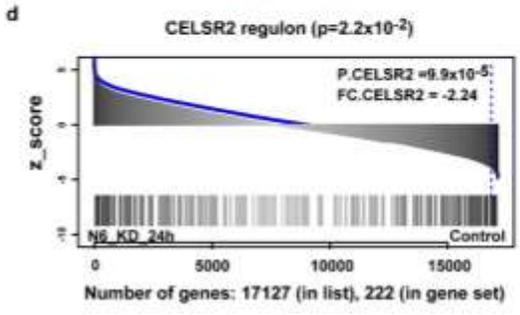
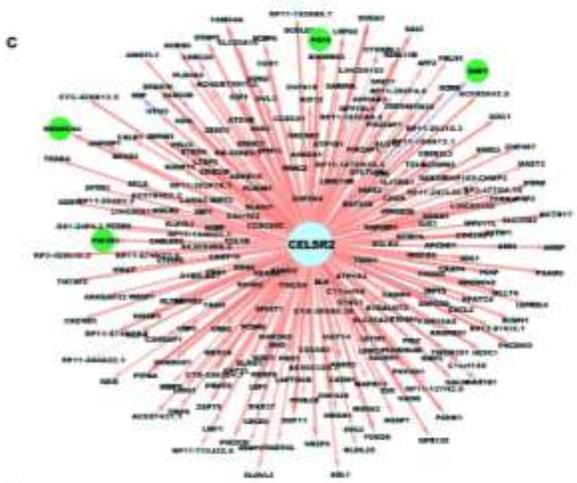
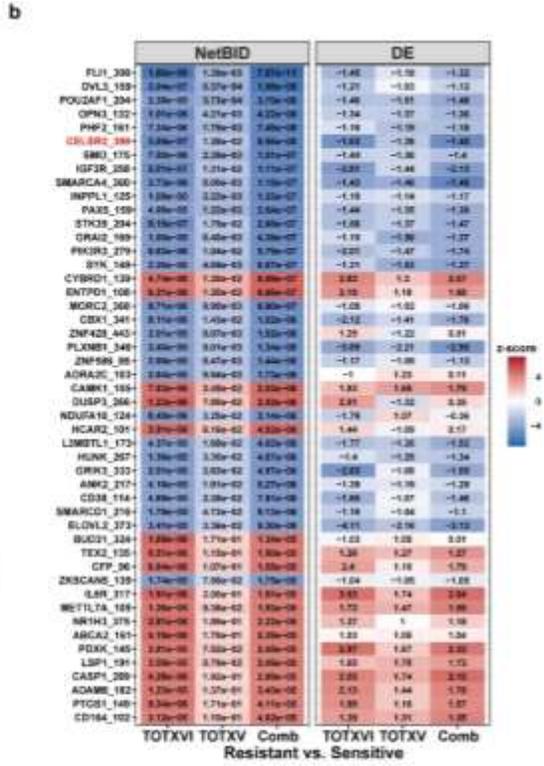
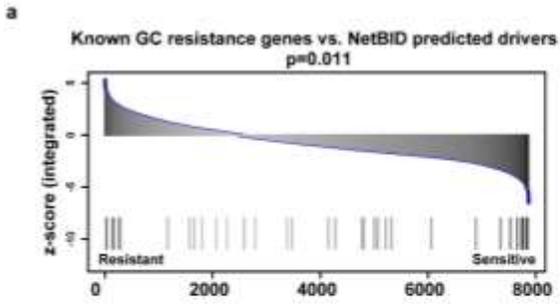
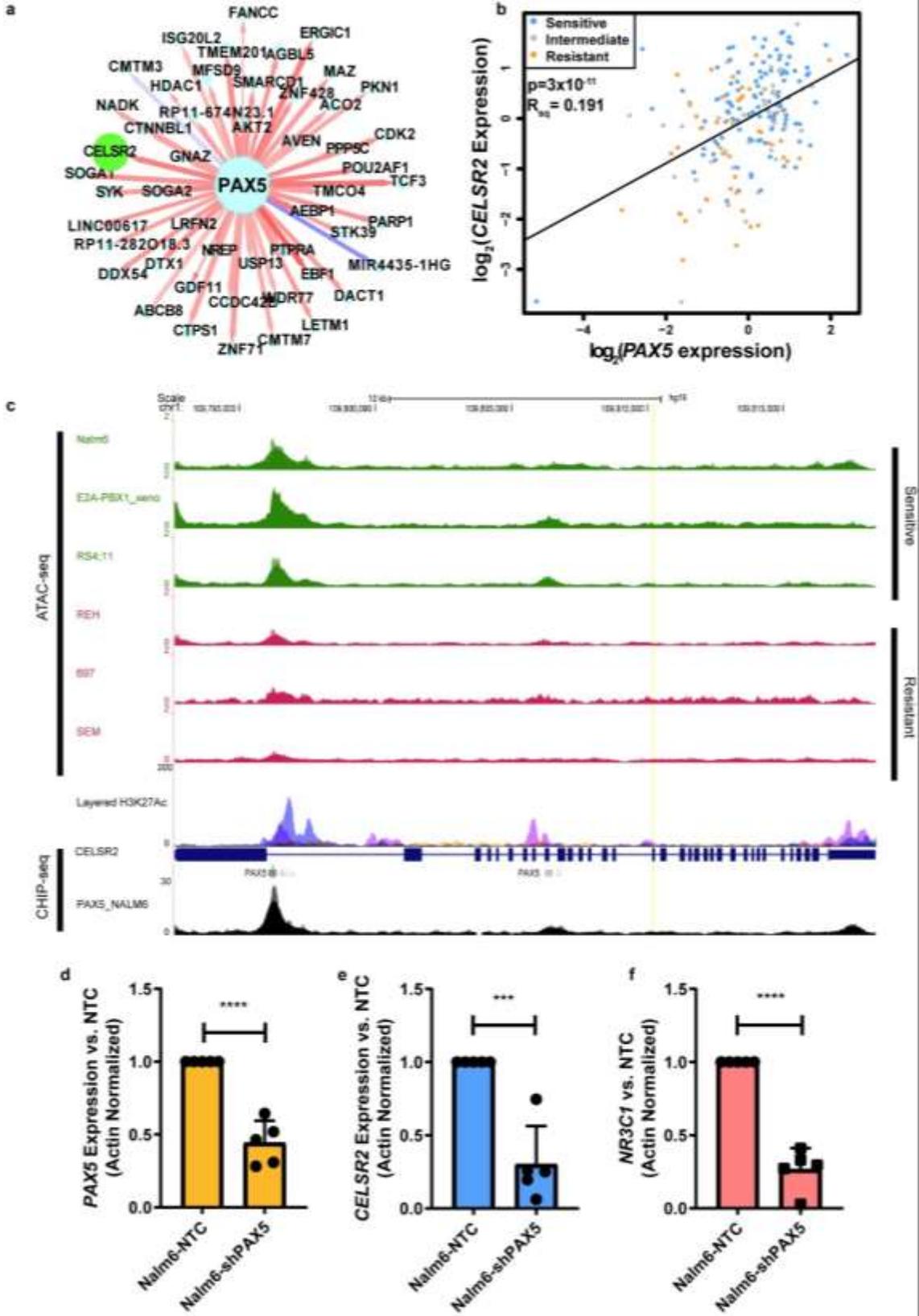


Figure 4-13. *CELSR2* mRNA expression is related to *PAX5* expression in primary ALL cells

(a.) Subnetwork (top 50 interactions ranked by mutual information) of *PAX5* and *CELSR2* from B-ALLi (n=185 patients). Legends of node and edge follow **Figure 4-11c**, except that nodes in green are those in top 48 drivers (**Figure 4-11b**). (b.) *CELSR2* expression positively correlates with *PAX5* expression in primary acute lymphoblastic leukemia cells (black line represents regression fit associated with linear model p-value and R_{sq}). (c.) Open chromatin regions defined by ATAC-seq in three sensitive and three resistant human leukemia cell lines and H3K27 acetylation from ENCODE in upstream 5' region of *CELSR2*. ENCODE binding site in GM12878 lymphoid cells for *PAX5* and CHIP-seq peaks from NALM-6 cells for *PAX5* binding are indicated at bottom of the plot. (d) *PAX5* (**** = 3.5×10^{-5}) (e.) *CELSR2* (** = 3.0×10^{-4}) (f.) *NR3C1* (**** = 3.2×10^{-5}) protein expression (mean \pm S.D.) in NALM-6 leukemia cell lines stably expressing shRNA knockdown constructs targeting *PAX5* (n=4 independent experiments; two-tailed t-test p-values).



PAX5 knockdown in NALM-6 cells (**Figure 4-13d**) showed significant reduction in both *CELSR2* (two-tailed t-test p-value =0.0003; **Figure 4-13e**) and *NR3C1* (two-tailed t-test p-value<0.0001; **Figure 4-13f**).

scRNA-seq Reveals Clonality of Resistance Genes

We performed single cell RNA-seq on primary ALL cells from a patient with prednisolone sensitive leukemia ($LC_{50} = 0.091\mu\text{M}$) and from a patient with prednisolone resistant leukemia ($LC_{50} = 1006\mu\text{M}$). Primary leukemia cells from each patient were treated *ex vivo* with $63\mu\text{M}$ prednisolone or incubated in media without prednisolone, and single-cell RNA sequencing was used to generate clusters of surviving cells after 96 hours, based on their gene expression profiles. As expected, the prednisolone sensitive leukemia had a pronounced reduction in CD19+ cells (**Figure 4-14a,d**; **Figure 3-5a-c**; $p < 2 \times 10^{-16}$), whereas the resistant leukemia retained a high percentage of CD19+ cells after prednisolone treatment (**Figure 4-14a**; **Figure 3-5d-f**; **Supplementary Table 9**). Single-cell RNA sequencing documented that the sensitive leukemia had higher *CELSR2* expression before treatment than the resistant leukemia, which had essentially undetectable de novo expression of *CELSR2* (**Figure 4-14b**; FDR = 0.009). BCL-2 expression was significantly higher in the resistant patient after prednisolone treatment compared to control, and greater than in the sensitive leukemia (**Figure 4-14c,f**; FDR = 0.005; **Supplementary Table 9**).

CELSR2 Is a Mediator of Non-canonical Wnt Signaling

To assess the potential effects of *CELSR2* on GR expression, we performed ATAC-seq to interrogate regulatory regions upstream of the *NR3C1* gene in three glucocorticoid sensitive and three glucocorticoid resistant ALL cell lines, revealing enriched open chromatin for the GR in the sensitive cell lines compared to resistant ALL cells, in regions overlapping H3K27-acetylation peaks from ENCODE (**Figure 4-15a-d**). The REH cell line has a known stop gain mutation in *NR3C1*, which leads to glucocorticoid resistance independent of *CELSR2*. ENCODE transcription factor binding data revealed binding sites for *TEAD4* (Hippo signaling), *NFATC1* and the AP-1 components cJun and fos within the upstream regulatory regions of *NR3C1* (**Figure 4-15d**).

Because *CELSR2* is known to regulate non-canonical WNT signaling,¹⁹⁵ we quantitated the expression of NFAT, pJNK, cJun, phos-cJun and *NR3C1* in *CELSR2* knockdown cells and control cells (**Figure 4-16**; **Figure 4-15e-h**). This documented significantly lower nuclear expression of phosphorylated JNK ($p=0.015$) and lower phosphorylation of its target cJun at serine 63 ($p=0.0017$), which was also evident in cells treated with prednisolone (24hr at $10\mu\text{M}$; $p=0.03$). We also documented decreased nuclear GR levels in *CELSR2* knockdown compared to control cells in both treated ($p=0.03$) and untreated ($p=0.005$) cells. Cytoplasmic levels of the GR were also

Figure 4-14. Single cell transcriptomic analysis verifies lower *CELSR2* and higher *BCL2* in glucocorticoid-resistant primary ALL cells

(a.) Clustering of single cells (n= 2 patients) based upon the top 1000 most highly expressed genes. Both patients are independent of the discovery and validation cohorts; leukemia cells from one patient are sensitive (left) and one resistant to prednisolone (right). Clusters annotated to show CD19+ cells; red denotes control (untreated) and blue depicts cells after treatment with prednisolone 63 μ M for 96h (b.) *CELSR2* expression from clustered single cell populations of sensitive and resistant patients either without treatment or after 96h prednisolone (c.) *BCL2* expression from clustered single cell populations of sensitive and resistant patients (d.) Bar plot depicting greater proportion of sensitive leukemia cells (n=2,427 control cells; n= 924 treated cells) killed after treatment with prednisolone for 96h compared to resistant patient (n= 686 control cells; n=759 treated cells; two proportion z-test p-value; **** = p <0.0001). (e.) Violin plot representing kernel density of gene expression (individual points represent single cells) of *CELSR2* or (f.) *BCL2* in CD19+ leukemia cell populations comparing prednisolone treated to untreated cells in sensitive (n=2,427 control cells ; n= 924 treated cells) or resistant patients (n= 686 control cells; n=759 treated cells; ** = p <0.01).

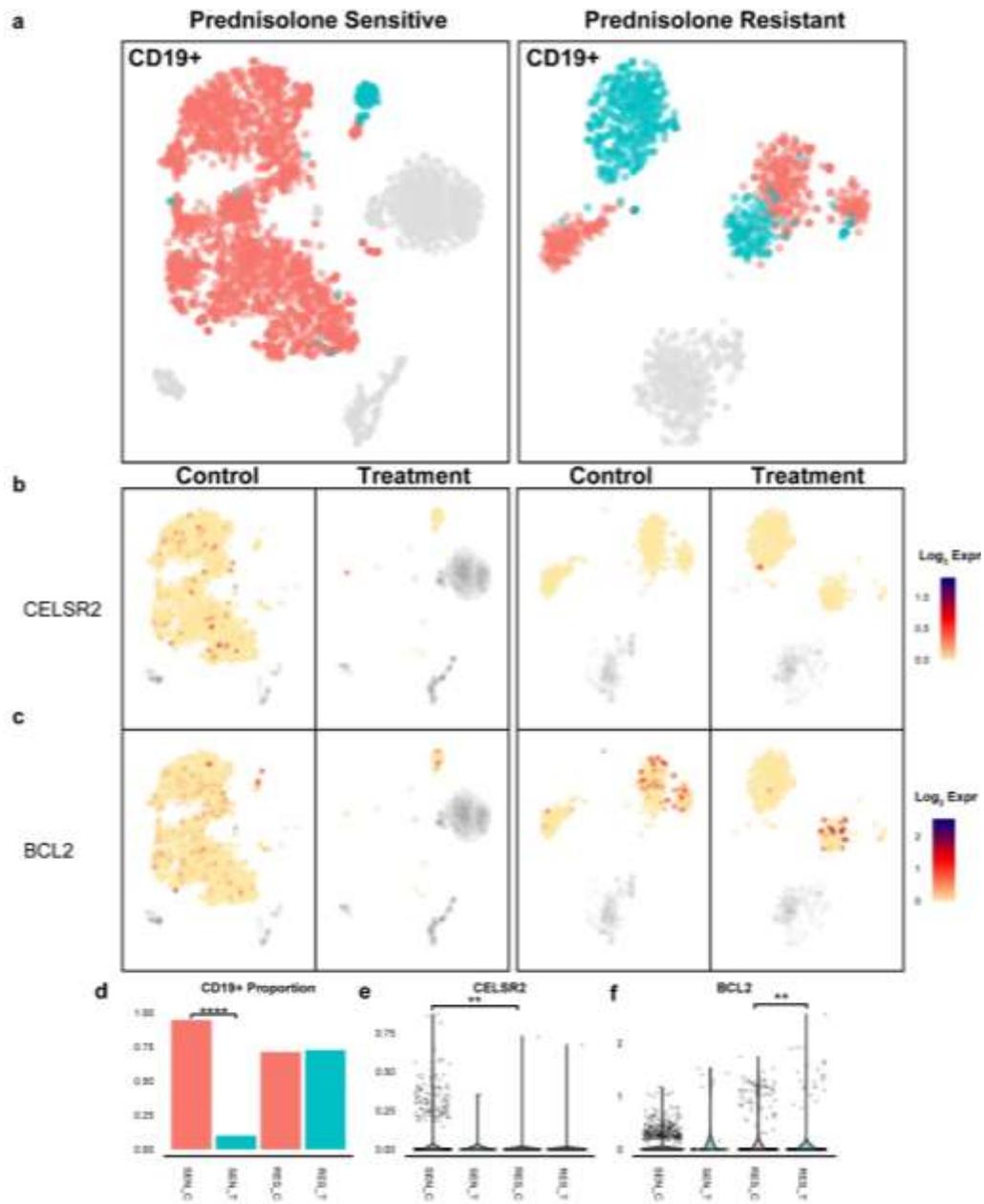
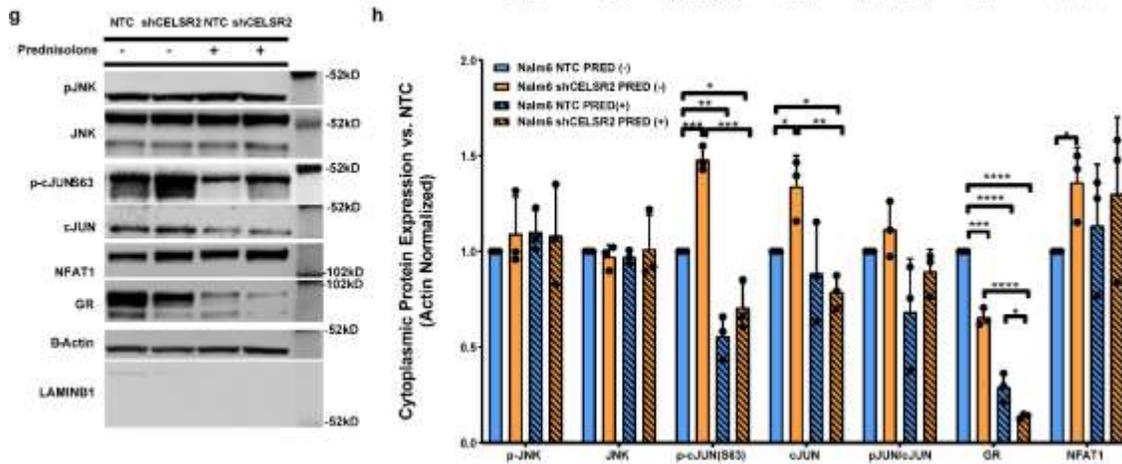
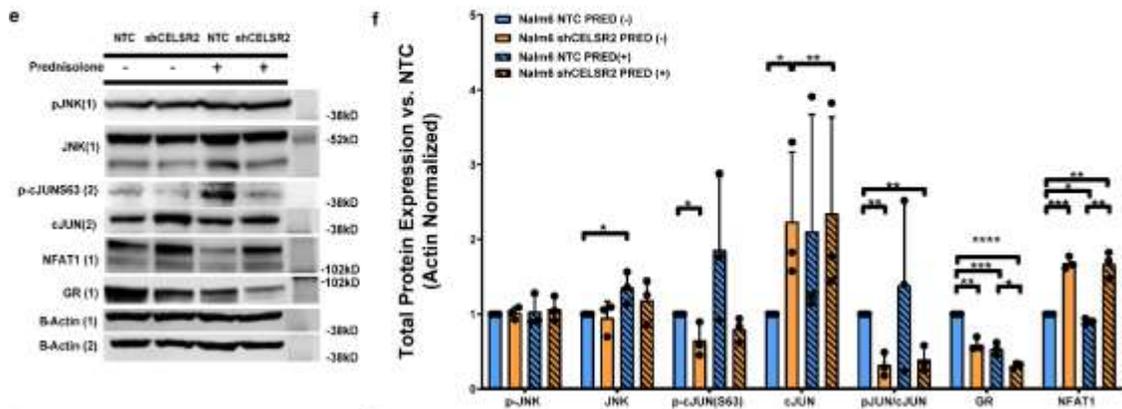
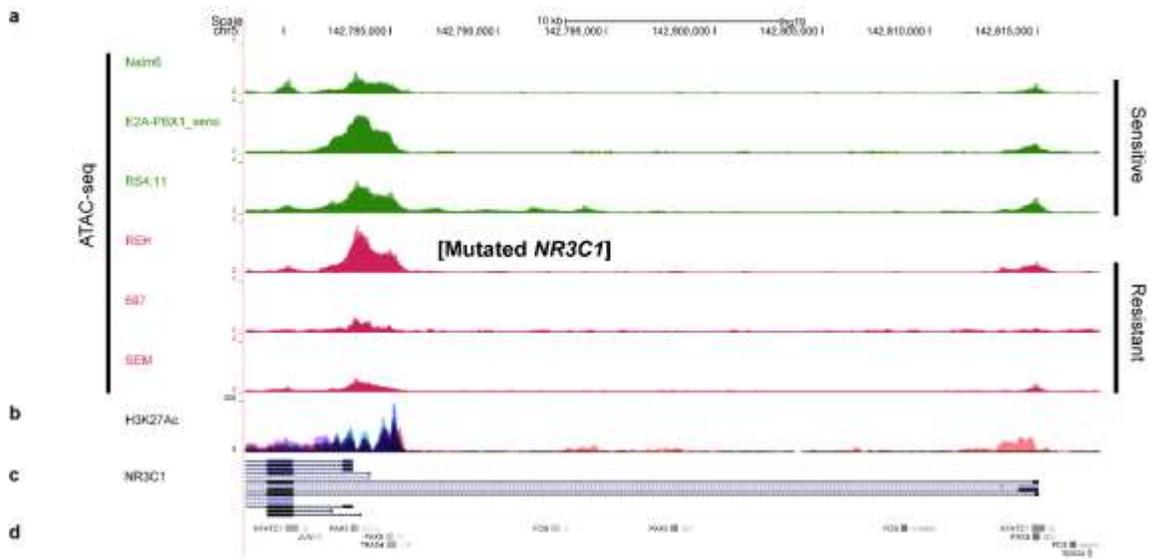


Figure 4-15. Chromatin status in glucocorticoid sensitive and resistant human ALL cell lines, and perturbation of non-canonical WNT signaling by reduction of *CELSR2* expression

(a.) ATAC-seq for six human leukemia cell lines, three prednisolone sensitive and three resistant cell lines depicting open chromatin in the region upstream of *NR3C1* (n= 2 independent experiments). (b.) H3K27Ac data from ENCODE (black box) showing lymphocyte regulatory region in GM12878 cell line (pink) (c.) RefSeq *NR3C1* transcripts (d.) ENCODE transcription factor binding sites for *PAX5*, *NR3C1*, *TEAD4* and non-canonical Wnt effectors (*NFATC1* and AP-1 [*JUN* and *FOS*]) (e.) Western blot and (f.) Barplot (mean \pm S.D.) depicting total cellular protein expression of signaling components from planar cell polarity and Ca²⁺/NFAT non-canonical Wnt signaling protein *CELSR2* knockdown vs. control cells with or without 10 μ M prednisolone treatment for 24hr. (g.) Western blot and (h.) Barplot (mean \pm S.D.) depicting cytoplasmic protein expression of signaling components from planar cell polarity and Ca²⁺/NFAT non-canonical Wnt signaling protein *CELSR2* knockdown vs. control cells with or without 10 μ M prednisolone treatment for 24hr.



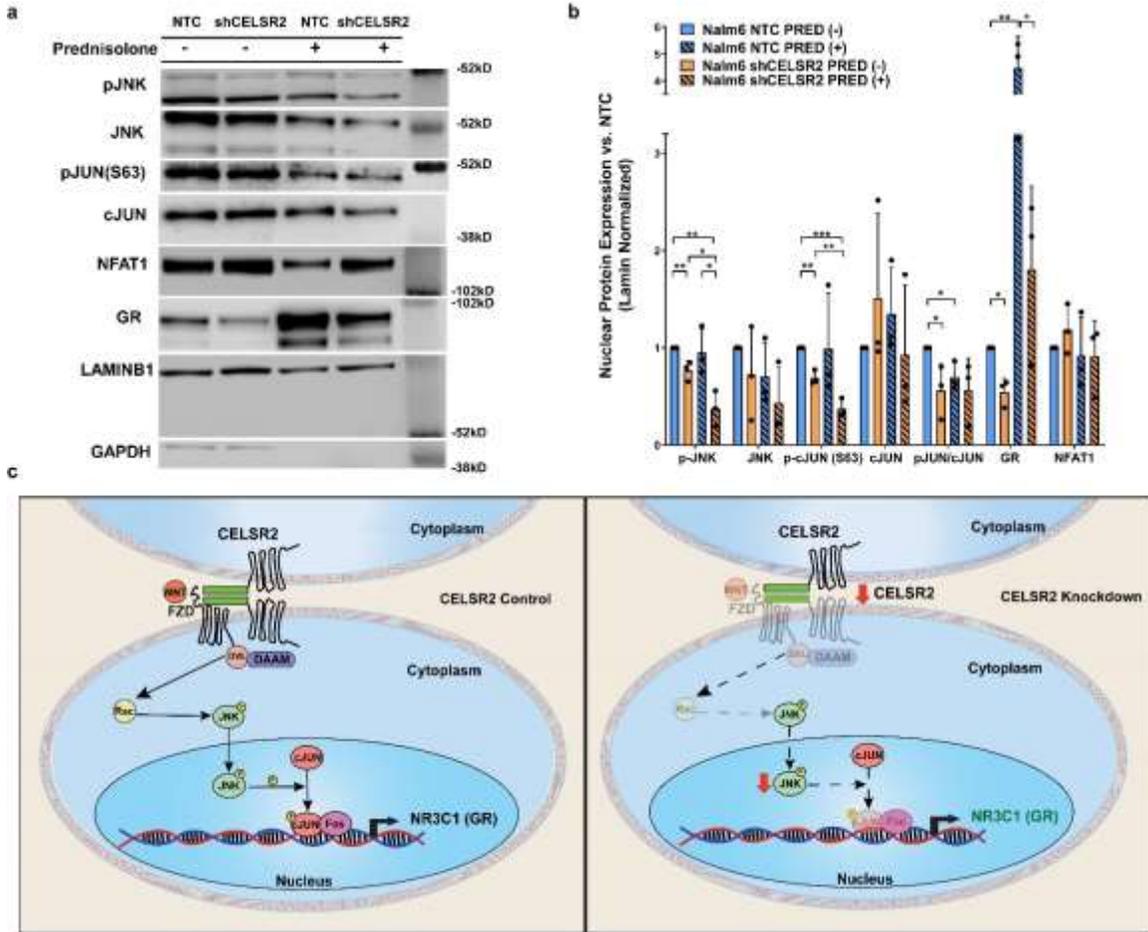


Figure 4-16. Perturbation of downstream non-canonical Wnt signaling leads to decreased GR expression and glucocorticoid resistance
 (a.) Representative western blot and (b.) Barplot (n= 3 independent experiments; mean \pm S.D.) depicting nuclear protein expression of signaling components from the planar cell polarity and Ca²⁺/NFAT non-canonical Wnt signaling pathway, *CELSR2* knockdown (sh*CELSR2*) vs. non-target control cells (NTC) with or without 10 μ M prednisolone treatment for 24hr (two-tailed t-test p-values; * P < 0.05; ** P < 0.01***; P < 0.001; **** P < 0.0001). (c.) Schematic representation of non-canonical Wnt signaling, depicting proposed mechanism by which low *CELSR2* expression leads to decreased expression of the GR (right panel).

significantly lower in *CELSR2* knockdown cells compared to controls, in both prednisolone treated ($p=0.03$) and untreated ($p=0.0002$) cells (**Figure 4-15g-h**).

CHAPTER 5. DISCUSSION*

Although there are several known genetic and epigenetic mechanisms of glucocorticoid resistance in ALL, many leukemias are resistant for reasons that have remained unknown. To assess the potential of agnostic genome-wide interrogation of multiple forms of genomic and epigenetic variants to identify mechanisms of drug resistance in human cancer, we assessed *de novo* prednisolone resistance in primary ALL cells from newly diagnosed patients. The *ex vivo* sensitivity of ALL cells to glucocorticoids is related to treatment outcome in ALL^{49,180} and was related to the persistence of residual leukemia (MRD) in our patient cohort. We identified 655 inter-related genomic features associated with 463 genes and 48 miRNAs that discriminated prednisolone sensitive and resistant ALL based on somatic variation in mRNA, miRNA, CpG methylation, SNPs, CNAs or SNVs/Indels. Notably, 94% of the mRNAs discriminating glucocorticoid sensitive and resistant ALL were statistically associated with one or more of the significant miRNA, cis CpG-methylation sites, SNPs, CNAs or SNVs/Indels within coding regions, indicating the interconnectivity of these genomic features.

To assess the increased utility of interrogating multiple data types simultaneously, we performed multivariable analysis using a forward selection method to generate a best fit model using all feature types, yielding a model that explained ~47% of the variability in prednisolone LC₅₀ (93% before bias correction). This model contained 32 features: 4 mRNAs (*CELSR2*, *FAM13A*, *NT5M* and *COBL*) two of which are in our top 15 genes, 12 methylation probes (including *BCL2*), 2 miRNAs and 14 SNPs (**Supplementary Table 1**), supporting the use of multiple data types together in genomic studies of complex phenotypes (e.g., drug resistance). Furthermore, many features not included in the multivariable model were significantly related to other features in the model, providing enhanced confidence in the genes identified. Gene-level integration of these six genomic features identified 118 genes that were significantly associated with prednisolone resistance by both the polygenomic and the TAP methods. Fifteen of these genes were also significant in a genome-wide CRISPR-knockout screen, 14 of which have not been previously associated with glucocorticoid resistance. The statistical probability of capturing 15 genes in all three analyses by chance is extremely small ($p=8.2 \times 10^{-79}$; see Chapter 3).

To assess the robustness of our approach, we compared genes identified in the current analyses with genes previously associated with glucocorticoid resistance in ALL.^{19,58,59,61,63,65,68,75,77,85-87,100,107,109,110,113,114,116-118,120,123,136,137,139-141,196,197} This revealed that 30 of 38 (79%) genes previously shown to confer glucocorticoid resistance in ALL were directly identified by our agnostic, integrative polygenomic strategy. Some

*Portions of chapter from previously published article; final submission modified with permission. Autry, R. J. et al. Integrative genomic analyses reveal mechanisms of glucocorticoid resistance in acute lymphoblastic leukemia. Nature Cancer, doi:[10.1038/s43018-020-0037-3](https://doi.org/10.1038/s43018-020-0037-3) (2020).¹⁴²

previously described genes such as *CREBBP* were not found directly, but genes known to be associated with their function (*CREB1*) were found. When we included genes in the same biological pathway, our method captured all 38 pathways previously shown to confer glucocorticoid resistance in ALL.

Re-discovery of this large number of known mechanisms of resistance gives confidence that many of the novel mechanisms are likely genuine, either as independent mechanisms or as members of common pathways.

CELSR2 was the top novel gene downregulated in glucocorticoid resistant ALL and decreasing *CELSR2* expression recapitulated glucocorticoid resistance in human leukemia cell lines. This also revealed genes that exhibited significantly altered expression as a consequence of reducing *CELSR2* expression, including markedly lower expression of the GR and higher expression of anti-apoptotic *BCL2* following prednisolone treatment. We showed that co-administration of a *BCL2* inhibitor (venetoclax) mitigated glucocorticoid resistance due to low *CELSR2* expression, documenting greater synergy in ALL cell lines in which *CELSR2* had been knocked down and in primary leukemia cells with low *CELSR2* expression. Low *CELSR2* expression was documented in approximately half of glucocorticoid resistant ALL patients (48%), suggesting that co-treatment with venetoclax could impact a large number of patients and this combination may have even broader utility since other mechanisms of glucocorticoid resistance involve lower GR expression or function.⁷⁵ We also observed a significant increase in survival *in vivo* in mice inoculated with *CELSR2* knockdown ALL cells when venetoclax (50 mg/kg) was given in combination with glucocorticoids (**Figure 4-9c-d**), consistent with our *ex vivo* findings of greater venetoclax sensitivity in primary leukemia cells and human ALL cell lines with low *CELSR2* expression (**Figure 4-9a-b**). NetBID network analyses using interactome data generated in the independent TARGET¹⁷⁰ cohort, corroborated many of the genes and pathways that we found significant, including *CELSR2*. This is consistent with *CELSR2* and its network of interacting genes acting as a master regulatory network influencing the sensitivity of leukemia cells to prednisolone.

CELSR2 is a membrane-bound G-protein coupled receptor that alters gene expression via non-canonical WNT signaling¹⁹⁵ and HIPPO signaling,¹⁹⁸ and is involved in cell-cell interactions. Manipulation of *CELSR2* in ALL cells led to alterations in downstream non-canonical Wnt targets, increasing the expression of NFAT1 and cJun and decreasing the phosphorylation of cJun at the total protein level,¹⁹⁹ consistent with the documented decreased activation of JNK via phosphorylation at Thr 183/Tyr 185. Increasing the level of cJun represses transcription of the GR,²⁰⁰ as we observed in primary ALL cells with low *CELSR2* expression. We further documented significantly lower nuclear levels of phosphorylated JNK and phosphorylated cJun, which forms a heterodimer with *FOS* (AP1) to drive expression of *NR3C1* (GR), consistent with lower *NR3C1* expression we observed in ALL with low *CELSR2* expression. In future studies, looking into targeting small molecules to *CELSR2* or other associated non-canonical Wnt pathway components could be a promising strategy to more directly target this pathway in mitigating glucocorticoid resistance. Clinical trials have been developed looking at an

anti-*ROR1* (which is involved in non-canonical Wnt signaling and also an mRNA hit in the polygenomic analysis) monoclonal antibody (cirmtuzumab) in B-cell malignancies (NCT03088878), and a number of other Wnt targeted therapies have been developed that are in various stages of clinical development.²⁰¹

Taken together, we have shown that integration of agnostic multi-dimensional somatic genome variants can identify discrete mechanisms of drug resistance in primary leukemia cells, reliably rediscovering known mechanisms of resistance and revealing mechanisms not previously reported. Our findings indicate that interrogating multiple types of genomic variation improves the ability to discover mechanisms of resistance, compared to interrogating only one type of genome variation. Applying this strategy, we discovered a previously undescribed mechanism involving low expression of *CELSR2* in approximately 50% of glucocorticoid resistant ALL patients, causing lower expression of GR and overexpression of *BCL2*, which can be mitigated by co-treatment with the BCL2-inhibitor venetoclax. These findings represent a broad strategy for discovering genetic and epigenetic mechanisms by which cancer cells develop resistance to chemotherapy, and for revealing new therapeutic strategies to mitigate resistance.

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