

Characterizing the Role of TLX1 in T-cell Acute Lymphoblastic Leukemia

Thomas Olender

Thesis submitted in partial fulfillment of the requirements for the
Doctorate in Philosophy (PhD) program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Preface

Contributions

Thomas Olender (TO) performed TLX1 immunoprecipitations, reciprocal immunoprecipitations, and all western blots presented here and prepared silver-stained gel samples for mass spectrometry. Dr. Lawrence Puente at the OHRI Proteomics Core processed these samples, ran them on the mass spectrometer (LC-MS/MS), and processed the raw data into a Scaffold file for analysis. TO analyzed the Scaffold data. ChIP-seq experiment and bioinformatic analysis of TLX1, BRG1, CTCF, and RAD21 in ALL-SIL. Bacteria protein expression, purification, and activity assay for pA-Tn5 transposase for CUT&Tag. Optimization of CUT&Tag experiment protocol and optimizing antibodies for CUT&Tag data used. CUT&Tag experiments for ALL-SIL, Patient 05H080, mouse tg-TLX1, Jurkat Dox-TLX1. CUT&Tag bioinformatic analysis for ALL-SIL, Patient-TLX1, mouse tg-TLX1, and Jurkat Dox-TLX1. HiC bioinformatic analysis of ALL-SIL, Patient-TLX1, mouse tg-TLX1. Prepared crosslinked cells for HiC for ALL-SIL, Patient-TLX1, and mouse tg-TLX1 for KH.

Qingzhou Zhang (QZ) developed the CUT&Tag read processing pipeline, which TO modified for additional functionality. QZ also developed pipelines for RNA-seq read processing, quantification, and scripts to generate clustering heatmaps and other RNA-seq figures. TO used QZ code to generate figures.

Manisha Jalan (MJ) harvested initial tg-TLX1 mice for downstream experiments and the leukemia survival curve. Generated Jurkat Dox-inducible flag-TLX1 stable clones, termed here as “Jurkat-TLX1,” as wild-type Jurkat does not express TLX1. Jurkat-TLX1 clone 13 was used for all experiments as it had the strongest TLX1 expression upon Dox induction. Performed Jurkat-TLX1 Dox Day 2 HiC experiment and cell crosslinking, and cells were sent to Arima Genomics for HiC kit experiment and sequencing. HiC data analysis by TO.

Dr. Karina Hamamoto from the Dr. Suming Huang Lab at Penn State (KH) used the Arima HiC+ kit for ALL-SIL, Patient T-ALL, and mouse tg-TLX1 sequencing. HiC samples were sequenced by the Penn State College of Medicine Genome Sciences Facility. TO developed the HiC pipeline and analysis.

Stephen Tur (ST) harvested most of the tg-TLX1 mouse thymus, spleen, and bone marrow for downstream sequencing experiments. He worked in parallel with TO during CUT&Tag for some wild-type mice presented. He also performed flow cytometry of thymus, spleen, and bone marrow cells and flow cytometry data analysis of tg-TLX1 mice, and his figures are presented.

Carmen Palii (CP) harvested the second batch of tg-TLX1 mouse thymus, spleen, and bone marrow for downstream experiments and did flow cytometry of the thymus, spleen, and bone marrow cells. ST did flow cytometry analysis of tg-TLX1 mice for these mice.

Audrey Kopp (AK): Preliminary drug testing in mice and IVIS (data not shown)

Magid Fallahi (M.F.): Mouse genotyping and handling at ACVS.

IRCM Molecular Biology and Functional Genomics Core Facility: Sequence library preparation, QC, and sequencing of RNA-seq, CUT&Tag, and ATAC-seq samples.

Dr. Adolfo Ferrando from Columbia University: Graciously provided the TLX1 transgenic mice for breeding.

Dr. Josée Hebert from the Quebec Leukemia Bank: Graciously provided the T-ALL patient samples.

Funding Sources

2017 – 2018 Walter Faigan Kiwanis Club Fellowship in Lymphoma and Transplant Research

2020 – 2021 University of Ottawa Excellence Scholarship

2020 – 2021 Ontario Graduate Scholarship

2021 – 2022 University of Ottawa Excellence Scholarship

2021 – 2022 Queen Elizabeth II - Graduate Scholarship in Science and Technology

2022 – 2023 University of Ottawa Excellence Scholarship

2022 – 2023 Queen Elizabeth II - Graduate Scholarship in Science and Technology

Thesis Advisory Committee

Dr. David Picketts

Dr. Theodore Perkins

Dr. David Atkins

Research Ethics Approval

This research has been conducted with the approval of the OHSN-REB and the University of Ottawa Animal Care Committee. See the **Appendix** for relevant forms.

Abstract

T-cell Acute Lymphoblastic Leukemias (T-ALLs) are aggressive hematological tumors caused by the aberrant expression of a subset of transcription factors (TFs) in T-cell progenitors¹⁻³. The identification of diverse sets of secondary mutations in T-ALL tumors suggests a complex process of clonal selection through which oncogenic TF-expressing pre-leukemic cells gradually transition along the path of oncogenic transformation^{3,4}. While transgenic mouse studies have established the causative role of primary oncogenic TFs in driving leukemia, the mechanisms through which these proteins contribute to disease progression remain unclear.

One subtype of T-ALL, representing 11% of T-ALL cases, is characterized by the translocation and ectopic expression of the homeobox family transcription factor TLX1⁵⁻⁷. TLX1 expression has been shown to stop thymocyte differentiation, dysregulate gene expression, and promote oncogenic transformation by accumulating genetic lesions and aneuploidy^{5,6,8-12}. Characterization of TLX1 T-ALLs has suggested recurrent secondary mutations and colocalization with several transcription factors on the genome^{5,6,8,13}. However, the precise mechanisms through which TLX1 transforms and maintains leukemic cells is currently unclear.

To decipher the mechanism of oncogenic transformation in the TLX1 subtype of T-ALL, we used a multi-omics approach in complementary model systems to characterize changes in gene expression, epigenetics, chromatin accessibility, and 3D chromatin conformation at multiple stages of oncogenic transformation. TLX1 T-ALL human samples translocated TLX1 to the TCR δ locus and had diverse secondary mutations, and TLX1 strongly colocalized with the BAF complex and chromatin loop anchors. Our mouse model of TLX1 T-ALL revealed the transformation of pre-leukemic cells over many weeks and gradual progression to overt leukemias with diverse phenotypes and gene expression. Ectopic expression of TLX1 in thymocytes alters genome topology early in transformation, partly by recruitment of the BAF complex and induction of aberrant DNA topology via CTCF repositioning. Ectopic expression of TLX1 in the Jurkat T-ALL cell line altered chromatin looping, and acute loss of BAF complex function by inhibition or degradation killed the TLX1-expressing ALL-SIL cell line.

Our results provide critical new insights into the molecular and cellular processes of leukemia transformation in TLX1 T-ALL and suggest a novel therapeutic approach through the disruption of chromatin remodeling factors.

Acknowledgments

I am writing this to express my sincere gratitude to all of you who have supported me throughout my doctoral journey. It has been a long and challenging road, but I could not have done it without your support and encouragement.

To Marjorie Brand, thank you for being my supervisor and mentor for nearly seven years. When I applied to your lab all those years ago, I did not think I would end up being doctorate material, but here we are. With your guidance, patience, and unwavering support, you helped me grow as a researcher and gave me the tools and opportunities to excel in my research career. You have made a lasting impact on me, and I will always remember you with gratitude and admiration.

To everyone in the Brand Lab and Dilworth Lab, past and present, thank you for your contributions to the project. No one can do it alone, and each of you brought skills and hard work that were instrumental to the project's success. Thank you for your friendships and all the laughs along the way.

To my Thesis Advisory Committee, Ted Perkins, Dave Picketts, and Harry Atkins, thank you for your insights and suggestions over the years. Your input helped shape the thesis into what it is today.

To my parents, thank you for your support and for housing Julia and me for the last few years. The rental situation in Ottawa has become a total mess, and many graduate students feel the cost-of-living pain. Your support, both emotional and financial, has set us up for success moving forward.

And to my fiancé, Julia Nunes, we started our master's journeys together as boyfriend and girlfriend, and we are finishing our doctorates as soon-to-be husband and wife. You have been a source of inspiration, motivation, and joy. You have shared my struggles, frustrations, doubts, and successes. You have celebrated my achievements and comforted me in my failures. We have both reached this milestone together, and we have many more milestones left to reach on the horizon.

I am proud of what I have accomplished here, but I know that it is not only my work. It is the result of a collective effort, a collaboration of many minds and hearts. I am deeply indebted to all of you for your contributions to my dissertation and my personal and professional growth. Thank you all for being a part of my journey. I hope that you will continue to be a part of my life as I embark on new adventures and challenges.

In the wise words of Dory, which have guided Julia and me throughout this journey,

“Just keep swimming.

Just keep swimming.

Just keep swimming, swimming, swimming.

What do we do? We swim, swim, swim.”

Table of Contents

Preface	ii
Abstract	iv
Acknowledgments	v
Table of Contents	vi
List of Abbreviations	ix
List of Figures	xi
List of Tables.....	xii
Chapter 1: Introduction.....	1
1.1 Hematopoiesis in the bone marrow	2
1.2 T-cell differentiation in the thymus.....	2
1.3 T-cell acute lymphoblastic leukemias	5
1.4 TLX sub-class of HOX transcription factors.....	7
1.5 TLX1 T-cell acute lymphoblastic leukemia	8
1.6 BAF complex.....	10
1.7 Chromatin organization and regulation.....	11
1.7.1 AB Compartments	13
1.7.2 TADs and chromatin looping.....	15
1.8 Rationale	19
1.9 Hypothesis	19
1.10 Main objectives	20
Chapter 2: Materials and Methods	21
2.1 Cell culture.....	22
2.2 Bradford protein assay.....	22
2.3 RIPA buffer whole-cell lysate protein extraction	23
2.4 Large-scale nuclear protein extraction	23
2.5 Crosslinking antibodies for immunoprecipitation.....	24
2.6 Immunoprecipitation of proteins in nuclear extract	25
2.7 Western blot.....	26
2.8 Mass spectrometry workflow.....	26
2.8.1 Silver-stain gel sample preparation.....	26
2.8.2 Mass spectrometry peptide identification by OHRI Proteomics Core.....	27
2.8.3 Mass spectrometry data analysis	27
2.9 ChIP-seq	28

2.10 Mouse dissection and isolation of cells.	31
2.11 Flow cytometry of mouse thymus, spleen, and bone marrow.	32
2.12 RNA-seq workflow	33
2.12.1 RNA-seq samples preparation	33
2.12.2 RNA-seq data analysis	33
2.13 Bacteria expression and purification of pA-Tn5 for use in CUT&Tag.....	34
2.13.1 Protein expression in E.coli.....	34
2.13.2 Protein purification	35
2.13.3 Pre-annealing MEDS adapters.....	37
2.13.4 Assembling transposome complex and activity validation.	37
2.14 CUT&Tag workflow	39
2.14.1 CUT&Tag experiment	39
2.14.2 CUT&Tag data analysis	41
2.15 ATAC-seq workflow	42
2.15.1 ATAC-seq sample preparation	42
2.15.2 ATAC-seq data analysis.....	43
2.16 HiC workflow	43
2.16.1 Cell crosslinking for HiC	43
2.16.2 Arima HiC+ experiment.....	43
2.16.3 HiC data analysis	44
2.17 BRG1 protein degradation assay using ACBI-1	45
2.18 Cell-Titer Glo cell proliferation drug assay.....	46
Chapter 3 - Results	47
3.1 Optimizing immunoprecipitation with TLX antibodies.	48
3.2 Identifying TLX1 cofactors by immunoprecipitation.	50
3.3 Western-blot verifies TLX1 protein-protein interactions with cofactors.	53
3.4 TLX1 colocalizes with CTCF and RAD21 using ChIP-seq.	55
3.5 CUT&Tag in ALL-SIL and Patient T-ALL shows strong colocalization of TLX1 and its cofactors at accessible chromatin.....	56
3.6 TLX1 binds similarly at regulatory regions in both T-ALL samples.	61
3.7 TLX1 binds at many TAD boundaries and loop anchors with its cofactors.	63
3.8 ALL-SIL and Patient T-ALL share the same breakpoint at the TCR δ locus.	65
3.9 TLX1 T-ALLs harbor recurring and unique secondary mutations.	68
3.10 Transgenic TLX1 mice models T-ALL progression.....	70
3.11 Flow cytometry analysis shows leukemic transformation and diverging leukemias. .	72

3.12 TLX1 disrupts gene expression early in transformation.	74
3.13 CUT&Tag differential analysis shows genomic changes early in transformation.	79
3.14 TLX1 colocalization alters the binding of CTCF, cohesin, and the BAF complex. ...	81
3.15 Early pre-leukemia shows minor AB compartment changes.....	83
3.16 TAD changes occur early in transformation.....	85
3.17 TLX1 binding perturbs chromatin loop formation early in transformation.	88
3.18 Ectopic expression of TLX1 results in differential loop formation.	91
3.19 ALL-SIL is sensitive to ACBI-1 and BRG1/BRM Inhibitor-1	93
Chapter 4 - Discussion	95
4.1 A note about antibodies.	96
4.2 Pursuing the BAF complex and chromatin looping in TLX1 T-ALL.....	97
4.3 Structural variants in human TLX1 T-ALLs.	99
4.4 TLX1 mouse modeling of T-ALL progression.....	101
4.5 Possible mechanisms of transformation promoted by TLX1.	103
4.6 Targeting the BAF complex in TLX1 T-ALL.	108
4.7 Limitations.....	109
Chapter 5 - Conclusion	111
References	112
Appendix.....	127
Ottawa Health Science Network Research Ethics Board Approval	128
Ethical Acceptance of Animal Research: Report of the Animal Care Committee.....	129
Supplemental Data	130
Curriculum Vitae.....	141

List of Abbreviations

Abbreviation	Full Length
APA	Aggregate peak analysis
ATAC-seq	Assay for transposase-accessible chromatin with sequencing
BAF complex	BRG-/BRM-associated factor complex
BCL11B	B-Cell CLL/lymphoma 11B
BRD	Bromodomain
BRG1	BRM/SWI2-related gene 1
BRM	Brahma homolog
BSA	Bovine serum albumin
cBAF complex	Canonical BAF complex
ChIP-seq	Chromatin immunoprecipitation sequencing
CLP	Common lymphoid progenitor
CTCF	CCCTC-binding factor
CUT&Tag	Cleavage under targets and tagmentation
DN	Double negative (thymocyte)
DNA	Deoxyribonucleic acid
Dox / DOX	Doxycycline
DP	Double positive (thymocyte)
EL	Elution
ESC	Embryonic stem cell
ETP	Early T-cell progenitor
ETS	Erythroblast transformation specific (TF family)
FDR	False discovery rate
HDAC	Histone deacetylase
HiC	High-throughput chromosome conformation capture
HMM	Hidden Markov model
HOX	Homeobox (TF family)
IKZF1	IKAROS family zinc finger 1
IP	Immunoprecipitation
LK	Leukemia
LMPP	Lympho-myeloid primed progenitor
LT-HSC	Long-term hematopoietic stem cell
MEIS	Myeloid ecotropic viral integration site (TF family)
MPP	Multipotent progenitor
ncBAF complex	Non-canonical BAF complex
NE	Nuclear extract (isolated proteins from cell nuclei)
NIPBL	Nipped-B-like
NKL	Neuronal Kruppel-like (TF family)

NPV	Nuclei pellet volume
NuRD complex	Nucleosome remodeling and deacetylase complex
PBAF complex	Polybromo-associated BAF complex
PBS	Phosphate-buffered saline
PBX	Pre-B cell leukemia homeobox (TF family)
PCA	Principal component analysis
PcG complex	Polycomb group complex family (PRC1 & PRC2)
PCV	Pellet cell volume
PDS5	PDS5 cohesin associated factor A
PIC	Protease inhibitor cocktail
PLK	Pre-leukemia
POL2	RNA polymerase II
PROTAC	Proteolysis-targeting chimera
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RAD21	RAD21 cohesin complex component
RBC	Red blood cell
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RT	Room temperature
RUNX	Runt-related transcription factor (TF family)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SMC1A	Structural maintenance of chromosomes 1A
SMC3	Structural maintenance of chromosomes 3
SP4	Single positive CD4
SP8	Single positive CD8
ST-HSC	Short-term hematopoietic stem cell
SV	Structural variant
T0	Time-zero
TAD	Topologically associated domain
TALE	Three amino-acid loop extension (TF family)
T-ALL	T cell acute lymphoblastic leukemia
TCR	T cell receptor
TF	Transcription factor
TLX1	T cell leukemia homeobox 1
WAPL	Wings apart-like protein homolog
WB	Western Blot
WT	Wild-Type

List of Figures

Figure 1. Overview of hematopoiesis toward T-cell differentiation lineage.	4
Figure 2. Representation of HiC compartmentalization.	14
Figure 3. Example of chromatin structures visualized in Juicebox.	16
Figure 4. Gating strategy for flow cytometry in thymus, spleen, and bone marrow samples....	33
Figure 5. pA-Tn5 activity validation assay.	38
Figure 6. Testing specificity of TLX1 and TLX3 antibodies in ALL-SIL, DND-41, and Jurkat nuclear extracts by western blot.	49
Figure 7. Immunoprecipitation of TLX1 and mass spectrometry analysis identifies candidate cofactors.	51
Figure 8. Western blot validation of TLX1 and cofactor interactions.	54
Figure 9. TLX1 binding sites are enriched for CTCF motifs and colocalize strongly with CTCF and cohesin.	56
Figure 10. TLX1 overlaps with CTCF, Cohesin, and BAF Complex at accessible chromatin.	58
Figure 11. TLX1 overlaps active regulatory regions in both TLX1 leukemias.	60
Figure 12. TLX1 overlaps cofactors at TAD boundaries and loop anchors.	62
Figure 13. Neo-TAD and neo-loop enhancer hijacking results in TLX1 expression.	65
Figure 14. Deletion of CDKN2A and CDKN2B in Patient and ALL-SIL cells.	67
Figure 15. Transgenic mouse model recapitulates T-ALL phenotype.	69
Figure 16. Flow cytometry analysis of mouse thymus across leukemia transformation.	71
Figure 17. Mouse RNA-seq analysis shows stepwise changes in gene expression.	75
Figure 18. Leukemic Mice have distinct gene expression signatures.	76
Figure 19. Differential analysis of CUT&Tag targets at WT, PLK-early, PLK-late, and LK...78	
Figure 20. Differential analysis of cofactors shows TLX1 colocalization in PLK-early.	80
Figure 21. Mouse differential AB compartment strength analysis using dcHiC.	82
Figure 22. TLX1 expression in thymocytes results in significant TAD reorganization.	85
Figure 23. TLX1 binding perturbs chromatin looping in PLK-early.	87
Figure 24. Example region of TLX1 binding perturbing cofactors and chromatin organization in PLK-early.	88
Figure 25. Expression of TLX1 results in differential loop formation.	90
Figure 26. ALL-SIL is sensitive to ACBI-1 and BRG1/BRM inhibitor-1.	92
Figure 27. Model of TLX1 driven leukemia transformation.	102
Supplemental Figure 1. NUP214-ABL1 mutation in ALL-SIL but not Patient T-ALL.	129
Supplemental Figure 2. Copy number variation calculated by NeoLoopFinder.	130
Supplemental Figure 3. Additional flow cytometry plots for WT and PLK-early mice.	135
Supplemental Figure 4. Additional flow cytometry data for PLK-late and LK mice.	136
Supplemental Figure 5. DiffBind PCA analysis of CUT&Tag targets in tgTLX1 mice.	137

List of Tables

Table 1. Significantly enriched complexes identified by ClueGO in TLX1 IP.	52
Supplemental Table 1. TLX1 IP mass spectrometry total spectra for proteins of interest.	128
Supplemental Table 2. Fusion-gene analysis of Patient 05H080.	131
Supplemental Table 3. Patient T-ALL genes affected by deletions and duplications.	132
Supplemental Table 4. Fusion-gene analysis of ALL-SIL.....	133
Supplemental Table 5. ALL-SIL genes affected by deletions and duplications.....	134
Supplemental Table 6. Additional Mouse Information.	138

Chapter 1: Introduction

1.1 Hematopoiesis in the bone marrow

Hematopoiesis is the process of hematopoietic stem cells differentiating into immune and red blood cells. This process is hierarchical in nature and was initially derived by characterizing cell surface markers. However, single-cell technologies have shown that it is a more continuous process, which can be delineated by gene expression^{14,15}. A general overview for cells headed to the thymus is provided in **Figure 1**. The process of hematopoiesis begins with quiescent LT-HSCs in the bone marrow. Upon a stress response stimulus, these cells enter the cell cycle to become ST-HSC/MPP1, which proliferate and support blood cell populations^{14,16}. This pool of cells then differentiates into parallel MPP2-4 cell populations. MPP2/3 cells commit to CMPs and the myeloid stream, whereas MPP4/LMPPs commit to the lymphoid lineage as CLPs¹⁴. CLPs that exit the bone marrow and migrate to the thymus commit to the T-cell lineage on contact with thymic epithelial cells (TECs)³.

1.2 T-cell differentiation in the thymus

CLPs enter the thymus to undergo several stages of differentiation and selection in the thymus, then exit as CD4⁺ or CD8⁺ T-cells. These stages are identified by the expression of different combinations of cell surface markers, which can be distinguished by flow cytometry. The earliest thymocytes are referred to as double negative (DN) cells, as they do not express CD4 or CD8 surface receptors but can be distinguished by CD44 and CD25 expression. DN cells go through four stages of development in humans: ETP/DN1, DN2, DN3, and DN4, and are characterized by multipotency and stages of T-cell Receptor rearrangement (TCR β , δ , γ)¹⁶. DN4 cells then express CD4 and CD8, marking the DP stage, which can become CD4⁺ or CD8⁺ T-cells. Each stage of differentiation is coordinated by interactions with thymic

epithelial cells and signaling factors in different compartments of the thymus. These interactions result in changes in gene expression within the thymocyte that regulate the stepwise maturation events. **Figure 1** provides an overview of the main steps, which are described in more detail below.

CLPs progress into the ETP/DN1 stage¹⁶. During this stage, DN1 thymocytes upregulate the NOTCH1 receptor and commit to the DN2a stage^{16,17}. When external ligands bind to the NOTCH1 receptor, it triggers the cleavage of the internal portion of NOTCH1, resulting in the formation of the transcription factor ICN1. ICN1 then moves to the nucleus and complexes with CBF1/MAML1/RBPJ to activate critical T-cell differentiation genes, including HES1, MYC, and TCF-1^{18,19}. TCF-1 expression upregulates GATA3 and BCL11B, which are vital for T-cell differentiation and maintaining identity¹⁹. Upregulation of LCK and recombination proteins RAG1 and RAG2, which initiates TCR rearrangement, signifying the DN2b stage¹⁷. Successful TCR rearrangement results in TCR expression¹⁷. Rearrangement of the TCR δ , then TCR β and TCR γ loci occurs during DN2b and DN3a, along with the initial expression of pre-TCR α (pT α)^{6,16,17,20}. Commitment toward the $\gamma\delta$ or $\alpha\beta$ lineage depends on successful TCR rearrangement and expression, Notch signaling strength, and IL-7R expression¹⁷. DN3a cells that express TCR δ and TCR γ commit to the $\gamma\delta$ lineage (TCR $\gamma\delta$, IL-7R^{hi}), while successful recombination of TCR β continues to DN3b (TCR β , IL-7R^{low}, pT α) and the $\alpha\beta$ lineage, where TCR α rearrangement completes later in DP^{6,16,17}. DN3a thymocytes that do commit to the $\alpha\beta$ -lineage enter DN3b and need to then pass β -selection. β -selection selects for cells that have a successfully rearranged TCR β and express pT α and CD3, which complex together on the cell surface^{17,21}. Together with Notch signaling, this activates anti-apoptotic and proliferation pathways as DN4 cells. DN4 cells re-express RAG1 and RAG2 to complete the TCR α rearrangement while expressing CD4 and CD8 surface proteins, indicating the DP

stage. DP cells with a fully rearranged TCR $\alpha\beta$ surface receptor go through positive and negative selection. Positive selection is mediated by MHC class-I and MHC class-II antigen-presenting cells. DP cells with unreactive receptors signal apoptosis (death by neglect), and cells with reactive receptors commit to either SP4 (CD4+) or SP8 (CD8+) T-cell lineage, respectively^{16,20}. Negative selection eliminates self-antigen-reacting TCR receptors²⁰. Surviving T-cells, about 3-5%¹⁷, then migrate out of the thymus.

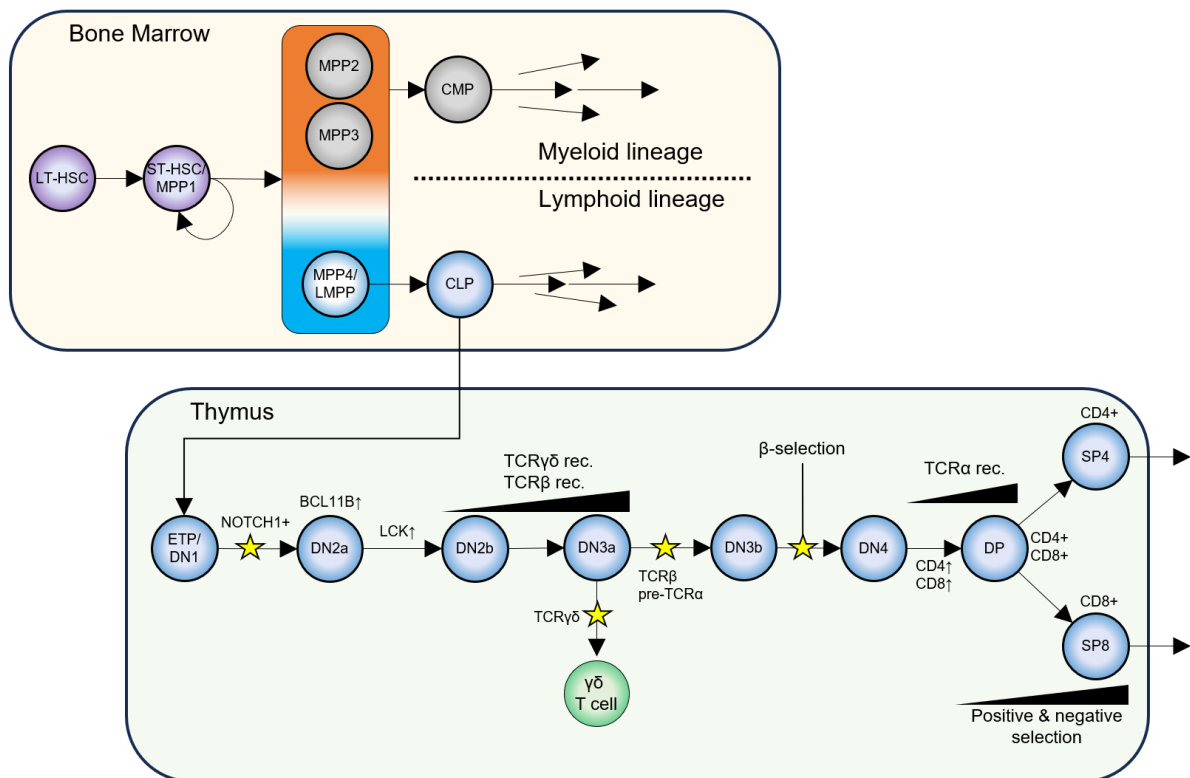


Figure 1. Overview of hematopoiesis toward T-cell differentiation lineage. Pathway of differentiation focusing on T-cells is shown. HSCs differentiate into MPP pools. MPP4/LMPPs bias toward lymphoid lineage and become CLPs. CLPs that migrate to thymus and interact with TECs and upregulate NOTCH1. DN1 cells expressing NOTCH1 commit to DN2a. LCK upregulation commits to DN2b where TCR β , γ , δ recombination begins and ends in DN3a. Successful TCR $\gamma\delta$ recombination and expression become $\gamma\delta$ T-cells, while recombination of TCR β and pre-TCR α expression commits to $\alpha\beta$ T-cell lineage as DN3b. Correctly recombined TCR β and pre-TCR α pass β -selection and proceed to DN4. DN4 cells express CD4 and CD8 receptors and complete TCR α recombination as DP cells. DP cells are positively selected toward SP4 or SP8 T-cells and negatively selected for self-antigens. SP4 and SP8 T-cells that survive selection exit the thymus. Stars indicate important regulatory checkpoints.

1.3 T-cell acute lymphoblastic leukemias

T-cell differentiation is a complex process that occurs in several stages and requires the expression of various factors, which need to be expressed at specific times¹⁷. DNA recombination is an essential part of T-cell differentiation but also makes these cells sensitive to abnormal recombination events^{3,22}. These events can result in the expression of specific oncoproteins that, in cooperation with proliferative and anti-apoptotic signaling and the accumulation of secondary mutations, synergize in a process called leukemia transformation, which gives rise to leukemia cells³.

T-ALL transformation is initiated by a primary translocation event, which ectopically expresses a transcription factor (TF) or oncogenic fusion protein^{3,23}. Indeed, it has been shown that chromosomal translocations are associated with nearly all leukemias and lymphomas^{3,22}. These translocations typically occur near non-B DNA, such as G-quadruplexes, hairpins, cruciform, and others, which are fragile regions of DNA²². These structures can arise when DNA is unwound by transcription or replication machinery and refold into highly stable non-B structures²⁴. These structures can pause polymerases and create single- and double-stranded breaks, which can be inadvertently repaired with another chromosome²⁴. They can also be non-specific targets of RAG-mediated recombination during differentiation, as they resemble DNA sequences found at recombination sites, such as BCR-ABL1^{22,24}.

For the case of TLX1 translocation, two regions immediately upstream of the TLX1 promoter have been identified as prone to G-quadruplex formation²⁴. It is suggested that these sites could be targets for early DN2 recombination, as the translocation breakpoint is consistently found at either the TCR β or δ loci^{6,23,25}.

Following ectopic expression of the primary oncoprotein, this leads to T-cell differentiation arrest at various stages, accumulation of additional genetic abnormalities

(structural variants, point mutations, aneuploidy), and recurring cooperating mutations^{5,23,26}. T-ALL primary oncoproteins are transcription factors categorized into subgroups based on their mutually exclusive expression, such as TAL1/2, TLX1/3, LMO1/2, BHLHB1, LYL1, NKX2-1, and MYB, of which TLX represents 30% of T-ALLs^{1,3,5,7,12,23,27}. The age of patients and prognoses vary between the TLX T-ALLs. Patients with TLX1 T-ALL are predominantly adults and have an 85% survival rate^{28,29}, while TLX3 T-ALL patients are predominantly children^{23,30} and have a survival rate of 45%³¹. However, while remission rates are considered relatively good, relapse has very poor outcomes.

Recurring cooperating mutations are found across T-ALLs and mostly appear to accelerate disease progression but are not always necessary^{5,7}. Leukemia-initiating cells can produce heterogeneous populations of leukemia cells with different combinations of secondary mutations and gene expression profiles³. This presents a challenge for treatment, as drug-resistant sub-populations can survive primary therapy and return over the long term³². The leukemia-initiating cells themselves may also be more resistant to therapy than their descendants³. TLX1 T-ALL also harbors recurring mutations. CDKN2A and CDKN2B are cell cycle checkpoint inhibitors that pause the G₁-S transition and are frequently deleted in T-ALLs^{7,23,26,33,34}. Activating NOTCH1 secondary mutations occur in 50-70% of T-ALLs and have been shown to cooperatively accelerate disease progression, as aberrant Notch signaling alone has poor leukemia initiating capacity³⁵⁻³⁷. These mutations can enhance the cleavage rate, extend the half-life of ICN1, or transcribe only the ICN1 segment, skipping regulation by cleavage altogether^{3,37}. Other mutations are found in lower frequencies or specific subtypes^{7,34}.

1.4 TLX sub-class of HOX transcription factors

HOX transcription factors play a critical role in embryonic development as they regulate the patterning of structures and tissues³⁸. HOX TFs bind to accessible DNA, and there is growing evidence that some HOX TFs can bind inaccessible chromatin and promote accessibility through several mechanisms³⁹. One of the proposed mechanisms involves interactions with chromatin remodeling complexes such as BAF, NuRD, INO80, and ISWI³⁹. The binding of HOX TFs to DNA promotes subsequent interactions with such complexes to promote the opening of previously inaccessible chromatin. HOX TFs are known to complex with TALE-family TFs PBX and MEIS, including TLX1⁴⁰. A subset of canonical HOX TFs (HOXD9-13) have also been found to colocalize with CTCF and RAD21 on the genome, suggesting a possible regulatory role in chromatin looping as well^{41,42}.

TLX1 and its paralogs, TLX2 and TLX3, have their own sub-class within the NKL class of homeobox transcription factors and play different roles during development^{38,43,44}. TLX1 regulates splenic anlage growth and is essential for spleen formation, as deletion results in asplenic mice^{45,46}. TLX1 and TLX3 direct neuron sub-type specification in the spinal cord with other HOX TFs⁴⁷⁻⁵⁰. TLX2 plays a critical role in intestinal nervous system development⁵¹. Importantly, these TFs are not expressed during T-cell differentiation. Their ~60% homology is mainly within their homeobox domains, with varied N-terminal and C-terminal ends. The TLX homeodomain is considered a "non-canonical" HOX protein and is not well-defined in motif databases. Previous research using in-silico modeling of TLX3 and EMSA assays of TLX1 and TLX3 suggest a conserved core motif of 5'-TAA(T/G)TA-3'⁵²⁻⁵⁴. However, TLX1 activity has also been suggested to interact independently of its DNA binding domain through interactions with other TFs^{6,55}. While previous research shows that TLX1 can interact with PBX and MEIS like other HOX TFs, it is unclear how TLX1 interacts with

cofactors on the genome to bring about changes in gene expression in its normal and T-ALL contexts.

1.5 TLX1 T-cell acute lymphoblastic leukemia

TLX1 is not expressed in thymocytes^{11,45,48}. TLX1 T-ALL leukemic transformation starts with aberrant translocation of t(10;14)(q24;q11) or t(7;10)(q35;q24)^{12,56}, which moves TLX1 to the highly active TCR δ or TCR β loci, resulting in its ectopic expression and a block in differentiation at the DN2 stage^{6,12,25,55,57}. Transgenic mouse models have shown TLX1 T-ALL has a long pre-leukemic development time of 6 to 12 months, suggesting cooperative secondary mutations are needed for disease progression^{5,10}. The pre-leukemic stage is characterized by changes in gene expression^{5,8}, an accumulation of secondary mutations^{5,8}, and an abnormal degree of aneuploidy compared to other T-ALLs^{10,58}. The mouse thymus is notably shrunk during this stage, with a high degree of cell death, but leukemia-initiating cells (LICs) eventually develop and aggressively proliferate^{3,10}. At the leukemic stage, mouse models show aggressive infiltration of the malignant cells into the lymph nodes, spleen, and liver and form mediastinal tumors^{1,5,10,29}. While TLX1 expression in thymocytes is necessary for leukemic transformation, its activity in leukemic cells is unclear. Dadi et al. (2012) showed that siRNA knockdown of TLX1 in ALL-SIL, a TLX1-expressing T-ALL cell line, caused redifferentiation of leukemic cells and massive apoptosis⁶. However, other papers have shown that the knockdown of TLX1 only slowed the proliferation of ALL-SIL with some degree of apoptosis^{5,12,27}. Additionally, these knockdown studies focused on the ALL-SIL cell line derived from relapsed leukemia, which harbors specific secondary mutations. The degree that which TLX1 activity is needed in T-ALL with other secondary mutations is not clear.

TLX1 T-ALL is associated with recurrent secondary mutations commonly seen in other T-ALLs. Transgenic mouse models have characterized frequent cooperating mutations found in TLX1 T-ALLs^{5,10}. Notably, these secondary mutations are not necessary for TLX1-driven leukemic transformation and instead synergize with TLX1 to accelerate progression^{5,12}. The fusion gene NUP214-ABL1 is frequently found in TLX1+ and TLX3+ patient T-ALLs, and forced expression in mice activated downstream TFs that synergized with TLX1 to accelerate leukemia development^{5,59}. Mouse models also identified frequent inactivating mutations of transcription factors RUNX1, BCL11B, IKZF1, and tumor suppressor PTEN^{9,10,33}. However, drug treatments targeting pathways associated with these mutations only produced transient responses^{5,12,60}.

Studies have shown TLX1 binding near various transcription factor motifs, such as ETS, RUNX, MEIS, and PBX family transcription factors, and TLX1 colocalizes with ETS1 and RUNX1 in ALL-SIL cells^{5,6,8}. When looking at changes in gene expression, Della Gatta et al. (2012) analyzed T-ALL patient samples and found TLX1 and TLX3 T-ALLs had a unique gene expression signature from other T-ALLs⁹. While many genes were commonly misregulated, many genes were also differentially expressed between the two subtypes, suggesting common and unique activities⁹. Notably, ChIP-chip of TLX1 in ALL-SIL and TLX3 in HPB-ALL revealed that 2-3 thousand genes were found to have TLX1 or TLX3 at their promoter⁹. However, only a fraction of those genes showed significant changes in gene expression⁹. Other studies have corroborated TLX1 binding at promoters, as well as enhancers and super-enhancers, and distal regulatory features^{5,8,61}. Taken together, this suggests that TLX1 can bind to many regulatory regions, but context-specific interactions may be needed to facilitate TLX1 activity. TLX1 activity in its normal neuron and spleen contexts are not well characterized and may not be applicable to the leukemia context as cofactors may not be

expressed. It has yet to be determined which cofactors interact with TLX1 to exert both repressor and activator activity on the genome.

1.6 BAF complex

The BAF complex is critical for many cell development lineages, such as stem cell differentiation, neuron differentiation, and hematopoiesis^{62–65}. Deletion of several subunits is embryonic lethal or precludes entry into specific differentiation branches^{64,66,67}. The BAF family of complexes, also known as the SWI/SNF family (switch/sucrose nonfermentable), are chromatin remodeling complexes that modulate nucleosome accessibility through nucleosome sliding, eviction, or exchange^{68,69}. These complexes are subdivided into 3 main groups (cBAF, PBAF, and ncBAF), with further cell-type specific compositions like esBAF, nBAF, and npBAF^{65,67,70}. These complexes contain either BRG1 or BRM, which have catalytic ATPase activity, as well as variable combinations of subunits that mediate interactions to the genome, histone PTMs, and additional cofactors like transcription factors and other epigenetic complexes^{62,71–73}. Expression of subunits is cell-type specific, and sub-complexes have unique but overlapping binding sites on the genome^{71,72,74}. The subunits of the BAF complex have diverse protein domains that can recognize DNA structures, binding motifs, and histone modifications⁷¹. The BAF complex is also recruited by transcription factors and coordinates with other complexes, such as histone deacetylases, histone acetyltransferases, and histone methyltransferases^{71,75}. As such, BAF is known to exert activating and repressing activity in a context-dependent manner, mainly at enhancers and promoters^{68,76–79}. The BAF complex has also been shown to antagonize the PcG complexes, which deposit repressive histone modifications^{70,78–80}. This is partially achieved by involvement in the maintenance of TAD boundaries by maintaining proper nucleosome occupancy around CTCF sites^{81–83}. The BAF

complex is implicated in interactions with several classes of transcription factors, such as AP1, CEBP, PBX, and BRD, ESC pluripotency factor OCT4, pancreatic homeobox TF PDX1, and T-cell transcription factors BCL11B and RUNX1^{64,71,79,84–86}.

The BAF complex is critical for thymocyte differentiation. BCL11B is associated with the reorganization of higher-order chromatin interactions at the DN2 to DN3 transition and is critical for T cell lineage commitment^{87–90}. Mouse studies have also shown that deletion of BRG1 in thymocytes results in a DN4 to DP block⁹¹, while mice with BAF47 deletion rapidly develop CD8+ T cell lymphomas⁹².

The BAF complex is considered tumor suppressive as mutations of its subunits have been identified in a broad range of cancers^{62,68,93,94}. In some cancers, BAF - PcG antagonism is imbalanced due to inactivating mutations in one of these complexes, leading to gene regulatory addiction and a synthetic-lethal condition^{70,71,83}. This addiction has been exploited by drug inhibition of the opposing complex, leading to a collapse of gene regulation and cancer cell death^{94,95}. Indeed, retargeting of BAF activity by oncogenic transcription factors has been shown in prostate cancer⁹⁶. Taken together, the BAF complex participates with many regulatory proteins to control the accessibility of DNA in a context-dependent manner, and aberrant recruitment can result in the misregulation of genes.

1.7 Chromatin organization and regulation

Chromatin organizes into 3-dimensional structures that range from several megabases down to several kilobases^{97,98}. Early microscopy research showed that chromosomes occupied individual territories in the nucleus and that chromatin had an ordered arrangement in the nucleus⁹⁹. Genome-wide higher-order structures explaining these observations were characterized along with the introduction of Hi-C, which found intrachromosomal interactions

formed an alternating “plaid” pattern termed AB compartments **(Figure 2)**¹⁰⁰. Since then, ultra-high-resolution sequencing HiC and other methods of chromatin capture describe several layers of organization that are mediated by several distinct but partially interdependent mechanisms. These include A/B compartments at the highest level, which are partitioned into topologically associated domains (TADs), nested sub-TADs, and chromatin looping, which mediates inter- and intra-TAD interactions^{97,101,102} **(Figure 3)**. Several processes, including chromatin looping, epigenetic histone modifications, enhancer-promoter interactions, transcription factors, DNA methylation, and chromatin accessibility, are involved in regulating these levels^{90,97,103}.

HiC provides a wealth of information to describe chromatin organization. It is the earliest sequencing experiment for interrogating genome-wide 3D chromatin interactions, and many programs have been developed to analyze the data. HiC provides information on AB compartments, TADs, and chromatin looping, and recent programs use HiC patterns to identify copy number variation¹⁰⁴ and structural variants¹⁰⁵. However, the amount of data that is extracted from HiC is a function of how many reads are sequenced and the quality of the HiC library¹⁰⁶. Higher read counts allow for higher resolution analysis with diminishing returns¹⁰⁶. Newer methods of extracting chromatin interaction information using relatively fewer reads have been developed, such as Micro-C¹⁰⁷, HiCAR¹⁰⁸, and Hi-TrAC¹⁰⁹.

Spatial chromatin regulation is critical to gene expression as these mechanisms coordinate the interactions of genes and their regulatory elements in 3-dimensional space¹¹⁰. They bring enhancers into the proximity of promoters or insulate them apart, and they stop heterochromatin from spreading into active gene regions^{111–113}. Chromatin structures play an important role in cell differentiation, as changes in structure are observed in many cellular contexts, and misregulation is implicated in disease and cancers^{90,114–116}.

1.7.1 AB Compartments

AB compartments are large segments of chromatin that alternate between active euchromatin and inactive heterochromatin¹⁰⁰. Generally, A compartments are gene-rich, contain active histone marks, and tend to be physically located closer to the center of the nucleus¹¹⁰. B compartments are gene-poor, have repressive histone marks, and tend toward the nuclear periphery or nucleoli (**Figure 2**). Notably, it is possible to have repressed regions in the A compartment and active genes in the B compartment, but it is not common. Compartments are further characterized and are subdivided into A1, A2, B1, B2, and B3 based on their location in the nucleus, associated histone PTMs, and cofactors¹¹⁷.

- A2: Euchromatin, POL2, active histones, and TFs, further from nuclear speckles
- A1: Euchromatin, POL2, active histones, and TFs, near nuclear speckles
- B1: Heterochromatin, Polycomb complexes, H3K27me3
- B2: Heterochromatin, H3K9me3, HP1, near nucleoli
- B3: Heterochromatin, H3K9me3, HP1, at nuclear lamina, bound to lamins

Compartment-regulating mechanisms are understood to be separate from TADs and chromatin looping as the formation of compartments is proposed to follow a phase-separation model (analogous to oil and water)^{118–120}, where sufficient concentrations of molecules involved in various mechanisms (active or inactive histone modifications, transcriptional machinery, promoter-enhancer interactions, HP1 packaging, RNA interactions) spontaneously separate into their phases, clustering like-compartments together (A with A, B with B) while opposing switches to the opposite compartment. Chromatin organization resets during mitosis due to chromatin compaction^{121,122}. Cells exiting mitosis into G1 show initial AB compartmentalization followed by TAD and chromatin loop formation in early G1 phase¹²².

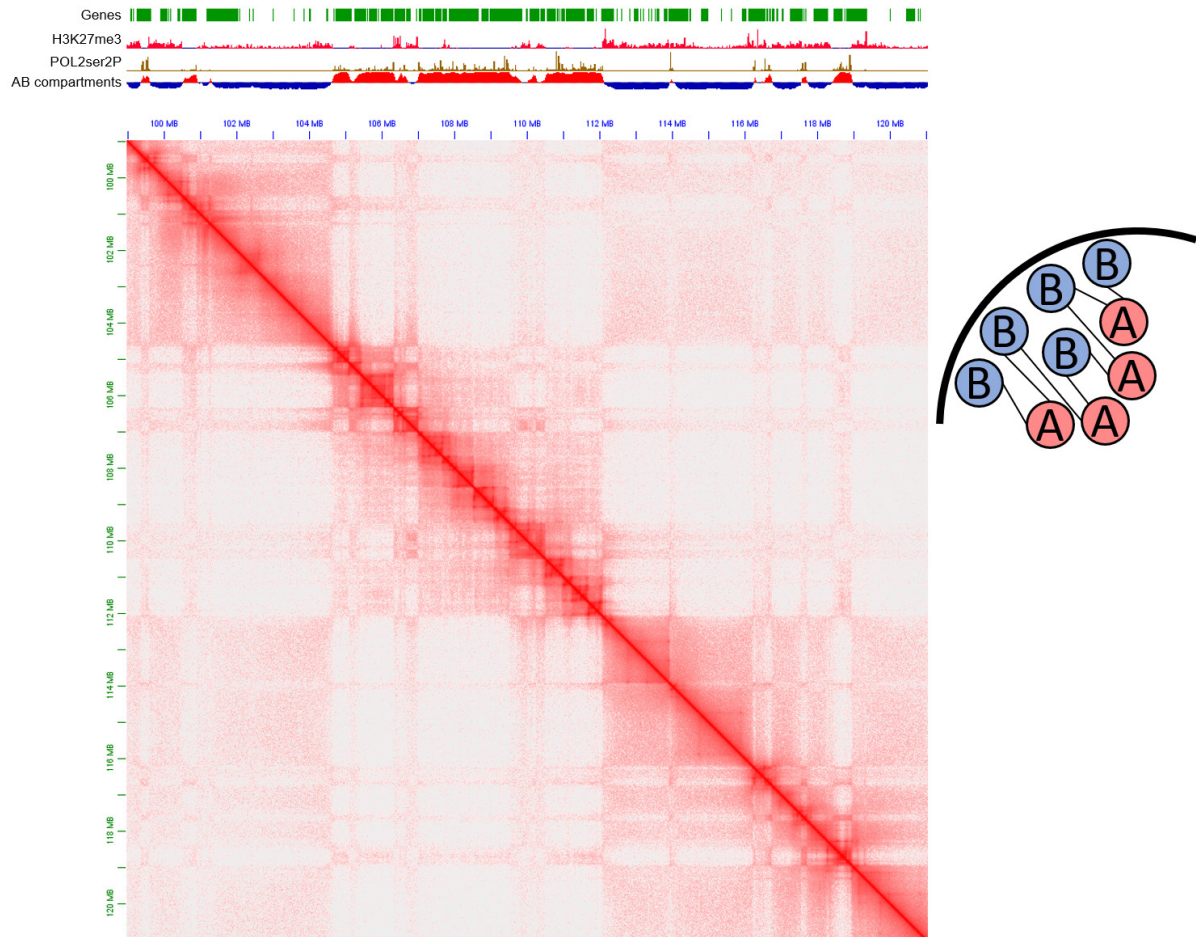


Figure 2. Representation of HiC compartmentalization. Left: Region of chr6:99-121Mb of ALL-SIL showing the “plaid” pattern seen in HiC data signifying spatial separation of A and B compartments. H3K27me3 correlates with repressed B segments of a chromosome. POL2-ser2P correlates with A compartments with actively transcribed genes. AB compartments track shows A compartments as red and B compartments as blue. HiC matrix is KR balanced and $\text{Log}(\text{observed}+1)$, visualized in Juicebox. Right: representation of a single chromosome plaid pattern and localization in the nucleus. B compartments tend to localize closer to the nuclear lamina, while A compartments localize closer to the nuclear center (not to scale).

1.7.2 TADs and chromatin looping.

Topologically associated domains (TADs) exist within compartments and are defined as segments of DNA that interact more with themselves than adjacent DNA^{98,101,123}. TADs can range anywhere from several tens of kilobases to about two megabases, with some TADs exceeding 2Mb, and can have within them one to many genes¹²⁴. TADs are mediated largely by CTCF, a DNA-binding transcription factor, and cohesin, a complex of RAD21, SMC3, and SMC1A, and STAG1 or STAG2, which forms a ring-like complex^{97,125–128}. Chromatin loops are defined as distinct interactions between pairs of distant loci (loop anchors) and appear as “dots” in HiC data (**Figure 3**). Loops act as boundaries between TADs and bring enhancers and promoters into spatial proximity within TADs, which appear as nested sub-TADs (**Figure 3**). This has a profound influence on gene expression, as expression of some genes has been shown to be metered by the number of enhancers that are looped to it¹⁰⁹. Housekeeping genes tend to have few enhancers needed for expression, while developmental genes tend to have many enhancers required for tight control of expression¹⁰⁹. CTCF has a well-characterized DNA-binding motif that only binds in accessible DNA, as it is not a pioneer factor. The BAF complex maintains nucleosome occupancy around CTCF motifs, and BRG1 has been shown to bind directly to CTCF¹²⁹. CTCF binding sites are found in distal regions and most enhancers and promoter regions as well^{130,131}. Of the multiple subcomplexes, the ncBAF complex colocalizes more frequently with CTCF than cBAF or PBAF⁸³. The formation of loops is characterized by a “loop-extrusion” model^{119,123}, where cohesin is loaded onto DNA by NIPBL & MAU2, and the DNA is held within the ring complex¹²⁸. Cohesin ATPase activity then pulls DNA through itself until it pauses at CTCF-bound DNA¹³¹. CTCF pauses cohesin for a period of time, then CTCF either dissociates from DNA or cohesin passes over CTCF to continue extrusion¹³¹. WAPL & PDS5 can unload cohesin from DNA at any point in the process^{131,132}.

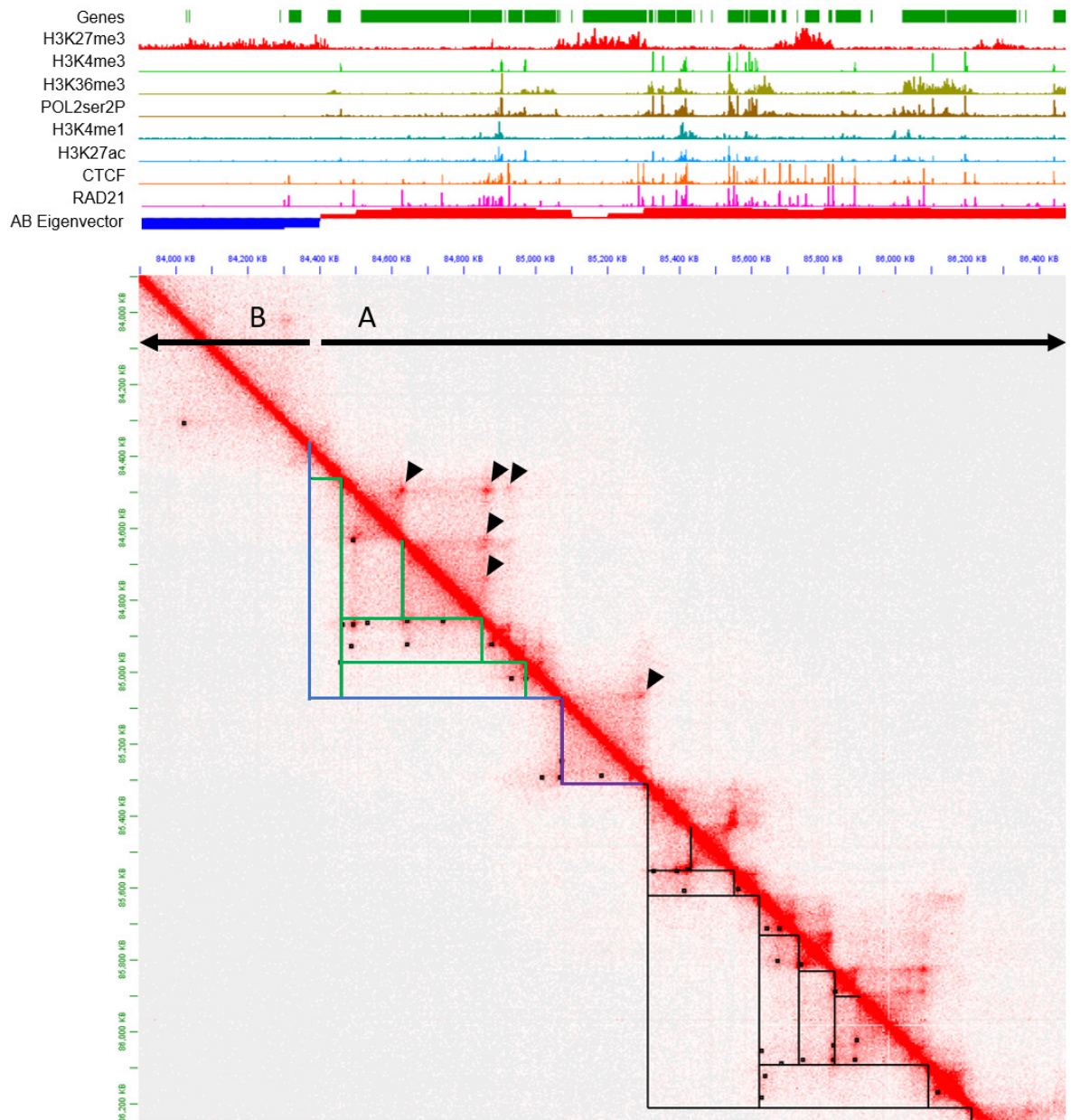


Figure 3. Example of chromatin structures visualized in Juicebox. Diagonal representation of ALL-SIL HiC at chr2:83.9-86.4Mb with accompanied CTCF & RAD21, POL2, and histone tracks. Upper diagonal shows HiC data without annotations for clarity. Lower diagonal shows TADs and sub-TADs identified by OnTAD (triangles), and chromatin loops (dots) identified by Mustache. Blue triangle shows an example of the main TAD, and green triangles show sub-TADs. Purple triangle shows example of repressed TAD within an A compartment. Black triangles point to example dots searched for and identified by Mustache.

As such, chromatin looping is highly dynamic as CTCF binds temporarily on DNA (1-4min), and cohesin is constantly being loaded and unloaded, with cohesin having a residence time on DNA of about 20min¹³³. Several studies have elucidated this dynamic equilibrium by modulating each of these components and how it alters chromatin topology (rapid depletion, over-expression, partial knockdown)^{109,127,132,134–137}. While CTCF positions cohesin, additional factors are at play regarding cohesin migration along DNA. Loop extrusion can be paused by bound TFs and complexes blocking extrusion at enhancers and promoters, and is also relocated by RNA polymerase as it transcribes DNA¹³⁸. The amount of cohesin present and rates of cohesin loading and unloading also influence the strength and length of TADs and compartments, as well as chromatin loop intensity¹³².

The relationship between chromatin looping and gene regulation is a hotly debated topic^{124,131}. It is generally agreed upon that chromatin looping plays an important role in regulating gene expression, as looping is involved in promoter-enhancer interactions, and deletion of RAD21 causes cell death¹³³. Studies have shown that inserting, removing, inverting, or partially mutating CTCF binding sites can have a direct effect on local gene regulation¹²⁴. However, how looping works together with and is influenced by other mechanisms of gene regulation, such as epigenetic marks, TFs, DNA accessibility, and transcription machinery, is an active area of research¹²⁰. Regarding differentiation, recent research suggests that the formation of chromatin loops coincides with increasing H3K27ac and DNA accessibility at and between loop anchors, which correlates with increased gene expression within the loop¹³⁹. However, loss of loops correlated with up- and downregulation of genes, suggesting that chromatin looping influences the activation of genes, but separate mechanisms maintain expression¹³⁹. It is unclear if these changes occur because of chromatin looping or if chromatin looping is the result of these other mechanisms¹³⁹. A ‘time-buffering’

model using rapid depletion time courses was recently proposed, which suggests that CTCF and cohesin have little direct effect on the maintenance of gene expression¹³³. Chromatin looping may instead establish enhancer-promoter interactions by spatially concentrating TFs and epigenetic complexes so they can then directly regulate gene expression¹³³. Additional factors that operate together and independently of CTCF and cohesin to form loops have also been identified. These mechanisms are not studied here but are mentioned for context. The ubiquitously expressed transcription factor YY1 homodimerizes to form loops by itself^{140,141}. LDB1 can also bridge transcription factor complexes to form loops with LHX2 and EBF^{102,142}. HCFC1 and ZNF143 have recently been found to mediate shorter-range and promoter-promoter loops, while CTCF and cohesin mediate enhancer-enhancer and longer-range loops¹⁰⁹. The PcG complex PRC2 has also been shown to mediate long-range interactions between PRC-bound loci and silenced genes^{102,143}. The mediator complex, which bridges enhancers and promoters, has also been implicated in interactions with cohesin and NIPBL^{102,144}. Transcription factories are regions with highly concentrated enhancer and promoter machinery that form condensates that hold themselves together. While more research is needed to tie these mechanisms together, regulation of CTCF binding sites results in changes in 3D chromatin structure that influence gene expression, and misregulation can dysregulate gene expression, resulting in cancer.

1.8 Rationale

TLX1 is a non-canonical HOX transcription factor whose ectopic expression in developing thymocytes drives leukemic transformation, resulting in T-ALL. TLX1 binds to promoters and enhancers and has been shown to have activator and repressor activity. While there is considerable evidence that TLX1 expression drives leukemia transformation, the cofactors and mechanisms that TLX1 interacts with to bring about changes in gene expression to drive transformation are currently unclear. Exploratory findings during my master's work suggest interactions with the BAF complex and CTCF/cohesin, which implicate TLX1 in epigenetic and chromatin looping mechanisms. Other HOX transcription factors have been shown to colocalize with CTCF and are involved in chromatin remodeling. TLX1 has not been suggested thus far to be involved in these mechanisms in its normal developmental roles nor its T-ALL context. Understanding the molecular and cellular processes involved in TLX1-driven leukemia transformation will provide insights into oncogenic TF function in thymocytes and identify possible avenues for targeted treatment for T-ALL.

1.9 Hypothesis

We hypothesize that TLX1 establishes and maintains leukemic cells partly through the recruitment of complexes that mediate chromatin loop formation, resulting in the dysregulation of 3D chromatin structures.

1.10 Main objectives

Master's Objective: Identify candidate proteomic and genomic mechanisms where TLX1 participation may drive the oncogenic transformation of T-cell progenitors into their leukemic state.

- This was the general objective of my Master's project that transitioned to the hypothesis above and objectives 2, 3, and 4.

Objective 2: Characterize the role of TLX1 in chromatin domain transformation and maintenance.

- Assess the degree to which TLX1 binds at TAD boundaries as well as AB compartment occupancy in human TLX1 T-ALLs.
- Characterize TAD and AB compartment organization across stages of mouse leukemic transformation.

Objective 3: Determine the mechanism through which TLX1 regulates chromatin looping across model systems.

- Characterize TLX1 occupancy at chromatin loops in human TLX1 T-ALLs with its cofactors.
- Characterize changes in chromatin looping sites across stages of mouse leukemic transformation and assess TLX1 occupancy with its cofactors at these sites.
- Determine whether ectopic expression of TLX1 in Jurkat T-ALL causes changes in chromatin loops.

Objective 4: Evaluate BAF complex inhibition as a potential T-ALL therapeutic strategy.

- Determine, in vitro, whether the TLX1 cell line ALL-SIL is sensitive to targeting the BAF complex through inhibition or degradation of BRG1 and BRM.
- Determine whether targeting the BAF complex with these drugs can be replicated in mice.

Chapter 2: Materials and Methods

2.1 Cell culture

Cell cultures were maintained at 37°C and 5% CO₂. ALL-SIL (DSMZ ACC 511) was cultured in RPMI-1640 media supplemented with 20% FBS, 1% Pen/Strep in a 37°C incubator (5% CO₂). Jurkat (DSMZ ACC 282) was cultured in RPMI-1640 media supplemented with 10% FBS, 1% Pen/Strep in a 37°C incubator (5% CO₂). The Jurkat-Dox-flag-TLX1 stable cell line (Jurkat-TLX1) was cultured in RPMI-1640 media supplemented with 10% FBS, 1% Pen/Strep, 400 µg/mL HygromycinB, and 20 µg/mL Blasticidin-HCl. For induced expression of TLX1 in Jurkat, 5 µg/mL Doxycycline was added and replaced every 48 hours.

2.2 Bradford protein assay

BSA standards and blanks were prepared in respective buffers for relevant experiments (ex. RIPA buffer, NE buffer D). A 1X working solution of Bio-Rad Protein Assay Dye Reagent Concentrate (cat #5000006) was prepared in dH₂O at RT. When measuring by cuvette, 2 µL of sample, standards, and blank was added to 1 mL working solution per cuvette. Cuvettes were mixed by inversion and incubated for 5-10 minutes, then measured using a spectrophotometer (Eppendorf BioPhotometer Model #6131). For the 96-well microplate measurements, 1 µL of sample, standards, and blank was mixed with 24 µL of dH₂O, briefly vortexed, then 250 µL of the working solution was added, incubated for 10 minutes, and measured using a BioTek Synergy HT plate reader. Sample concentration was determined by BSA standard curve with both methods.

2.3 RIPA buffer whole-cell lysate protein extraction

Cells were pelleted at 600g for 5 minutes at 4°C. Media was aspirated, and cells were resuspended in cold 1X RIPA buffer (50 mM Tris pH 7.4; 150 mM NaCl, 1% Triton X-100, 0.5% Na-Deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 0.5 mM DTT, 1X PIC) supplemented with 5 mM MgCl₂ and 0.5 ng/μL Benzonase[®] nuclease (Millipore 70746). Note: Benzonase degrades RNA and DNA, which greatly reduces the viscosity of whole-cell lysates. Samples were placed in a thermomixer set to 25°C and 1400rpm for 15 minutes, then kept on ice. Protein concentration was measured by Bradford assay. Samples then had added 1X final dilution of 4X SDS loading dye (200 mM Tris pH 6.8; 40% glycerol, 8% SDS, 80.4 mM DTT, 0.4% Bromophenol Blue), boiled for 10 minutes, and stored at -20°C.

2.4 Large-scale nuclear protein extraction

Prior to nuclear extraction, cultured cells (ALL-SIL, DND-41, Jurkat) were grown in 1L spinner flasks supplied with medical oxygen (21% O₂, 5% CO₂) on a magnetic stirring platform in a 37°C incubator. Cells were harvested and pelleted at 600g at 4°C for 15 minutes. Media was aspirated, and cell pellets were resuspended in cold MS30 buffer (50 mM Tris pH 8.0; 2.5 mM EDTA, 30% glycerol, 0.1 mM DTT), then snap-frozen in liquid nitrogen and stored at -80°C.

Cells were thawed on ice and combined in a 50 mL tube. Cells were pelleted at 1500rpm for 15 minutes at 4°C, and pellet cell volume (PCV) was estimated. Pellet was resuspended in 5X PCV of cold PBS and re-pelleted, then resuspended in 5X PCV of NE Buffer A (10 mM HEPES-KOH pH 7.9; 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1X PIC) and incubated for 15 minutes on ice. Cells were pelleted, resuspended in 2X PCV of NE Buffer A, and lysed using a Kontes B-type homogenizer with 10 strokes. Lysate was transferred to

high-speed centrifuge tubes and spun at 25,000g for 30 minutes at 4°C. The supernatant (cytosolic fraction) was removed, and cells were resuspended in 1X nuclei pellet volume (NPV) of NE Buffer C (10 mM HEPES-KOH pH 7.9; 1.5 mM MgCl₂, 600 mM KCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1X PIC) and lysed using a Kontes B-type homogenizer with 10 strokes. Lysate was then transferred to high-speed centrifuge tubes, rocked for 30 minutes at 4°C, and spun at 25,000g for 30 minutes at 4°C. The supernatant (nuclear fraction) was transferred to a 10,000 MWCO Slide-A-Lyzer™ Dialysis Cassette (ThermoFisher 66380) and dialyzed for 2 hours in 1 L of cold NE Buffer D (25 mM Tris pH 7.9; 5 mM MgCl₂, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) with gentle stirring. NE Buffer D was replaced and dialyzed overnight with gentle stirring. The nuclear extract was transferred to a 15 mL tube and completed with 1X PIC, 1 mM PMSF, 1 mM Na-Orthovanadate, and 0.05% NP-40. Protein concentration was measured by Bradford assay, and the nuclear extract was aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C.

2.5 Crosslinking antibodies for immunoprecipitation

Dynabeads Protein A magnetic beads (ThermoFisher 10001D) were washed twice with Wash buffer (0.5M K-phosphate pH 8.2; 0.1% chicken albumin). Antibodies were incubated using 25 µg antibody and 100 µL of Dynabeads in 0.1 M K-phosphate pH 8.2 and rotated at RT for 1 to 3 hours. Beads were washed once with K-phosphate buffer and then washed twice with 0.2 M triethanolamine pH 8.2. Beads were then crosslinked by resuspending in 500 µL of 0.2 M triethanolamine pH 8.2 with 10 mg/mL DMP and rotated for 30 minutes. Crosslinking was quenched by washing once with 50 mM Tris pH 7.2. Beads were then washed three times using PBS with 0.1% chicken albumin, washed once with IP150 buffer (25 mM Tris pH 7.9; 5 mM MgCl₂, 10% glycerol, 150 mM KCl, 0.1% NP-40, 0.3 mM DTT, 1X PIC), washed once

with 100 mM glycine pH 3.0, washed twice with IP150 buffer, then stored at 4°C. Crosslinking was verified by Coomassie gel using 5% aliquots of post-incubation supernatant, pre-crosslinking, and pre-storage.

2.6 Immunoprecipitation of proteins in nuclear extract

Nuclear extract (NE) was thawed on ice, then ethidium bromide (100 µg/mL final) was added and incubated for 30 minutes on ice. The NE was centrifuged for 15 minutes at 14,000rpm at 4°C to pellet insoluble proteins, and the NE supernatant was retained. Dynabeads A used for pre-clearing were washed once with IP150 buffer. The NE was then pre-cleared for 2 hours at 4°C with an equivalent volume of Dynabeads A used for the IP. An aliquot was retained for western blot “Input.” Pre-cleared NE was then added to the crosslinked target and control antibody tubes and incubated overnight. An aliquot of post-incubation supernatant “SN” was aliquoted for western blot.

For TLX1 IP mass spectrometry samples, beads were stringently washed using cold IP300 buffer (25 mM Tris pH 7.9; 5 mM MgCl₂, 10% glycerol, 300 mM KCl, 0.1% NP-40, 0.3 mM DTT, 1X PIC) and IP150 (25 mM Tris pH 7.9; 5 mM MgCl₂, 10% glycerol, 150 mM KCl, 0.1% NP-40, 0.3 mM DTT, 1X PIC). Beads were then eluted using 5% acetic acid, vortexed for 30-45 minutes, then evaporated in an Eppendorf 5301 Vacufuge Concentrator. Samples were resuspended in 2X SDS Loading Dye and migrated on SDS-PAGE gel for western blot or silver-stain gel.

For reciprocal IPs, beads were washed four times with IP100 buffer (25 mM Tris pH 7.9; 5 mM MgCl₂, 10% glycerol, 100 mM KCl, 0.1% NP-40, 0.3 mM DTT, 1X PIC), then eluted using 2X SDS loading dye, boiled for 5 minutes, and then migrated on SDS-PAGE gel for western blot.

2.7 Western blot

Prior to western blot loading, samples had added 1-2X final ratio of 4X SDS loading dye, then boiled at 95°C for 5-10 minutes depending on the experiment. Samples were then migrated on 8-12% acrylamide/bis SDS-PAGE gel in a BioRad Mini Trans-Blot Cell in SDS running buffer (0.2% SDS, 192 mM glycine, 25 mM Tris-base). Gels were transferred onto nitrocellulose membrane for 60-90 minutes at 150V in Transfer buffer (192 mM glycine, 25 mM Tris-base). Membranes were blocked with PBS (5% skim milk) for 1 hour, then incubated with primary antibody for 60-90 minutes at RT or overnight at 4°C. Membranes were washed at least three times with PBS or PBS-T, then incubated for 60-90 minutes at RT with secondary antibody. Membranes were incubated with HRP substrate according to manufacturer instructions. X-ray exposures used ECL™ Western Blotting Detection Reagent (Cytiva RPN2209). ChemiDoc-MP images used SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher 34580).

2.8 Mass spectrometry workflow

2.8.1 Silver-stain gel sample preparation

The immunoprecipitated eluate from the IPs was migrated on 10% SDS-PAGE gel and stopped before the dye front reached the bottom. The gel was fixed in a glass beaker using Fixing solution (50% EtOH, 5% glacial acetic acid) for 1 hour. The gel was then washed in 50% EtOH for 10 minutes. The gel was then washed with dH₂O twice for 10 minutes. Gel was sensitized for 90 seconds using Sensitizer solution (0.02% Na-Thiosulfate, dH₂O), washed three times for 30 seconds with dH₂O, and then stained for 30 minutes using Stain solution (0.1% silver nitrate, dH₂O). Gel was developed using Developer solution (0.04% formalin, 2% Na-Carbonate, dH₂O) until bands were visible, and then stopped using Stop solution (5%

acetic acid). The gel was washed three times for 15 minutes with dH₂O and stored in 1% acetic acid. Gel bands were excised according to OHRI Proteomics Core protocol in a laminar flow hood.

2.8.2 Mass spectrometry peptide identification by OHRI Proteomics Core

Scaffold (version Scaffold 4.11.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 21.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al. *Anal. Chem.* 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from NCBI (downloaded 7-Mar-2018) and NCBI (downloaded 20-Apr-2018). (Ashburner, M et al. *Nat. Genet.* 2000;25(1):25-9).

2.8.3 Mass spectrometry data analysis

Proteins that passed a 99% protein threshold, 1.0% FDR peptide threshold, and a minimum of 2 peptides were exported from the Scaffold for further filtering. Proteins that were removed included non-human, keratins, decoy peptides, incomplete fragments, alternate isoforms with the same ID, and proteins with more spectra in IgG control. Proteins were compared to CRAPome¹⁴⁵, where proteins belonging to known families of frequent IP contaminant proteins were removed (ex. HNRNP, heat-shock (HSP), ribosomal proteins). The

final protein list was inputted into ClueGO¹⁴⁶ for gene ontology (GO) analysis. Protein interaction networks were visualized using Cytoscape¹⁴⁷.

2.9 ChIP-seq

Cells were harvested from culture (1×10^8) and centrifuged for 10 minutes at 1500rpm at RT; media was aspirated. Cells were resuspended in 39 mL of pre-warmed RPMI-1640 with 10% FBS, then 37% formaldehyde (1% final) was added and rotated for 30 minutes. Crosslinking was quenched with 125 mM glycine, and cells were centrifuged for 10 minutes at 1500rpm at 4°C. Cells were resuspended in PBS and centrifuged again, then resuspended into 2 mL PBS and split into 2x 1.5 mL tubes (50 million cells per tube). Cells were centrifuged at 500g for 3 minutes at 4°C, and the supernatant was aspirated. Cells were snap-frozen in LN2 and stored at -80°C.

Antibody-bound magnetic beads were prepared prior to sonication. Dynabeads Protein A was washed twice in IP100 Buffer (25 mM Tris pH7.9, 5 mM MgCl₂, 10% glycerol, 100 mM KCl, 0.1% NP-40, 0.1 mM DTT, 1X PIC), then 10 ug of antibody was combined with 40μL Dynabeads Protein A (30 μg/μL) in IP100 buffer and rotated at RT for at least 2 hours.

To prepare sonicated chromatin for ChIP, 50 million cells were thawed on ice and resuspended in 1mL Swelling buffer (25 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 1X PIC) on ice for 15 minutes. Cells were lysed with a Dounce B-type pestle with 40x strokes and checked with trypan-blue under the microscope. Cells were pelleted at 2000rpm for 5 minutes at 4°C, and the supernatant was aspirated. The cell pellet was then resuspended in 400 μL Sonication buffer 1% SDS (50 mM HEPES pH 7.9; 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 1% SDS, dH₂O, Roche PIC). Cells were sonicated using a 4°C actively-cooled Bioruptor with settings: high power, 65

cycles of 30sec ON, 90sec off. Note: Cycles were determined empirically prior to the ChIP protocol to produce ~300bp sheared DNA. Sonicated lysates were combined in a 10 mL polypropylene tube and diluted with Sonication buffer without SDS (50 mM HEPES pH 7.9; 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, Roche PIC) to a final 0.1% SDS concentration. Lysate was separated into 1.5 mL tubes and spun at 14000rpm for 15 minutes at 4°C. The cleared supernatant (sheared DNA) was combined in a 10 mL polypropylene tube. DNA concentration was measured with a Qubit-1 Fluorometer and Qubit dsDNA High-Sensitivity assay kit (Q32851). Sonicated λ DNA (0.5 μ g/mL final) and chicken albumin (1 μ g/ μ L final) were added to each sample. Dynabeads Protein A was washed twice with Sonication Buffer 0.1% SDS, and then chromatin was pre-cleared for 2 hours by rotating at 4°C using 10 μ L Dynabeads/mL chromatin. The tubes were placed on a magnet and cleared for 2 minutes; then, the supernatant was transferred to a new 10 mL tube and used immediately for ChIP; the remaining was stored at 4°C. This is the Input DNA.

For each ChIP sample, 1 mL of chromatin was incubated for 3 hours at 4°C with rotation using the pre-bound 10 μ g antibody per 40 μ L Dynabeads. The samples were then placed on a magnet, allowed to clear, and the supernatant was aspirated. This was repeated for the following washes that were each rotated at 4°C for 10 minutes. Two washes with cold Sonication buffer 0.1% SDS. Two washes with cold Wash buffer A (50 mM HEPES pH 7.9; 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS, 1X PIC). Two washes with cold Wash buffer B (20 mM Tris pH 8.0; 1 mM EDTA 250 mM LiCl, 0.5% NP40, 0.5% Na-Deoxycholate, 1X PIC). They were then quickly washed two times with cold TE Buffer (10 mM Tris pH 8.0), where the resuspended beads were transferred to new 1.5 mL tubes to minimize carryover proteins and DNA stuck to previous tube walls. Samples were then eluted with 200 μ L RT Elution Buffer (50 mM Tris pH 8.0; 1 mM EDTA, 1% SDS) and

vortexed for 10 minutes at max speed using a thermomixer set to 65°C. The first elution was transferred to a new 1.5 mL tube, and elution was repeated for a total of 400 µL. To reverse-crosslink the ChIP DNA, NaCl was added for a 200 mM final concentration along with 10 µg/mL final concentration of RNase-A (DNase-free) and incubated overnight at 37°C.

The next day, EDTA (12.5 µL final) and Proteinase-K (62.5 ng/µL final) were added and incubated at 42°C for 2 hours. DNA was then measured again by Qubit. For phenol-chloroform extraction, 400 µL of phenol/chloroform/IAA 25:24:1 pH 8.0 (Fisher cat. BP1752I400) was added to each tube and vortexed at max speed for 30 seconds, then centrifuged at 8000rpm for 10 minutes. The aqueous upper phase was transferred to a new tube, and then 400 µL of chloroform was added. Samples were vortexed at maximum speed for 30 seconds and then centrifuged at 8000rpm for 10 minutes. The upper aqueous phase was transferred to a new tube, and glycogen (20 ug/µL final), Na-Acetate pH 5.5 (0.3 M final), and 800 µL ethanol was added, then vortexed for 10 seconds. Samples were incubated overnight at -20°C. The next day, samples were centrifuged at 14,000rpm for 30 minutes at 4°C. White DNA/glycogen pellets were visible at the base of each tube, and the supernatant was carefully removed. Samples were washed with 70% ethanol and centrifuged at 14,000rpm for 30 minutes at 4°C. Ethanol was removed, and pellets were vacuum centrifuged to remove the remaining liquid. Pellets were resuspended with 45 µL of 10 mM Tris pH 7.4 and dissolved for at least 30 minutes at RT. The final DNA concentration was determined by Qubit. An aliquot of ChIP DNA was used for qPCR validation of enrichment, and the rest was sent to the IRCM for library preparation, quality control, and sequencing.

To prepare “Input DNA” for sequencing (an alternative control to IgG), an aliquot of 25 µL of input DNA was diluted into 375 µL of Elution Buffer. These tubes were reverse-

crosslinked and purified in parallel with the ChIP samples and sequenced. For qPCR validation of enrichment, a 1:50 dilution of “Input DNA” was used as a control.

ChIP-seq reads for ALL-SIL were mapped to the human hg38 reference genome using Bowtie2. Properly paired, uniquely mapping reads with MAPQ > 30 were retained. MACS2¹⁴⁸ was used for peak calling using Input DNA as background control and $q=1e-2$. Homer¹⁴⁹ was used for peak overlapping. MEME-ChIP¹⁵⁰ was used for motif analysis centered on peak summits. Heatmaps were generated with DeepTools¹⁵¹.

2.10 Mouse dissection and isolation of cells.

Animals were maintained and experimental protocols were conducted in accordance with and ethically approved by the University of Ottawa Animal Care Committee (see **Appendix**). Transgenic TLX1 mice and littermate control mice were sacrificed to isolate cells inside the thymus, spleen, and bone marrow. Mice were euthanized in a CO₂ chamber, weighed, and disinfected. For cell handling, we learned early on that thymocytes removed from the thymus quickly start dying in warm RPMI-1640, and sequencing experiments are very sensitive to dead cells. We remedied this by using cold media, and isolation was handled on ice. For each mouse, 9x cryotubes and 9x 1.5 mL tubes were prepared, as well as 3x 50 mL tubes with a 40 μ m filter and 20 mL cold media. Spleen, thymus, and leg bones were harvested, and the spleen and thymus were weighed and imaged next to a ruler. The organs were placed in a 6-well plate with 2 mL cold media. The thymus and spleen were crushed between glass coverslips, and released cells were transferred into wells. Bone marrow was recovered by cutting open leg bones on each end and flushing with 1 mL media into wells. Cells were treated with RBC lysis buffer; 1 minute for thymus and bone marrow, and 3 minutes for spleen, and then inactivated with cold media. Cells were then passed through the 40 μ m filter into their

respective 50 mL tubes containing 20 mL cold media. Cells were centrifuged for 5 minutes at 300g and then resuspended in 5-10 mL of media. Total cells were counted for thymus, spleen, and bone marrow. Each organ was aliquoted for flow cytometry. Thymus cells were aliquoted for CUT&Tag, ATAC-seq, RNA-seq, and western blot, and some mice had to be prioritized for HiC as that protocol needs 10-30 million cells for cross-linking. All thymus cells were used for WT and PLK mice as they had significantly fewer cells than LK. Extra cells were frozen in 90% FBS+10% DMSO, cryo-cooled at -80°C, then stored in liquid nitrogen.

2.11 Flow cytometry of mouse thymus, spleen, and bone marrow.

Samples were handled on ice. Aliquoted cells were spun at 1500rpm for 5 minutes, and the media was aspirated. Cells were resuspended in 200 μ L flow cytometry solution (PBS with 2% FBS). For unstained and 6x single-stain controls, 100 μ L of thymus, spleen, and bone marrow cells were combined and diluted to 700 μ L with flow cytometry solution, then distributed into 7 vials. For each vial, 100 μ L of flow cytometry solution and respective antibodies were added. A master mix of conjugated antibodies (Thy1.2, CD8, CD4, CD44, CD25, Live/Dead marker) was prepared in 300 μ L flow cytometry solution, and 100 μ L was added to thymus, spleen, and bone marrow vials. All vials were incubated for 30 minutes at 4°C, then diluted with 1 mL PBS and centrifuged at 1500rpm for 5 minutes. The solution was aspirated with 100 μ L remaining, then 400 μ L of 10% formalin was added. Flow cytometry was run in a BD LSR Fortessa and analyzed with FlowJo. The gating strategy (**Figure 4**) characterizes the sub-populations of thymocytes at each stage in differentiation for each organ.

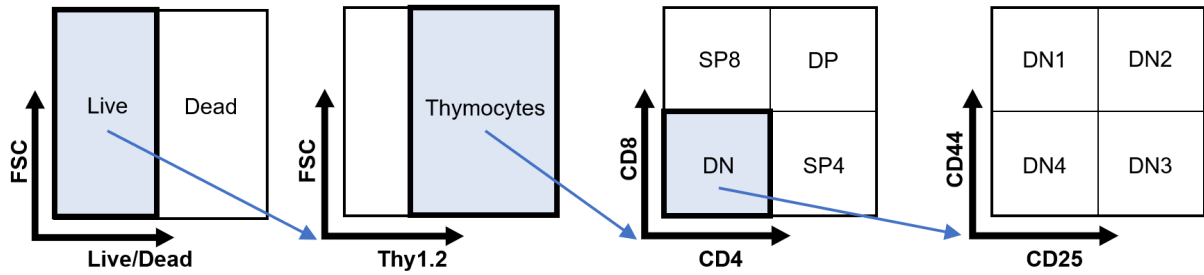


Figure 4. Gating strategy for flow cytometry in thymus, spleen, and bone marrow samples. Cells are first gated for living and dead cells. Thymocytes are then identified with Thy1.2. CD4 vs. CD8 then distinguishes sub-populations of DN, DP, SP4, SP8 thymocytes. The DN sub-population is then gated into DN1, DN2, DN3, DN4 using CD25 and CD44. Blue arrows indicate the population subset for the next gate.

2.12 RNA-seq workflow

2.12.1 RNA-seq samples preparation

Cells were harvested from mice or culture flasks and immediately placed on ice and centrifuged at 500g for 5 minutes at 4°C. Media was promptly aspirated by pipette, and the pellet was immediately resuspended in RNeasy buffer. RNA extraction was performed using the Qiagen RNeasy Mini kit (Qiagen 74104) according to manufacturer instructions. RNA was eluted into 50 µL of nuclease-free water, and RNA concentration was measured by Nanodrop 6000. Samples were sent to IRCM for mRNA enrichment (poly-A capture) and sequencing.

2.12.2 RNA-seq data analysis

Reads were processed by Salmon¹⁵² to generate gene-count tables. Read counts were tallied based on the reference transcriptome obtained from the Gencode database (Mouse M25, Human V39). The counts matrix underwent count filtering by removing genes with CPM less than 0.2 in at least 4 of 19 samples used, then removing rows with fewer than 200 total counts across all samples. Counts were then upper quartile normalized by EDA-seq¹⁵³. RUV-seq¹⁵⁴

was used to calculate covariates using RUVs and $k=2$. Note that conditions WT, PLK-early, PLK-late, and individual LKs were verified to cluster separately before RUVs unwanted variation correction. Conditions were also externally corroborated by CUT&Tag, ATAC-seq, and flow cytometry results. RUVs covariates were used in EdgeR¹⁵⁵ for differential analysis. R code was adapted from these sources^{153–156}. EdgeR *glmTreat* with $lfc=\log_2(1.1)$ and genes with an adjusted p-value < 0.05 were reported as differentially expressed for each condition comparison. Normalized counts were converted to log2 scale for heatmaps and normalized expression graphs. Heatmaps were generated using pheatmap and clustered using K-means. Clustered genes were used for functional analysis using ClueGO¹⁴⁶.

2.13 Bacteria expression and purification of pA-Tn5 for use in CUT&Tag

2.13.1 Protein expression in E.coli

We recently published a STAR Protocols paper where I contributed an optimized protocol which was adapted from the CUT&Tag protocol¹⁵⁷ for the expression of pA-Tn5 transposase in E.coli, column-based protein purification, pre-annealing adapters to the transposase, and activity assay¹⁵⁸. The 3XFlag-pA-Tn5-F1 plasmid (Addgene #124601) was transformed into T7 Express lysY/Iq E. coli (NEB C3013I) and plated onto agar containing ampicillin, then incubated overnight at 37°C. A single colony was transferred to 20 mL of LB Miller broth containing 100 µg/mL ampicillin and incubated for 6 hours at 37°C on an orbital shaker. The 20 mL starter culture was used to inoculate 1 L of LB Miller broth containing 100 µg/mL ampicillin, then split into 5x 200 mL flasks. The flasks were monitored until the OD₆₀₀ reached 0.6, after which the flasks were placed in an ice bath for 30 minutes. A 1 mL aliquot was taken for “pre-induction,” centrifuged at 4000g for 5 minutes, media was aspirated, and the bacteria pellet was resuspended in 2X SDS-loading dye and boiled for 10 minutes.

Expression was induced with 500 μ M IPTG and incubated overnight for 26 hours. The OD₆₀₀ was measured, and an aliquot was taken for “post-induction,” which was calculated to have the same size pellet as the “pre-induction” based on OD₆₀₀. The aliquot was centrifuged at 4000g for 5 minutes, the media was aspirated, and the bacteria pellet was resuspended in 2X SDS-loading dye and boiled for 10 minutes. The flasks were combined into 2x 500 mL centrifuge tubes and centrifuged at 5000g for 20 minutes at 4°C. Media was aspirated, and then the bacteria pellets were frozen and thawed twice, with 2 minutes in a dry-ice ethanol bath and then 10 minutes in an RT water bath. Pellets were stored at -20°C until purification, and pA-Tn5 expression was verified by Coomassie gel staining.

2.13.2 Protein purification

For transposase purification, each bacteria pellet (from 500 mL media) was thawed and resuspended in 40 mL HEGX Buffer (20 mM HEPES-KOH pH 7.2, 800 μ M NaCl, 10% glycerol, 0.2% Triton X-100, 1 mM EDTA, and 1 Roche Complete EDTA-free protease inhibitor tablet per 50mL). Lysozyme (0.5 mg/mL final) was added and incubated on ice for 30 minutes to break down the cell wall. The lysates were then sonicated in ice water using a Sonic Dismembrator Model 500 for 13 cycles of 45 seconds at a 50% duty cycle, allowing for 1-2 minutes to cool down the lysates. Lysates were then centrifuged at 31,000g at 4°C for 30 minutes using an Avanti centrifuge and JA25.50 rotor. During centrifugation, chitin columns were prepared using 4x 30 mL disposable columns (Bio-Rad 7321010) with 2.5 mL chitin slurry resin (NEB S6651S) in each. Each column was washed with 30 mL dH₂O, then 20 mL HEGX buffer, and drained to the top of the resin bed. After centrifugation, an “SN” aliquot of 100 μ L was retained for SDS-PAGE gel, and 20 mL of the supernatant was distributed into each of the 4 columns. Columns were sealed and rotated overnight at 4°C. The lysate pellet

was resuspended in 10 mL HEGX, and 100 μ L was retained as “pellet” for SDS-PAGE gel. The next day, columns were allowed to settle, and the unbound flow-through was collected, with 100 μ L being retained as “FT” for SDS-PAGE. Columns were washed once with 20 mL HEGX buffer and drained to the top of the resin bed ($\sim$ 1mL). The remaining HEGX buffer above the resin bed was exchanged by adding 1 mL Chitin Elution buffer (HEGX buffer with 100 mM DTT) and draining to the top of the resin bed. The resin from all 4 columns was combined by adding 6 mL Chitin Elution buffer to the first column, resuspending the resin, then transferring the contents to the next column; and this was repeated until all the resin was collected, then transferred to a 15 mL conical tube. The slurry was rotated at 4°C for 48 hours, then transferred to a clean 30 mL disposable column and allowed to settle. The first elution fraction was drained to the top of the column bed, and 50 μ L was retained as “EL1”. Chitin Elution buffer (5 mL) was added slowly and drained to the top of the column bed as the second fraction, and 50 μ L was retained as “EL2”. EL1 was transferred to 2x 10,000 MWCO Slide-a-Lyzer™ 3 mL dialysis cassettes and dialyzed in 800 mL cold Dialysis buffer (100 mM HEPES-KOH pH 7.2, 200 mM NaCl, 20% glycerol, 0.2% Triton X-100, 0.2 mM EDTA, 2 mM DTT) for 2 hours at 4°C on a magnetic stirrer. The dialysis buffer was replaced and dialyzed overnight at 4°C on a magnetic stirrer. EL1 eluate was combined from the cassettes and stored at 4°C.

Protein concentration was measured with parallel BSA standard by Coomassie gel stain. If the pA-Tn5 concentration was above 826 μ g/ μ L (11 μ M), then the eluate was diluted using Dialysis buffer. If the concentration was below the target concentration, then it was concentrated using an Amicon Ultra-4 Centrifugal 30 K filter tube (Millipore UFC803024) at 4000g at 4°C and corrected to the target concentration. After correcting the eluate concentration, an equivalent volume of 80% glycerol was added, so the final pA-Tn5

concentration was 5.5 μM in a 50% glycerol buffer. A 50 μL aliquot was retained for the activity assay (without MEDS adapters).

2.13.3 Pre-annealing MEDS adapters

To prepare ME-A and ME-B pre-annealed adapters, lyophilized MED oligonucleotides (ME-A, ME-B, and ME-rev) were each resuspended in annealing buffer (10 mM Tris-HCl pH 8.0; 50 mM NaCl, 1 mM EDTA) to a concentration of 200 μM . Annealed-Pair A was prepared with 50% ME-A + 50% ME-rev (100 μM final), and Annealed-Pair B was prepared with 50% ME-B + 50% ME-rev (100 μM final). These were distributed into PCR tubes (50 μL max). PCR tubes were placed in a SimpliAmp Thermal Cycler (Applied Biosystems) with a heated lid, heated to 95°C for 5 minutes, then cooled over 45 minutes to RT at a slow ramp rate. Annealed-Pair A and B tubes were consolidated in separate 1.5 mL tubes.

2.13.4 Assembling transposome complex and activity validation.

To prepare the transposome complex, 80 μL of Pair A and 80 μL of Pair B were combined first to have a 1:1 ratio. The total 160 μL was then added to 1000 μL of pA-Tn5, briefly vortexed, and then rotated for 1 hour at RT. This is the Tn5 glycerol stock, which has 4.74 μM pA-Tn5 pre-annealed with an equal ratio of ME-A and ME-B for use in CUT&Tag paired-end sequencing. A 25 μL aliquot was retained to verify Tn5 activity, and the Tn5 glycerol stock was stored at -20°C. A transposome activity assay was used to verify that the pA-Tn5 only cuts DNA and ligates adapters in the presence of MgCl_2 (**Figure 5**). This was set up using 40 μL reactions with any large plasmid we had on hand; I used pTRIPz (~13kb). NEB 3.1 was used as it contained 10 mM MgCl_2 and 100 mM NaCl, which is a salt concentration

that allows pA-Tn5 to ligate adapters non-specifically to naked DNA. The following reactions were prepared:

- A. 1ug Plasmid + 1X NEB3.1; no activity without pre-annealed Tn5.
- B. 1ug Plasmid + NEB3.1 and un-annealed Tn5; no activity without adapters.
- C. 1ug Plasmid + dH₂O + pre-annealed Tn5; no activity without MgCl₂.
- D. 1ug Plasmid + NEB3.1 + pre-annealed Tn5; tagmentation expected.
- E. 1ug Plasmid + NEB3.1 + pre-annealed Tn5 (heat inactivated); no activity.

The reactions were then incubated for 15 minutes at 55°C to accelerate the reaction (pA-Tn5 is very thermostable). Afterward, 0.4 µL of 10% SDS was added and incubated for 10 minutes at 55°C to release pA-Tn5 from the DNA. Proteinase-K (0.63 µL of 20 mg/mL) was added to degrade the pA-Tn5 and was incubated for 20 minutes at 55°C. Finally, 6X DNA loading dye was added (1X final), and 250 ng of DNA per reaction was loaded and migrated on a 1.2% agarose gel to assess tagmentation (**Figure 5**).

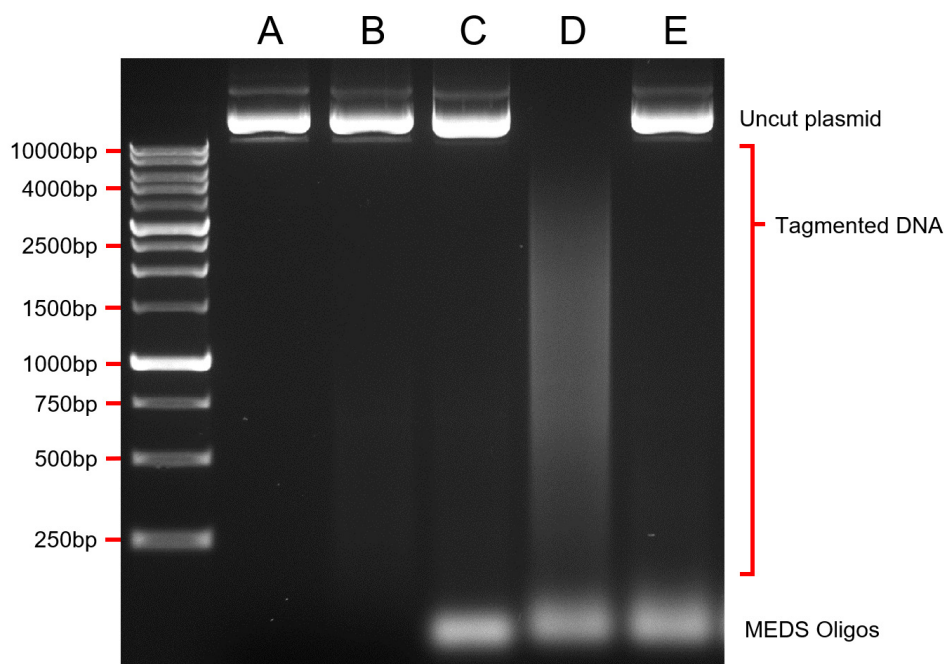


Figure 5. pA-Tn5 activity validation assay. Agarose gel (1.2% w/v) loaded with 250 ng of each reaction (A - E) described above. Migrated at 120 V for 20 minutes. Tn5 activity is evident as a streak of tagmented plasmid DNA.

2.14 CUT&Tag workflow

2.14.1 CUT&Tag experiment

CUT&Tag was performed according to the original protocol¹⁵⁷ with several modifications. Buffers were prepared fresh for each day of the experiment. Cells were harvested and counted, then centrifuged at 600g at RT. Media was aspirated, and cells were resuspended in 1 mL PBS. Formaldehyde (0.1% final) was added under light vortexing, and cells were inverted gently for 2 minutes. Crosslinking was quenched with 1.25 M glycine (6% v/v). Samples were then centrifuged at 800g for 5 minutes at 4°C, then resuspended in Wash buffer (20 mM HEPES-KOH pH 7.5; 150 mM NaCl, 0.5 mM spermidine, 0.1% BSA, 1X PIC). BioMag[®] Plus Concanavalin A magnetic beads (ConA beads) (Bangs Laboratories BP531) were washed with Binding buffer (20 mM HEPES-KOH pH 7.9; 10 mM KCl, 1 mM CaCl₂, 1 mM MnCl₂, 0.1% BSA). Cells were recounted and rotated with ConA beads for 20 minutes at a ratio of 10 µL beads to 125k-175k cells per tube. The bead-bound cells were placed on a magnet stand, and the buffer was replaced with Antibody buffer (20 mM HEPES-KOH pH 7.5; 150 mM NaCl, 0.5 mM spermidine, 0.1% BSA, 0.5% digitonin, 2 mM EDTA), then distributed into Lo-bind Eppendorf tubes with 125k-175k cells per tube. Primary antibodies were added at 1:100 or 1:50 dilution (determined empirically), and tubes were rotated overnight at 4°C. The next day, Antibody buffer was replaced with Dig-wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 0.1% BSA, 0.5% digitonin) containing 1:100 secondary antibody (guineapig- α Rabbit), then rotated at RT for 1 hour. Bead-bound cells were washed 3x with Dig-wash buffer, then rotated for 60 minutes in Dig-300 buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM spermidine, 0.1% BSA, 0.5% digitonin) with 1:250 pre-annealed pA-Tn5. Cells were washed three times with Dig300 buffer, then resuspended in 300 µL Tagmentation buffer (20 mM HEPES pH 7.5, 300 mM

NaCl, 0.5 mM spermidine, 0.1% BSA, 0.5% digitonin), then rotated for 60 minutes at 37°C. Tagmentation was stopped with 10 µL of 0.5 mM EDTA, 3 µL 10% SDS, and 5 µL Proteinase K (10 µg/µL), then incubated overnight at 37°C in a thermomixer at 300rpm. Samples were mixed with 300 µL phenol/chloroform/IAA 25:24:1 pH 8.0 (Fisher cat. BP17521400), pulse vortexed at max for 15 seconds, then transferred to 1.5 mL MaXtract High-Density Tubes (Qiagen Cat.129046). Tubes were spun at 16,000g for 7 minutes, then 300 µL chloroform was added to the aqueous phase, mixed by inversion, and then spun at 16,000g for 7 minutes. The aqueous phase was transferred to 1.5 mL DNA LoBind® Tubes (Eppendorf Cat. 022431021), then 750 µL EtOH (anh.) and 30 µL 3M Na-Acetate pH 5.2 were added and chilled at -20°C for 1 hour. DNA was pelleted at max speed for 1 hour at 4°C, washed once with anh-EtOH, centrifuged for 20 minutes at 4°C, and then the EtOH was poured off. The pellet was dried for 10 minutes in a vacuum centrifuge (Eppendorf 5301 Vacufuge Concentrator). The DNA pellet was solubilized in 27 µL of 1 mM Tris pH 8.0 overnight at 4°C.

Library cycles were determined by test amplification using a QuantStudio-3 (Applied Biosystems). A master mix of 200 nM i5 index primer and 200 nM i7 index primer in 1X PowerUp™ SYBR™ Green Master Mix (A25778) was prepared and distributed in a 96-well PCR plate (23.69 µL per well), and 1.31 µL of sample was added to each well. Conditions for qPCR: (1) 50°C for 5 min, (2) 72°C for 5 min, (3) 98°C for 1 min, (4) then 40 cycles of; 98°C for 10 sec, 63°C for 10 sec, (5) then final extension at 72°C for 1 min, (6) 98°C for 30 sec, (7) then melt curve from 60°C to 98°C. Library amplification cycles were determined by subtracting 3 cycles near the Ct.

For library amplification, 21 µL of the sample was combined with 2 µL i5 index (400 nM), 2 µL of i7 index (400 nM), and 25 µL NEBNext® High-Fidelity 2X PCR Master Mix (NEB M0541L). PCR amplification in a SimpliAmp with a heated lid was carried out with

these steps: (1) 72°C for 5 min, (2) 98°C for 30 sec, (3) then cycles of; 98°C for 10 sec, 63°C for 10 sec, (4) then final extension at 72°C for 1 min. DNA concentration was measured with a Qubit-1 Fluorometer and Qubit dsDNA High-Sensitivity Assay Kit (Q32851). Downstream sample processing (size selection, bioanalyzer, etc.) was conducted by the IRCM and sequenced using Illumina NextSeq 500.

2.14.2 CUT&Tag data analysis

Sequenced data was processed as described in Kaya-Okur 2019¹⁵⁷ with minor modifications. Reads were trimmed using CutAdapt¹⁵⁹ against Nextera transposase adapters with parameters: *-m 25 -e 0 --no-indels*. Reads were aligned to mm10 or hg38 and E.coli-K12 using Bowtie2 as recommended¹⁵⁷. Picard was used to mark and remove duplicates, mitochondrial reads were removed, and properly paired reads with MAPQ > 30 were retained for downstream analysis. TLX1, BRG1, CTCF, RAD21 Peaks were called using MACS2 with parameters: *-f BAMPE -gsize mm10/hg38 --nolambda --nomodel --keep-dup all*. Sharp histones were called using with parameters: *-f BAMPE --gsize mm10/hg38 --nolambda --nomodel --keep-dup all --max-gap 1000*. Broad histones were called using with parameters: *-f BAMPE --gsize mm10/hg38 --broad --nolambda --nomodel --keep-dup all*. The MACS2 “*--qval*” and “*--bval*” were adjusted per sample to account for signal-to-noise variability. MEME-ChIP¹⁵⁰ was also used for motif analysis centered on peak summits. Poor-quality samples were removed before differential analysis due to very low read counts or poor signal-to-noise. DiffBind^{160,161} differential analysis using DESEQ2¹⁶² and RLE reads-in-peaks normalization was found to work best with our CUT&Tag data. Regions that had an adjusted p-value < 0.05 were reported as differentially enriched. Homer was used for overlap analysis and visualized using UpsetR¹⁶³. ChipPeakAnno¹⁶⁴ was used for genome annotations. Bigwigs for track visualization were

created using deepTools¹⁵¹ with CPM normalization for ALL-SIL and Patient T-ALL, or scaling factors calculated by DiffBind for mouse data¹⁶⁰. Average signal bigwigs were made using WiggleTools¹⁶⁵ for visualization in figures and heatmaps. K-means clustered heatmaps were generated with deepTools¹⁵¹. Bigwigs were visualized using IGV¹⁶⁶.

2.15 ATAC-seq workflow

2.15.1 ATAC-seq sample preparation

Crosslinked cells from the CUT&Tag protocol (0.1% formaldehyde for 2 minutes) were also used for ATAC-seq on the same day using a protocol developed by the IRCM. We found that ATAC-seq with lightly crosslinked cells had superior signal-to-noise than non-crosslinked cells. This protocol used the Illumina Tagment DNA Enzyme and Buffer Small Kit (20034197), followed by DNA column purification using Zymo DNA Clean & Concentrator™-5 kit (R1013) according to manufacturer instructions.

Briefly, 50,000 cells were centrifuged at 800g for 5 minutes at 4°C. Wash buffer was aspirated, and cells were resuspended in Lysis buffer (10 mM Tris pH7.4; 10 mM NaCl, 3 mM MgCl₂, 0.1% NP-40). Nuclei were spun at 500g for 10 minutes at 4°C, and the supernatant was removed. Nuclei were resuspended in 25 µL Transposase buffer (12.5 µL 2X TD buffer, 5 µL Transposase TDE1, 7.5 µL nuclease-free dH₂O) and incubated for 30 minutes at 37°C. Following incubation, 180 µL of DNA Binding buffer was added to the 25 µL sample, washed according to kit instructions, and then eluted with 16.5 µL Elution buffer. Downstream sample processing (index adapter ligation, PCR amplification, fragment size selection, bioanalyzer) was conducted by the IRCM and sequenced using Illumina NextSeq 500.

2.15.2 ATAC-seq data analysis

Reads were processed similarly to CUT&Tag as they both use Tn5 transposase tagmentation to generate DNA fragments. MACS2 was used for peak calling using default settings and *--local 50000*. Downstream analysis was performed in the same way as CUT&Tag.

2.16 HiC workflow

2.16.1 Cell crosslinking for HiC

Between 10-30 million cells were centrifuged at 500g for 5 minutes. Media was aspirated, and cells were resuspended in 10 mL PBS and immediately crosslinked using 1% formaldehyde for 10 minutes with rotation. Crosslinking was quenched with 200 mM glycine and incubated for 5 minutes at RT, then on ice for 15 minutes. Cells were centrifuged at 800g for 5 minutes at 4°C, PBS was aspirated, and cells were resuspended with 5 mL cold PBS with 0.1% BSA. Cells were counted and distributed into 1.5 mL tubes at 1×10^6 cells per tube. Tubes were centrifuged at 800g for 5 minutes at 4°C, and the supernatant was removed. Tubes were snap-frozen in liquid nitrogen and stored at -80°C until Arima HiC.

2.16.2 Arima HiC+ experiment

The Arima HiC+ kit (Arima Genomics A410031) was used for HiC library preparation. First, 0.5M crosslinked cells were lysed, and the DNA yield per 1M cell was estimated. Approximately 3.5 ug of total DNA was used for input. The cross-linked cell pellet was treated with lysis buffer and incubated for 15 minutes at 4°C. The extracted DNA from lysed cells was digested with the restriction enzyme cocktail included in the kit for 30 minutes at 37°C and then incubated for 20 minutes at 65°C. The 5'-overhangs were end-repaired and labeled

with biotinylated nucleotides. The digested end of DNA was treated with ligase and incubated at 25°C for 15 minutes. The ligated DNA was purified with DNA purification beads (AMPure XP Beads, Beckman Coulter), and the DNA concentration was measured by Qubit. Purified DNA (1 µg) per sample was sheared by sonication using a Bioruptor with default parameters (30 seconds ON, 30 seconds OFF pulse intervals) to obtain an average 400 bp fragment length. Fragmented DNA was size-selected to have a size distribution between 200-600 bp. After the enrichment of biotinylated DNA, library preparation was performed with the KAPA Hyper Prep Kit (Roche KK8504). Final libraries were checked by BioAnalyzer (Agilent) and submitted for paired-end sequencing of 100 bp length on an Illumina Novaseq.

2.16.3 HiC data analysis

Reads were trimmed using CutAdapt against Illumina Truseq adapters with parameters: `-e 0 --no-indels -m 40 -u 5 -U 5`. Trimmed reads were aligned to mm10 or hg38 using BWA with parameters: `-v 2 -T0 -SP5M`, and *bam* files were generated using SAMtools¹⁶⁷ with parameters: `-m 3G -h -b -S`. Pairtools was used to parse ligations, then duplicated ligations were marked and removed. Unique and rescued ligations were retained as a *pairs* file. Regions with low mappability or high background were removed. Retained reads were then converted to pre-cooler or pre-juicer formatted files.

Juicer¹⁶⁸ *pre* was used to make *hic* files with canonical chromosomes excluding chrY and chrM and reads with MAPQ > 10 with parameters: `-r 5000, 10000, 20000, 25000, 40000, 50000, 100000, 250000, 500000, 1000000, 2500000, 5000000, 10000000 -q 10 -k VC, VC_SQRT, KR, SCALE`. Juicebox¹⁰¹ and GENOVA¹⁶⁹ were used for visualization. Juicer *hic* files were used for calling and differential analysis of AB compartments, loops, TADs, and visualization. Loops were called at 5kb using Mustache¹⁷⁰ with parameters: `--r 5000 -d`

2000000 --pThreshold 0.1 --sparsityThreshold 0.80 --normalization SCALE, and mustache-diff was used for differential looping with an FDR of 1e-4. OnTAD¹⁷¹ was used to call TADs and sub-TADs with parameters: “10000 -hic_norm NONE -penalty 0.20 -ldiff 1.96 -maxsz 200 -sz 3 -lsize 5”. DiffDomain¹⁷² was used at 25kb resolution with otherwise default settings. Differential AB compartment analysis was performed with dcHiC¹⁷³ at 50kb resolution and otherwise default settings.

Cooler *cool* files were made using reads with MAPQ > 10, and cooler matrix *mcool* files were balanced with parameters: “--balance --balance-args '--tol 0.00001 --nproc 20 --ignore-diags 0 --mad-max 10 --min-nnz 1000'”, with the same resolutions as the HIC files. The *mcool* files were used for SV analysis using EagleC¹⁰⁵ and NeoLoopFinder¹⁰⁴. Note: blacklisted regions were not removed prior to this analysis but were checked afterward and omitted if they were identified as false positives.

2.17 BRG1 protein degradation assay using ACBI-1

ALL-SIL cells were seeded on Day 0 in a 24-well plate in 1 mL media. Seeding was calculated to have enough cells for RIPA buffer extraction for subsequent days. On Day 0, each well had 200 nM of ACBI-1 added, and T0 cells were harvested. For subsequent days 1 to 7, cell concentration and media volume were recorded, and cells were harvested. Harvested cells were centrifuged at 600g for 5 minutes at 4°C, washed once with cold PBS+PIC, and centrifuged at 800g for 5 minutes at 4°C. The supernatant was aspirated, and the pellet was frozen at -20°C. RIPA buffer extraction of pellets was done together. Western blot lanes were loaded with equal total protein. Western blot images were incubated with BRG1, TLX1, and TFIIB antibodies.

2.18 Cell-Titer Glo cell proliferation drug assay

The drug assay was performed in a 384-well plate, where wells containing cells in 50 μ L media were centered, and a 3-well border of dH₂O with 1% Pen-Strep was added to stop evaporation. On Day 0, ALL-SIL cells were seeded at 12,500 cells per well, which was determined empirically beforehand to not over-grow during the assay. Plates were incubated for 24 hours, and drugs ACBI-1 (MedChemExpress HY-128359) and BRG1/BRM Inhibitor-1 (MedChemExpress HY-119374) were added on Day 1 in 3-fold serial dilutions (0.5 nM to 10 μ M). Plates were incubated for the next 7 days in an incubator designated not to be opened for that time to minimize evaporation (cells were checked briefly on Day 5 under a microscope).

CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega G7571) was used according to manufacturer instructions. Briefly, assay reagents were warmed to RT in a water bath for 30 minutes, and plates were removed from the incubator for 30 minutes on Day 8. Assay reagents were combined into a master mix, and 50 μ L of the master mix was added to each well. Plates were incubated for 10 minutes and then centrifuged at 100g for 2 minutes to remove any bubbles. Luminance was measured using a SpectraMax M at a range of gain settings, ensuring media blank and max signal were within the dynamic range. GraphPad was used for statistical analysis. Outliers were removed using ROUT < 5%, and then absolute IC₅₀ was calculated using the average media blank signal as 0% and average max concentration signal as 100% for each drug.

Chapter 3 - Results

3.1 Optimizing immunoprecipitation with TLX antibodies.

We first had to find an efficient TLX1 antibody for immunoprecipitation experiments, as the antibody used in previous TLX1 studies (Santa Cruz sc-880) was discontinued. What we had remaining would not be enough for future experiments, so we had to find a replacement. I tested several antibodies for TLX1 and TLX3 by western blot first, as I was studying both T-ALL subtypes at the time. I tested several antibodies with nuclear extracts from ALL-SIL (TLX1+), DND41 (TLX3+), and Jurkat (TAL1+) T-ALL cell lines.

ALL-SIL is a cell line derived from a 17yo male with relapsed T-ALL. These cells have a TLX1-TCR δ translocation and express full-length TLX1. DND-41 is a cell line derived from a 13-year-old male with T-ALL and expresses full-length TLX3 due to its translocation to the highly active BCL11B locus⁵⁷. Jurkat is a cell line derived from a 14-year-old male with relapsed T-ALL that ectopically expresses TAL1 and was used here as a negative control as it did not express TLX1 or TLX3. These transcription factors are specific to each cell line, with no expression in the others.

TLX1 antibodies ab78498 and NB100-56424SS worked similarly to the discontinued sc-880 (**Figure 6A**). I then optimized antibody crosslinking to Dynabeads and TLX1 IPs. I found that using ab78498 and NB100-56424SS together during co-IP efficiently bound TLX1, as seen in the western blot IP verification for the mass spectrometry sample (**Figure 7B**). Notably, TLX3 17454-1-AP enriched both TLX3 in DND-41 and TLX1 in ALL-SIL by western blot (**Figure 6B**). This made sense as TLX3 17454-1-AP is a polyclonal rabbit antibody targeting full-length TLX3, which shares 56% homology with TLX1. At the time, I proceeded with mass spectrometry using the two TLX1 antibodies. I later found it more effective to use TLX3 17454-1-AP in ALL-SIL nuclear extract for western blot cofactor validation (**Figure 8A**), as it worked efficiently for TLX1 IPs. Using this antibody was

appropriate in ALL-SIL nuclear extracts because TLX2 and TLX3 were not confounding variables. I did a similar antibody test when optimizing sets of antibodies for CUT&Tag, and TLX3 17454-1-AP worked effectively for TLX1 CUT&Tag as well.

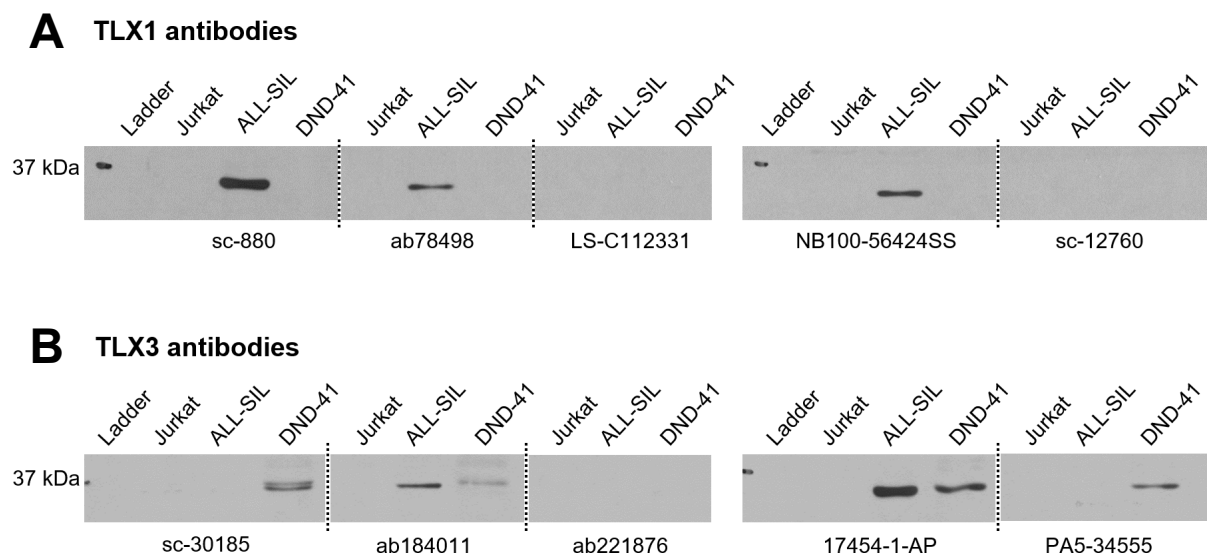


Figure 6. Testing specificity of TLX1 and TLX3 antibodies in ALL-SIL, DND-41, and Jurkat nuclear extracts by western blot. A) Nuclear extracts incubated with TLX1 antibodies. B) Nuclear extracts incubated with TLX3 antibodies. Each lane was loaded with 40ug of nuclear extract for Jurkat, ALL-SIL, or DND-41. After western blot migration and transfer, blots were cut and incubated with their respective antibodies and then re-aligned for imaging. Dotted lines represent membrane cut lines. Optimal exposures per antibody are shown.

3.2 Identifying TLX1 cofactors by immunoprecipitation.

After identifying appropriate antibodies for TLX1 IP, I proceeded with IP mass-spectrometry to identify possible cofactors that may be interacting with TLX1 (**Figure 7A**). This involved western-blot verification of TLX1 pull-down using part of the eluted proteins from the TLX1 and IgG control IPs (**Figure 7B**). The rest of the eluted proteins were separated by SDS-PAGE gel and then silver-stained to verify cofactor co-immunoprecipitation (**Figure 7C**). The silver-stained gel was then cut and sent for mass spectrometry at the OHRI Proteomics Core.

After filtering proteins for known contaminants and higher abundance in IgG, I used ClueGO to identify possible candidate complexes (**Table 1**). Previously identified TFs were identified, like PBX2 and RUNX1 (**Supplemental Table 1**). We found several epigenetic complexes were enriched at lower quantities, like the repressive NuRD and PcG complexes and the H3K4 methylating MLL complexes. Notably, many subunits of the BAF complex were highly enriched in the TLX1 IP. I reasoned that it would be most effective to decide on a candidate complex using both the completeness of the complex and the abundance of spectra for each subunit. Previous research also suggested that ALL-SIL was not sensitive to HDAC inhibition⁶, and changes in H3K4me1 were not driven by TLX1⁵, which is expanded on in the discussion. The completeness of the subunits identified and their high abundance pointed to the BAF complex as the best candidate to pursue further. We also identified CTCF and cohesin, which are involved in chromatin looping. Chromatin looping is partially regulated by DNA accessibility, which involves the BAF complex.

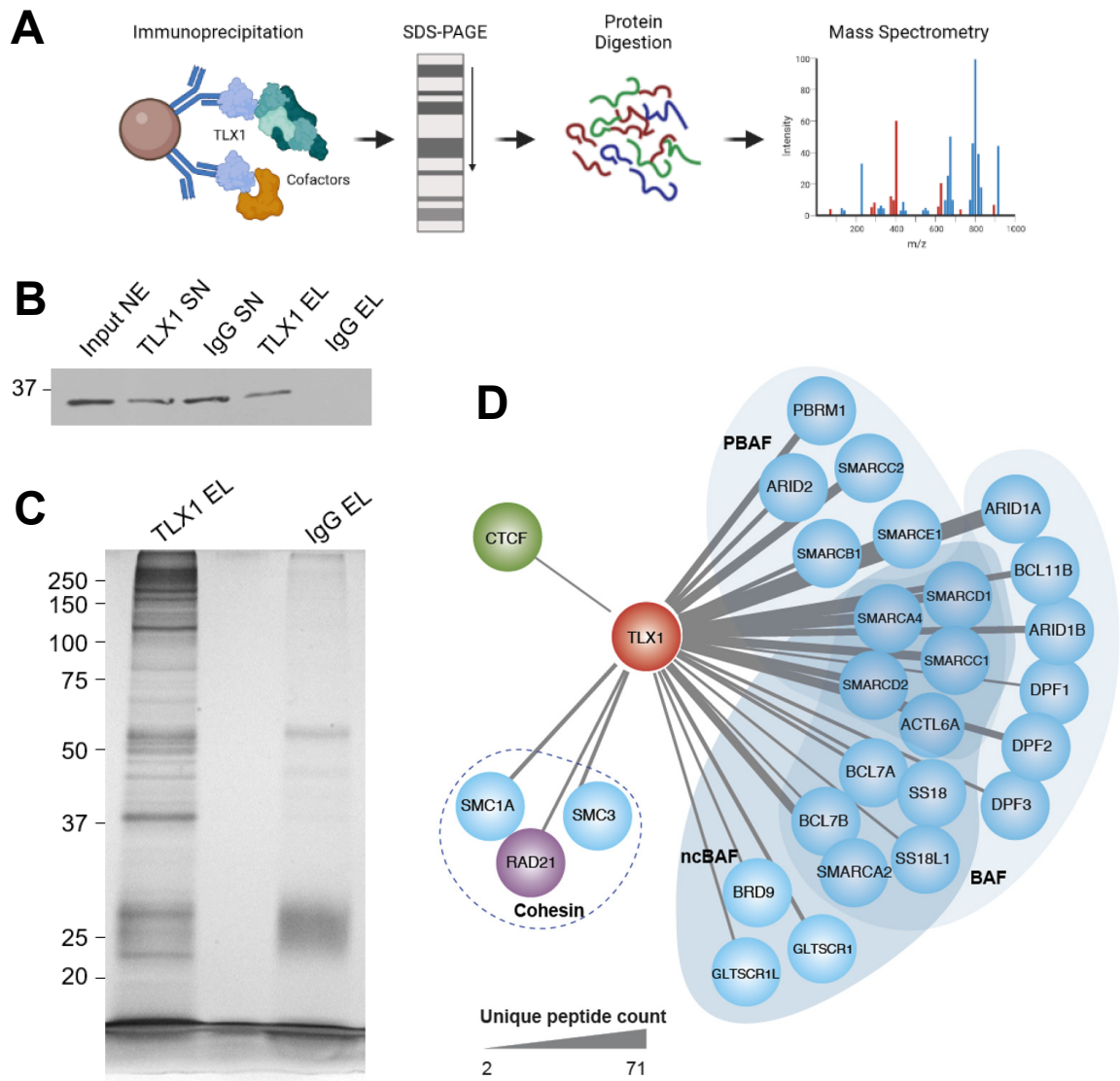


Figure 7. Immunoprecipitation of TLX1 and mass spectrometry analysis identifies candidate cofactors. A) Depiction of workflow, IP proteins are eluted and migrated on SDS-PAGE gel. Gel is silver-stained then processed and analyzed by mass spectrometry. B) Western blot validation for TLX1 immunoprecipitation samples sent for mass spectrometry. C) Silver stain gel image of mass spectrometry samples. D) Summary of proteins of interest, line thickness indicates total unique peptide counts.

Table 1. Significantly enriched complexes identified by ClueGO in TLX1 IP. All proteins found exclusively or with 2-fold higher abundance in the TLX1 IP (910 total) were inputted into ClueGO using the GO_CellularComponent database GO terms. A total of 155 GO terms with an adjusted pValue < 0.05 were output. P-values are corrected with Bonferroni-Hochberg (p<0.05). Terms identifying specific complexes are shown, and BAF complexes are in bold.

Term	Proteins identified	% Associated Proteins	BH corrected p-Value
BAF superfamily-type complex	68	66.0	1.24E-63
MLL1 complex	27	79.4	4.00E-29
SAGA-type complex	28	70.0	1.44E-27
BAF complex	23	74.2	9.11E-24
Sin3 complex	20	76.9	2.48E-21
PcG protein complex	24	49.0	1.11E-18
NuRD complex	16	72.7	1.43E-16
SAGA complex	17	65.4	2.36E-16
nBAF complex	13	76.5	4.39E-14
Ino80 complex	13	76.5	4.39E-14
npBAF complex	12	80.0	1.84E-13
Swr1 complex	11	78.6	2.78E-12
GBAF complex	11	73.3	9.60E-12
transcription factor TFIID complex	17	39.5	1.09E-11
bBAF complex	8	72.7	9.39E-09
NSL complex	8	72.7	9.39E-09
Set1C/COMPASS complex	9	56.3	2.38E-08
PRC1 complex	8	50.0	5.21E-07
MLL3/4 complex	7	50.0	2.81E-06
ISWI-type complex	6	42.9	4.42E-05
SLIK (SAGA-like) complex	3	100.0	1.82E-04
U2AF complex	4	50.0	4.94E-04
NURF complex	4	50.0	4.94E-04
RNA polymerase II, core complex	5	33.3	7.51E-04
cohesin complex	4	25.0	7.99E-03

3.3 Western-blot verifies TLX1 protein-protein interactions with cofactors.

The mass spectrometry data analysis suggested that TLX1 interacted with the BAF complex, as well as CTCF and cohesin. Reciprocal IPs and western blotting are important for mass-spectrometry validation of interactions, as interactions should be identified when immunoprecipitating the opposite protein.

To confirm TLX1-cofactor interactions, I validated binding by western blot from the TLX1 IP and reciprocal IPs for BRG1, CTCF, and RAD21. The TLX1 IP was enriched for BRG1, CTCF, and RAD21, and the reciprocal IPs all verified interactions with TLX1 (**Figure 8A-D**). These data strongly suggest that TLX1 physically interacts with BRG1, CTCF, and RAD21 in nuclear extracts. However, this does not necessarily mean they interact together on the genome. We next focused on genomic colocalization in the TLX1-expressing cell line ALL-SIL to see if the proteomic analysis translated to colocalization on the genome.

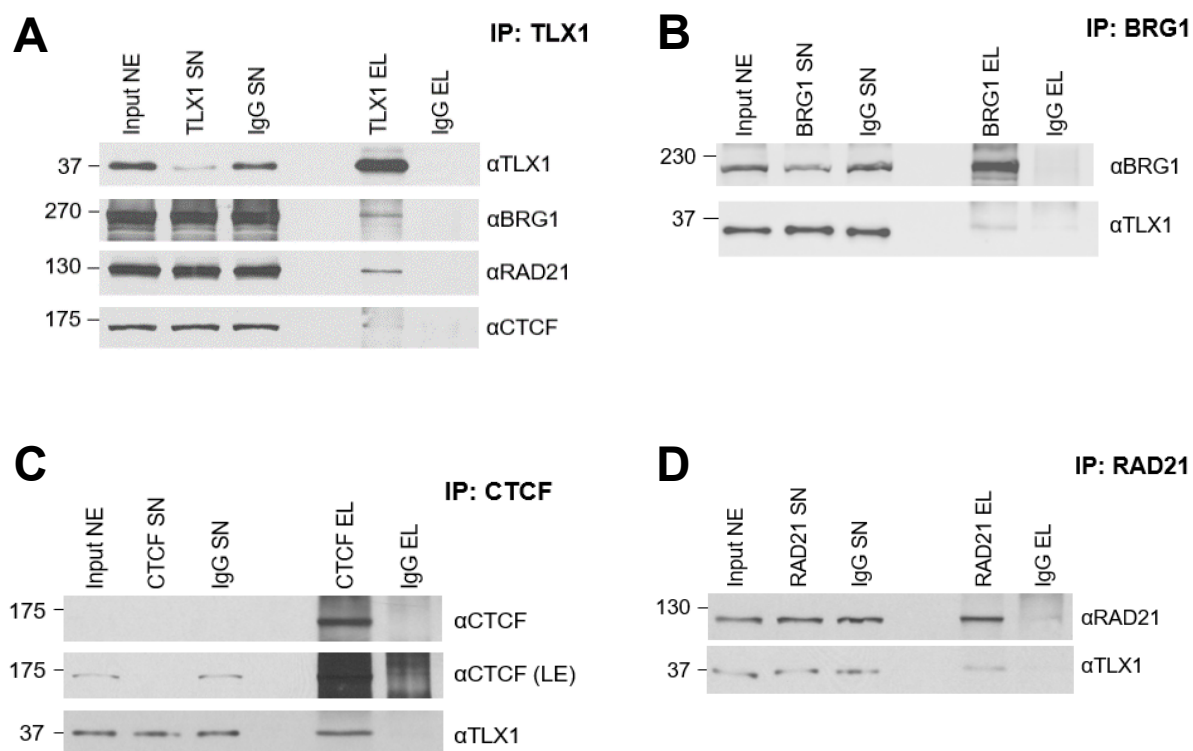


Figure 8. Western blot validation of TLX1 and cofactor interactions. A) TLX1 IP with validation for TLX1, BRG1, RAD21, CTCF. B) BRG1 reciprocal IP with validation of BRG1 and TLX1. C) CTCF reciprocal IP with validation of CTCF and TLX1. LE = Long exposure, which was needed to see depletion of CTCF in “CTCF SN”. D) RAD21 reciprocal IP with validation of RAD21 and TLX1.

3.4 TLX1 colocalizes with CTCF and RAD21 using ChIP-seq.

I wanted to see if TLX1 colocalized on the genome with CTCF, RAD21, and BRG1. I performed ChIP-seq on TLX1 in ALL-SIL and then did a motif analysis to identify which transcription factors likely colocalized with TLX1.

Motif analysis of TLX1 peaks in ALL-SIL identified RUNX1 and ETS1 motifs, which were identified in previous literature⁸, but CTCF was notably the most enriched motif (**Figure 9A**). I then performed ChIP-seq for CTCF, RAD21, and BRG1. The BRG1 ChIP-seq did not work well, as the BAF complex does not have a defined DNA binding motif, and sonication likely sheared it from its interacting sites. CTCF and RAD21 ChIP-seq worked very well. Combined CTCF and RAD21 peaks colocalized at 30% of TLX1 peaks, RAD21 alone colocalized at 24% of TLX1 peaks, while 44% of TLX1 peaks did not overlap with CTCF or RAD21 (**Figure 9B**). CTCF alone only colocalized at 3% of TLX1 peaks.

Taken together, this reinforced the mass-spectrometry data suggesting that TLX1 interacts with both CTCF and RAD21 on the genome. Notably, TLX1 also colocalized to a high degree with RAD21 independent of CTCF, suggesting that TLX1 may be binding in regions that block cohesin loop extrusion independent of CTCF. With this preliminary evidence, we modified the hypothesis and objectives to investigate whether TLX1 establishes and maintains leukemic cells partly through the recruitment of complexes that mediate chromatin loop formation, resulting in the dysregulation of 3D chromatin structures.

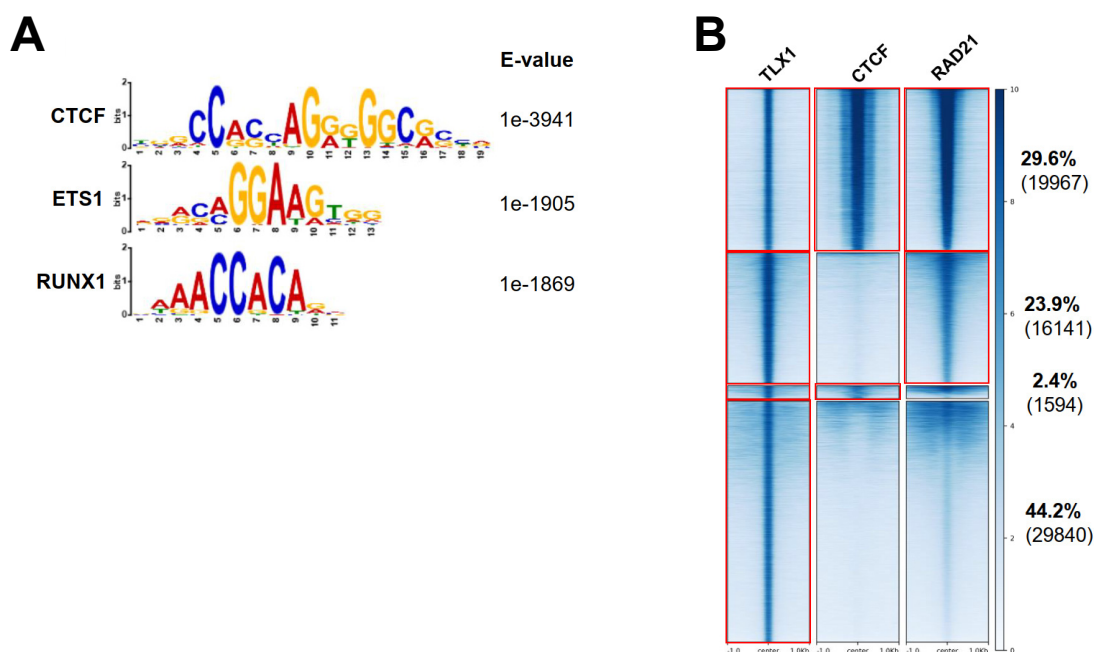


Figure 9. TLX1 binding sites are enriched for CTCF motifs and colocalize strongly with CTCF and cohesin. A) MEME motif analysis of all TLX1 binding sites. B) Peak overlap analysis for TLX1, CTCF, and RAD21. Red outlines show which peak files were used for each cluster. Heatmap is CPM normalized.

3.5 CUT&Tag in ALL-SIL and Patient T-ALL shows strong colocalization of TLX1 and its cofactors at accessible chromatin.

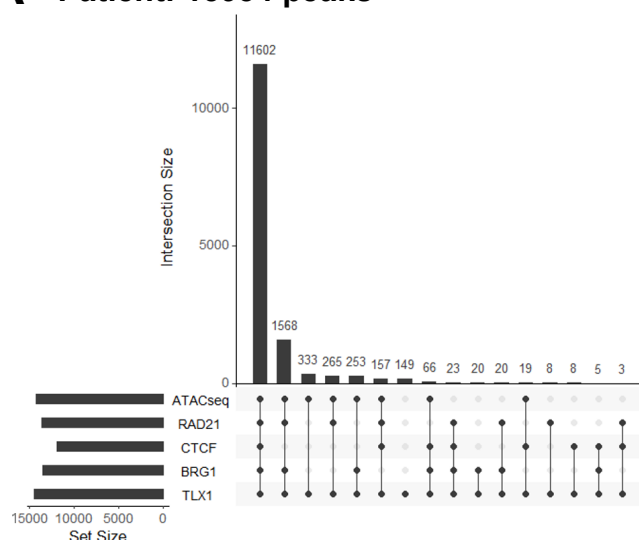
We decided to continue the project with CUT&Tag as it had several advantages over ChIP-seq. CUT&Tag requires far fewer cells per antibody target (50k - 500k), so I could prepare fewer cells and use a larger panel of antibodies. CUT&Tag is performed in situ with cells that are lightly cross-linked and bound to magnetic beads, then incubated with primary and secondary antibodies, and then with pA-Tn5. Excess pA-Tn5 is washed away, so DNA sites enriched for the target are ligated with library adapter sequences. No sonication is necessary, so targets like the BAF complex were effectively interrogated. The signal-to-noise ratio of CUT&Tag is far better than ChIP-seq, so we were able to get similar information with

fewer sequenced reads. We also found that it worked well for both TFs and histone modifications, and the lightly crosslinked cells could be used for ATAC-seq as well.

Therefore, I expressed and purified the pA-Tn5¹⁵⁸ (**Figure 5**), optimized antibodies for CUT&Tag using the ALL-SIL cell line, and made some modifications to the original CUT&Tag protocol¹⁵⁷. We included a second TLX1 T-ALL in the analyses going forward. Patient T-ALL 05H080 was acquired from a 39-year-old male with 99% of blasts expressing TLX1³³. I then characterized the binding of TLX1 with its cofactors and a panel of histones encompassing the main regulatory states, including repressed regions (H3K27me3), promoters (H3K4me3), enhancers (H3K4me1), active states (H3K27ac), and actively transcribed gene bodies (POL2-ser2P).

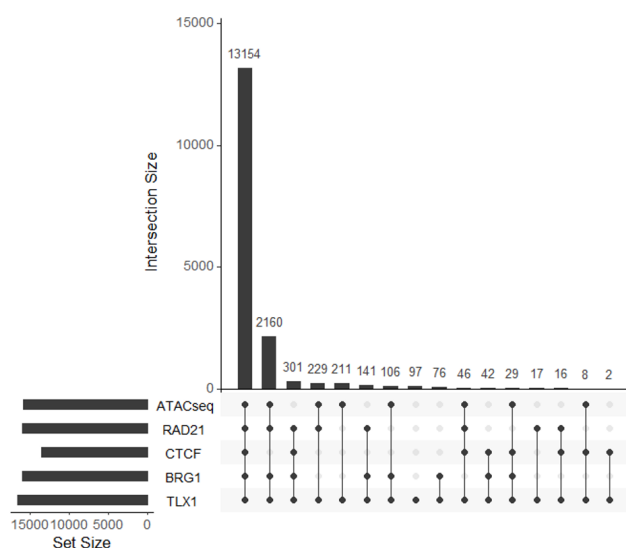
TLX1 peaks in ALL-SIL have very strong colocalization with CTCF, cohesin, and the BAF complex at accessible regions (**Figure 10A**). Colocalization was also consistent in the Patient T-ALL (**Figure 10B**). Notably, motif analysis is consistent with the ChIP-seq data as RUNX and ETS family TF motifs were enriched along with CTCF (**Figure 10A/B**). These CUT&Tag data validated the initial findings of the ChIP-seq data where TLX1 colocalizes with CTCF and RAD21 on the genome. CUT&Tag allowed the characterization of BRG1 as well, which also strongly colocalized with TLX1. Almost all TLX1 peaks were present in accessible chromatin based on ATAC-seq.

A Patient: 15954 peaks



Family	Motif	E-value
ETS		7.2e-282
KLF/SP		7.2e-245
RUNX		1.8e-166
FOX/NFY		9.3e-141
NRF		2.9e-132
CTCF/L		6.4e-121

B ALL-SIL: 19043 peaks



Family	Motif	E-value
ETS		1.5e-334
KLF/SP		5.7e-263
RUNX		1.2e-218
EGR		2.0e-171
NRF		4.3e-155
CTCF/L		9.4e-143

Figure 10. TLX1 overlaps with CTCF, Cohesin, and BAF Complex at accessible chromatin. A) Upset plot of TLX1 peaks and CTCF, RAD21, BRG1, and ATAC-seq in TLX1-expressing patient leukemia (left), and significant motif families at TLX1 peaks (right). B) Upset plot of ALL-SIL TLX1 peaks overlapping with CTCF, RAD21, BRG1, ATAC-seq (left), and significant motif families (right). Upset plots only show cofactor combinations that overlap with TLX1 peaks.

3.6 TLX1 binds similarly at regulatory regions in both T-ALL samples.

TLX1 has been characterized as predominantly binding to promoters and enhancers⁸. I used the CUT&Tag histone data and genome annotations to see if this was consistent in both natively expressing TLX1 T-ALLs.

Genome annotations at TLX1 binding sites in both T-ALLs show that about half of TLX1 peaks are at or near promoters (**Figure 11A/B**). Another quarter appear in introns, and about 12% in distal intergenic regions. Almost all TLX1 binding occurs at poised or active promoters and enhancers, and about half of these regions have TLX1 present (**Figure 11C/E**). K-means clustering of TLX1 peaks showed binding at active promoters (C3/C5) and active enhancers (C4/C6). TLX1 also does not appear to overlap repressed H3K27me3 regions (**Figure 11D/F**).

Taken together, these results agree with previous research showing that TLX1 predominantly binds to enhancers and promoters and does not bind often within repressed regions. It is well understood that most chromatin loop anchors and TAD/sub-TAD boundaries exist near promoters and enhancers as part of their gene regulatory function. To interrogate this aspect, I next analyzed the 3D chromatin organization of ALL-SIL and the patient T-ALL.

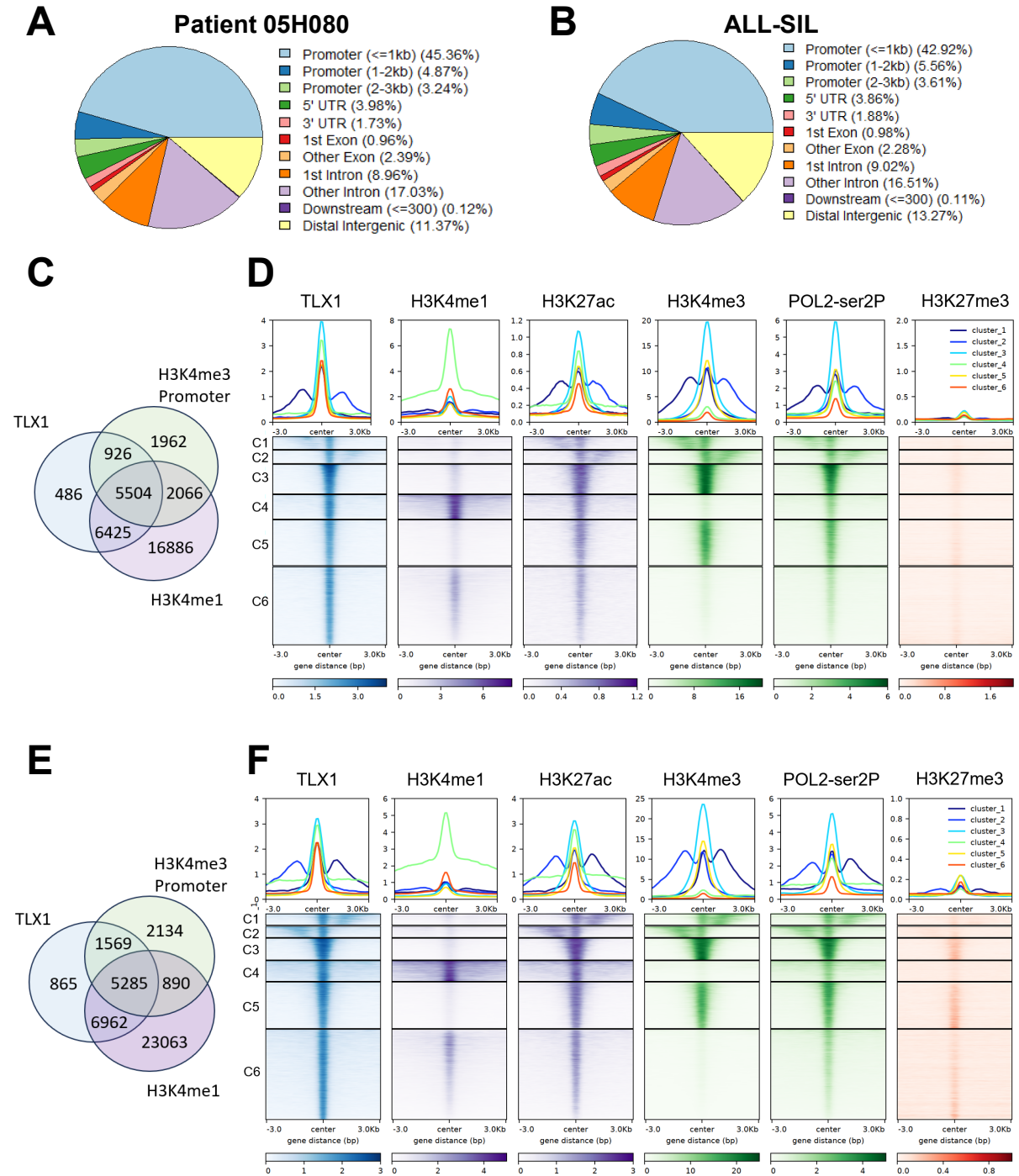


Figure 11. TLX1 overlaps active regulatory regions in both TLX1 leukemias. A) Genome annotations for Patient TLX1 binding sites. B) Genome annotations for ALL-SIL TLX1 binding sites C) Venn diagram of Patient TLX1 at H3K4me3-promoters and H3K4me1 D) Patient T-ALL K-means clustered heatmap at TLX1 sites and histone marks. E) Venn diagram of ALL-SIL TLX1 at H3K4me3-promoters and H3K4me1. F) ALL-SIL K-means clustered heatmap at TLX1 sites and histone marks.

3.7 TLX1 binds at many TAD boundaries and loop anchors with its cofactors.

Chromatin loop anchors and TAD/sub-TAD boundaries exist near promoters and enhancers as part of their involvement in gene regulation. TADs are usually demarcated on both ends by a CTCF-RAD21 site and tend to have one to several genes within them. TADs tend to have several nested sub-TADs, which signifies regions of high promoter-enhancer interactions relative to other sub-TADs and tend to be demarcated by CTCF-RAD21 sites as well (see section 1.7).

I used HiC to characterize ALL-SIL and Patient 05H080 chromatin organization to determine the extent of TLX1 binding at TAD boundaries and loop anchors. OnTAD¹⁷¹ was used to identify TADs and sub-TADs, and Mustache¹⁷⁰ was used to identify chromatin loops (example region Figure 3). For the Patient T-ALL, TLX1 bound near 37% of TAD and sub-TAD boundaries and 39% of loop anchors (Figure 12A). Most of the remaining boundaries and loop anchors overlapped with some combination of CTCF, RAD21, and BRG1. Similarly, TLX1 bound near 41% of TAD and sub-TAD boundaries and 27% of loop anchors in ALL-SIL (Figure 12B).

Taken together, TLX1 consistently colocalized with CTCF, RAD21, and BRG1 at a large proportion of TAD boundaries and loop anchors. However, these CUT&Tag and HiC analyses are correlative at the leukemia stage, which also have additional secondary mutations. These data show that TLX1 binds at regulatory regions relevant to chromatin looping. However, to show whether TLX1 alters chromatin looping during transformation, we used the TLX1 transgenic mouse model to characterize these changes. HiC tools provided additional information regarding structural variants in cancers, which we thought were important to characterize for TLX1 T-ALLs in the next section.

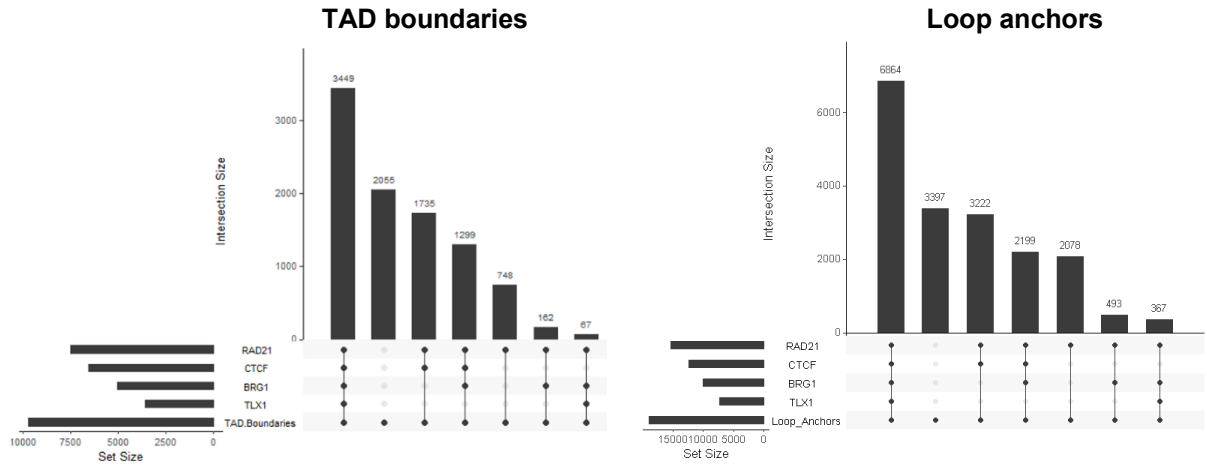
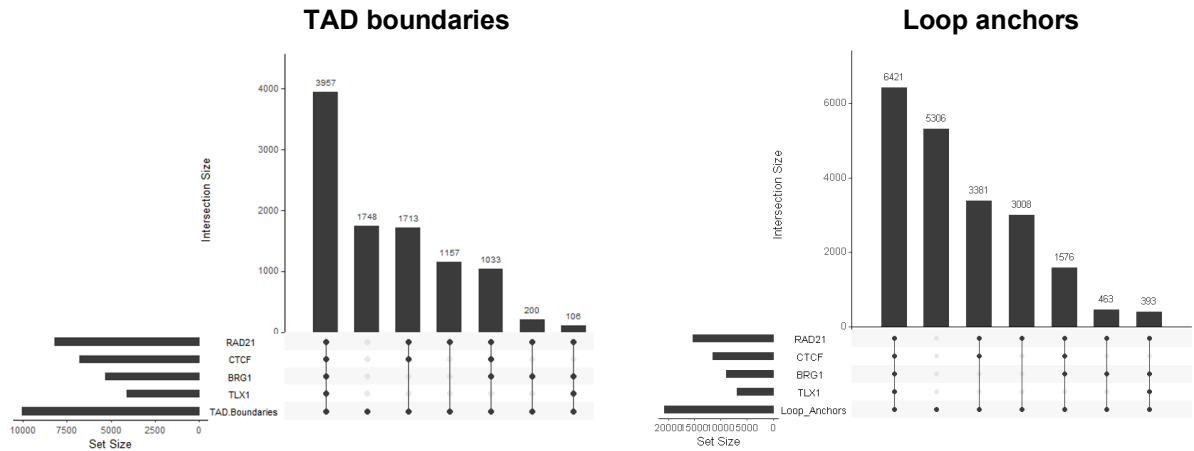
A**Patient****B****ALL-SIL**

Figure 12. TLX1 overlaps cofactors at TAD boundaries and loop anchors. A) Upset plots of TAD and sub-TAD boundaries (left), and loop anchors (right) overlapped with TLX1 and cofactors in Patient sample. B) Upset plots of TAD and sub-TAD boundaries (left), and loop anchors (right) overlapped with TLX1 and cofactors in ALL-SIL. Upset plots are sub-sets for only TAD boundaries or only loop anchors. Upset plots are in descending order and limited to first 7 largest groups for brevity.

3.8 ALL-SIL and Patient T-ALL share the same breakpoint at the TCR δ locus.

TLX1 T-ALLs have been shown to consistently translocate to the TCR δ , TCR β , or TCR α loci as the primary mutation that initiates TLX1 expression and leukemic transformation (**Figure 13A**). Additionally, the long latency of TLX1 T-ALL in mice⁸ suggests that secondary mutations are needed to progress leukemia, and recurring mutations are attributed to driving malignancy¹⁰.

We used EagleC to interrogate possible structural variants (SVs), which include deletions, insertions, inversions, duplications, and translocations. HiC is visualized using a reference genome, but alignment to the genome does not inform how a cancerous genome is necessarily arranged. EagleC scans HiC maps for characteristic patterns that are indicative of SVs¹⁰⁵. NeoLoopFinder, developed by the same lab as EagleC, is built to work in conjunction with EagleC. NeoLoopFinder calculates copy number variation, which is used by EagleC along with the HiC map to identify SVs. The results are then passed back to NeoLoopFinder, which identifies neo-TADs and neo-loops, which result from local regulatory regions and CTCF sites reorienting themselves¹⁰². I used these tools for ALL-SIL, Patient T-ALL, and the mouse HiC data.

As a check for EagleC and NeoLoopFinder, I made sure the translocation event that places TLX1 at TCR δ , TCR β , or TCR α was identified by the programs. Strikingly, the translocation breakpoint on chr14 for both T-ALLs was within 10kb of each other at the TCR δ locus (**Figure 13B/C**), between TRDV2 and TRDV3, suggesting this translocation happens in the DN2b/DN3a stage during TCR δ recombination. On chromosome 10, the breakpoints upstream of TLX1 were 25kb apart. Importantly, the recombination event also formed neo-TADs and neo-loops between the TLX1 gene and TCR δ (**Figure 13B/C**). TLX1 itself appears to be a part of these interactions, signified by the TLX1 & RAD21 peaks at neo-loops.

Additionally, **Figure 13D/E** shows that the reciprocal translocation event also forms neo-loops. This translocation event completely segregated the upstream and downstream ends of the TCR α locus by placing them on separate chromosomes, which may explain the block in differentiation.

The breakpoint upstream of the TLX1 gene in ALL-SIL is also consistent with previous literature that shows upstream sequences form G-quadruplex structures²⁴. The patient T-ALL is farther upstream relative to the ALL-SIL breakpoint, suggesting additional G-quadruplex structures could exist near TLX1. This data shows where TLX1 translocation occurs at the TLX1 and TCR δ locus, and its presence in both ALL-SIL and Patient T-ALL underlines TCR δ recombination as an important mechanism in TLX1 T-ALL development.

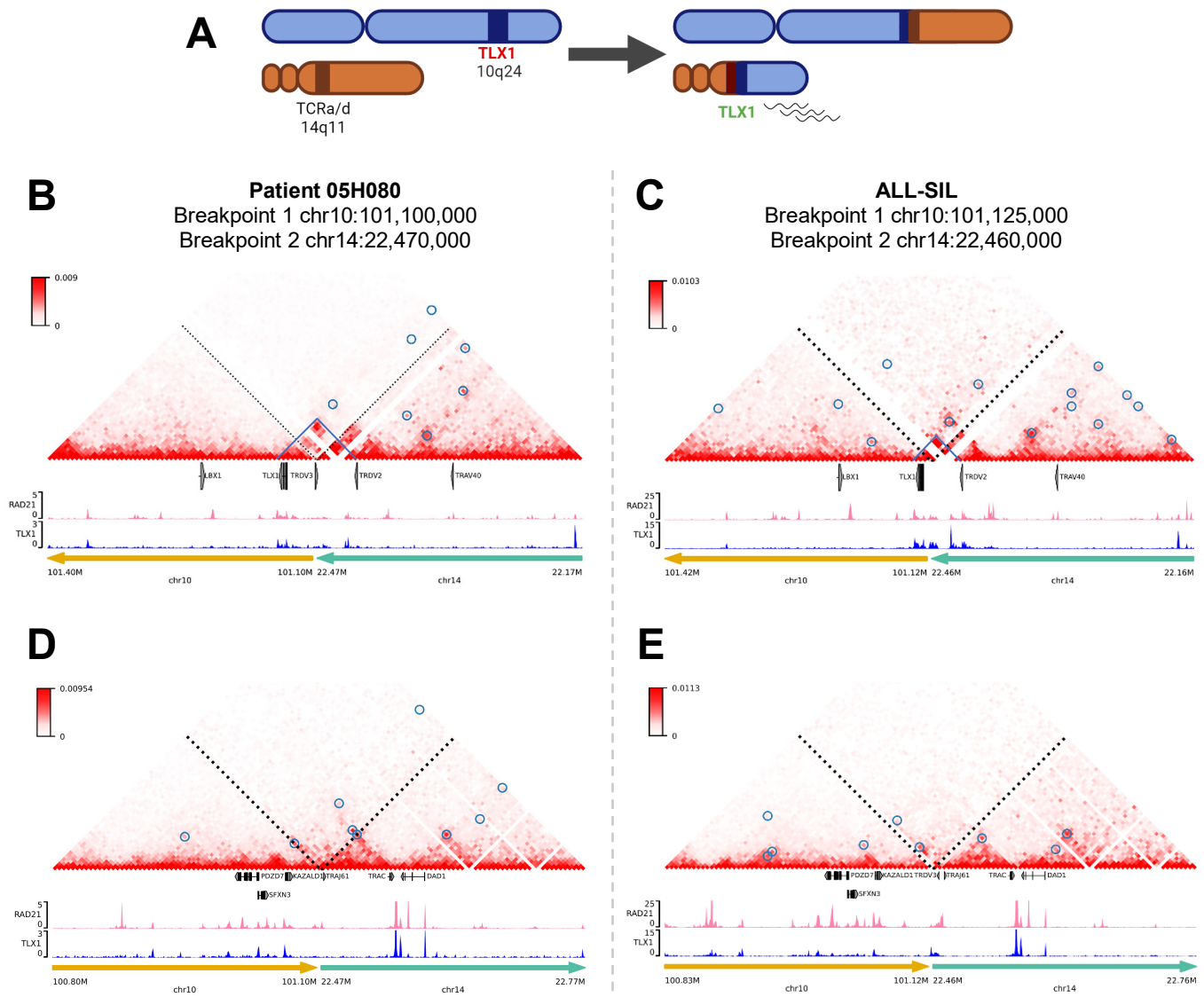


Figure 13. Neo-TAD and neo-loop enhancer hijacking results in *TLX1* expression. A) Diagram of chr10 and chr14 translocation resulting in *TLX1* expression. B) Translocation A results in neo-TADs and neo-Loops forming between *TCRδ* and *TLX1* loci in Patient T-ALL. C) Translocation A results in neo-TADs and neo-Loops forming between *TCRδ* and *TLX1* loci in ALL-SIL. D) Opposite translocation in Patient T-ALL results in *TCRAJ*-*TRAC* interacting with genes upstream of *TLX1*. E) Opposite translocation in ALL-SIL results in *TCRDV3* to *TRAC* interacting with genes upstream of *TLX1*. Blue circles show neo-loops, blue lines show neo-TADs. Black dashed line represents breakpoint.

3.9 TLX1 T-ALLs harbor recurring and unique secondary mutations.

With the TLX1-TCR δ translocation identified by the programs, we looked at other SVs identified by EagleC and NeoLoopFinder. CDKN2A and CDKN2B are considered tumor-suppressing genes that are frequently deleted in T-ALLs as they inhibit G1-S cell cycle progression²³.

Both ALL-SIL and the patient sample had a complete deletion of CDKN2A and CDKN2B, but the region deleted in the patient T-ALL was much larger (**Figure 14**). In both cases, deletion of these cell cycle inhibitors may be an important step in leukemic transformation. Notably, the NUP214-ABL1 mutation was identified correctly in ALL-SIL but did not occur in patient T-ALL, underlining previous research that suggests it is a cooperating mutation but is not necessary for transformation⁵ (**Supplemental Figure 1**). Additional SVs are described in **Supplemental Table 2-5**, which include partial deletions of epigenetic complex subunits (such as MBD3 and DOT1L) in Patient T-ALL, as well as deletion of other cell cycle regulatory genes in ALL-SIL (RB1).

Diverse aneuploidy was also evident in ALL-SIL, Patient T-ALL, and the leukemia mouse (LK3) we did HiC analysis on as well (**Supplemental Figure 2**). Except for chr10 triploidy and evidence of rearranged TCR β and TCR $\alpha\delta$ loci in LK3, no additional SVs were found in the pre-leukemia or LK3 mice that may have contributed to disease development (data not shown).

Taken together, the transformation process of T-ALLs produces varied secondary mutations that present challenges in treatment. Specific cooperating mutations have been studied previously⁵, and these additional mutations could be studied in the future.

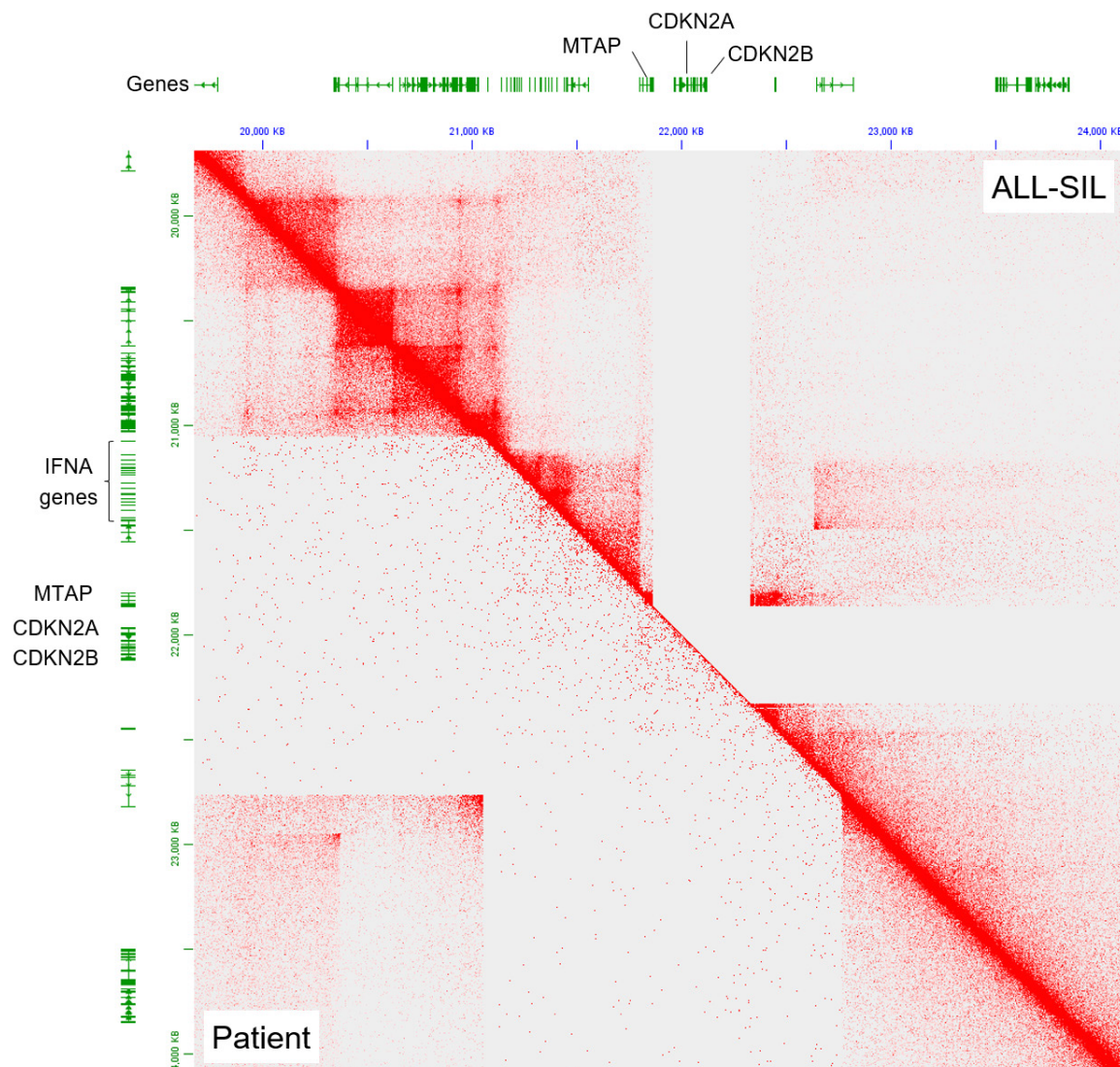


Figure 14. Deletion of CDKN2A and CDKN2B in Patient and ALL-SIL cells. Example region that is deleted in both Patient 05H080 and ALL-SIL. Labelled genes are deleted from respective samples. Visualized using Juicebox, KR normalized, 5kb resolution.

3.10 Transgenic TLX1 mice models T-ALL progression

To study how cell populations and chromatin conformation changes in the thymus during leukemia progression, we harvested thymocytes from transgenic C57BL/6 mice that express p56^{Lck}-TLX1 specifically in T-cell progenitors¹⁰. Here, the TLX1 gene is expressed under the dependence of the p56^{Lck} proximal promoter, which is activated in DN2 cells onwards due to LCK upregulation.

We used a range of time points based on previous studies to characterize leukemia transformation^{8,10}. We defined “early pre-leukemia” (PLK-early) as the early phase of the disease where transformation is likely not driven by secondary mutations and only TLX1 contributes to altered gene expression (8 to 20 weeks). The leukemic phase (LK) was defined when the mouse showed severe signs of tumor burden and had to be harvested (**Figure 15A**). Age-matched littermate wild-type mice are used as the control condition (WT). The mouse thymus, spleen, and bone marrow were harvested, and thymus cells were isolated for RNA-seq, HiC¹⁰¹, flow cytometry, CUT&Tag¹⁷⁴, and ATAC-seq.

Nearly all TLX1+ mice developed leukemia, with the median age of overt leukemia at 50 weeks, with TLX1+ mice developing leukemia as early as ~33 weeks and as late as 65 weeks (**Figure 15B**). TLX1 expression was verified by western blot in mouse thymocytes (**Figure 15C**), consistent with RNA-seq expression (**Figure 17B**). WT and PLK thymus were approximately the same size and weight, while LK mice had an enlarged thymus and spleen (**Figure 15D and Supplemental Table 6**), consistent with the proliferation of leukemic cells and infiltration into other organs.

These data are consistent with previous literature that previously used transgenic mice expressing TLX1 in early thymocytes¹⁰. Median death age is about 50 weeks, with leukemia occurring as early as 33 weeks. Pre-leukemic mouse thymi were the same size as Wild-type.

Leukemic thymi and spleens were enlarged due to the massive expansion of leukemic cells. The cell yields from WT and PLK thymus caused some issues when separating the cells for different experiments. Some experiments like HiC required 10M+ cells, so some mice were prioritized for HiC over other experiments. Taken together, the mouse model recapitulated TLX1 T-ALL development.

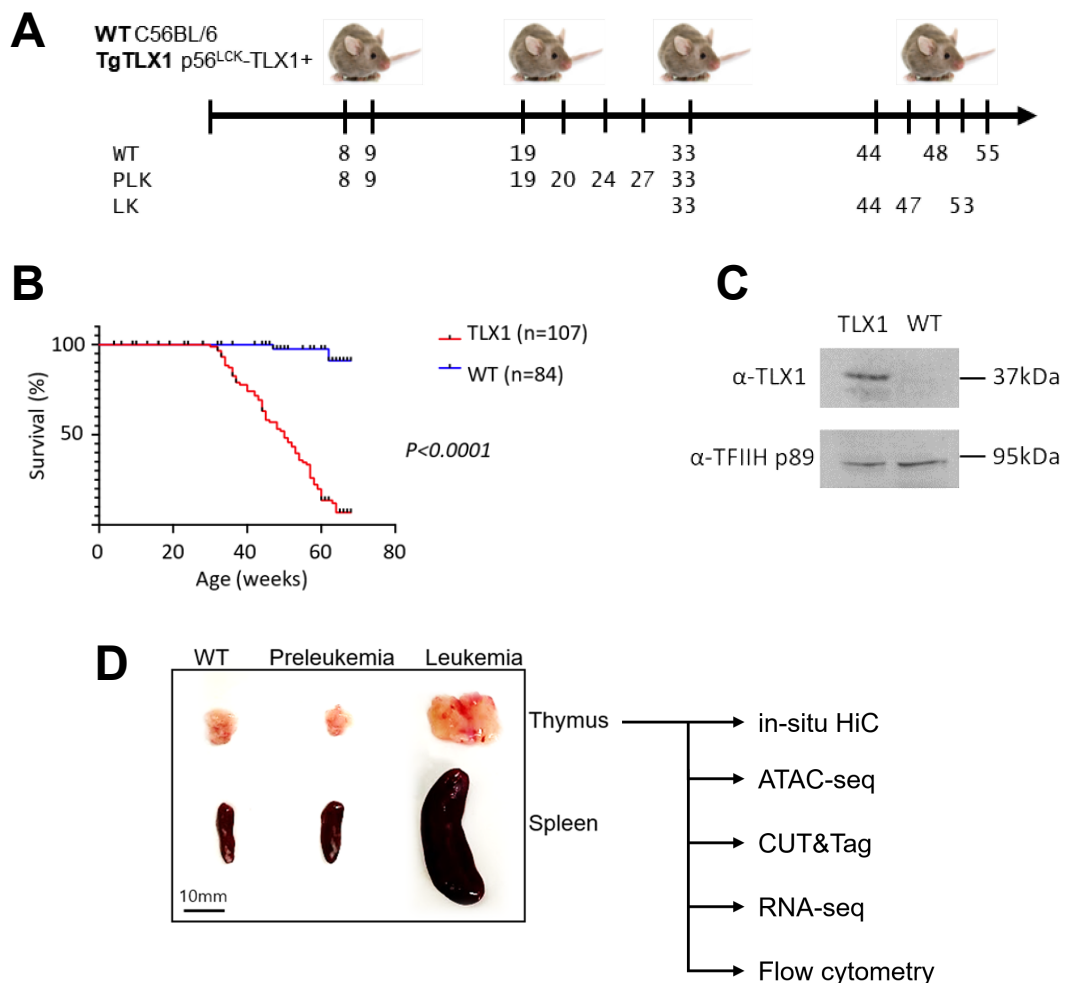


Figure 15. Transgenic mouse model recapitulates T-ALL phenotype. A) Time course of mouse cohort used for sequencing experiments. PLK mice ranged from 8 to 33 weeks. LK mice were sacrificed at signs of tumor burden. WT littermate control mice were sacrificed across entire range. B) Survival curve of transgenic mice. Black hatches signify mice removed from study due to unrelated circumstances. C) Validation of TLX1 expression in mouse thymocytes, normalized to TFIIH-p89. D) Representative images of thymus and spleen from WT, PLK, and LK mice.

3.11 Flow cytometry analysis shows leukemic transformation and diverging leukemias.

Previous literature has shown that TLX1 T-ALL has a characteristic block in DN2¹⁰. Using flow cytometry, we characterized thymocyte populations in the thymus across the stages of transformation to see if this was consistent in the mice as well. **Figure 16** shows representative mice showing the progression of leukemia in the thymus at different time points; additional plots are shown in **Supplemental Figure 3** and **Supplemental Figure 4**. WT and PLK-early had the same distribution of sub-populations, with most thymic cells in the DP state. The sub-populations are unchanged, but the PLK-early cells express a high level of TLX1 (**Figure 15** and **Figure 17B**). While differentiating thymocytes show changes in gene expression at each stage, bulk-cell analysis between WT and PLK-early thymocytes represents changes driven by TLX1 expression, as the sub-populations are unchanged, but TLX1 expression is high in PLK-early (**Figure 17B**). Among the mice that were analyzed further, we saw increased DN2 as a proportion of DN around 20 weeks for PLK6 and PLK2 (**Supplemental Figure 4**). Additionally, flow cytometry also highlights that leukemias tend to metastasize at either the DP or CD4⁺ stage. We did have one instance of leukemia that accumulated at the CD8⁺ stage (data not shown).

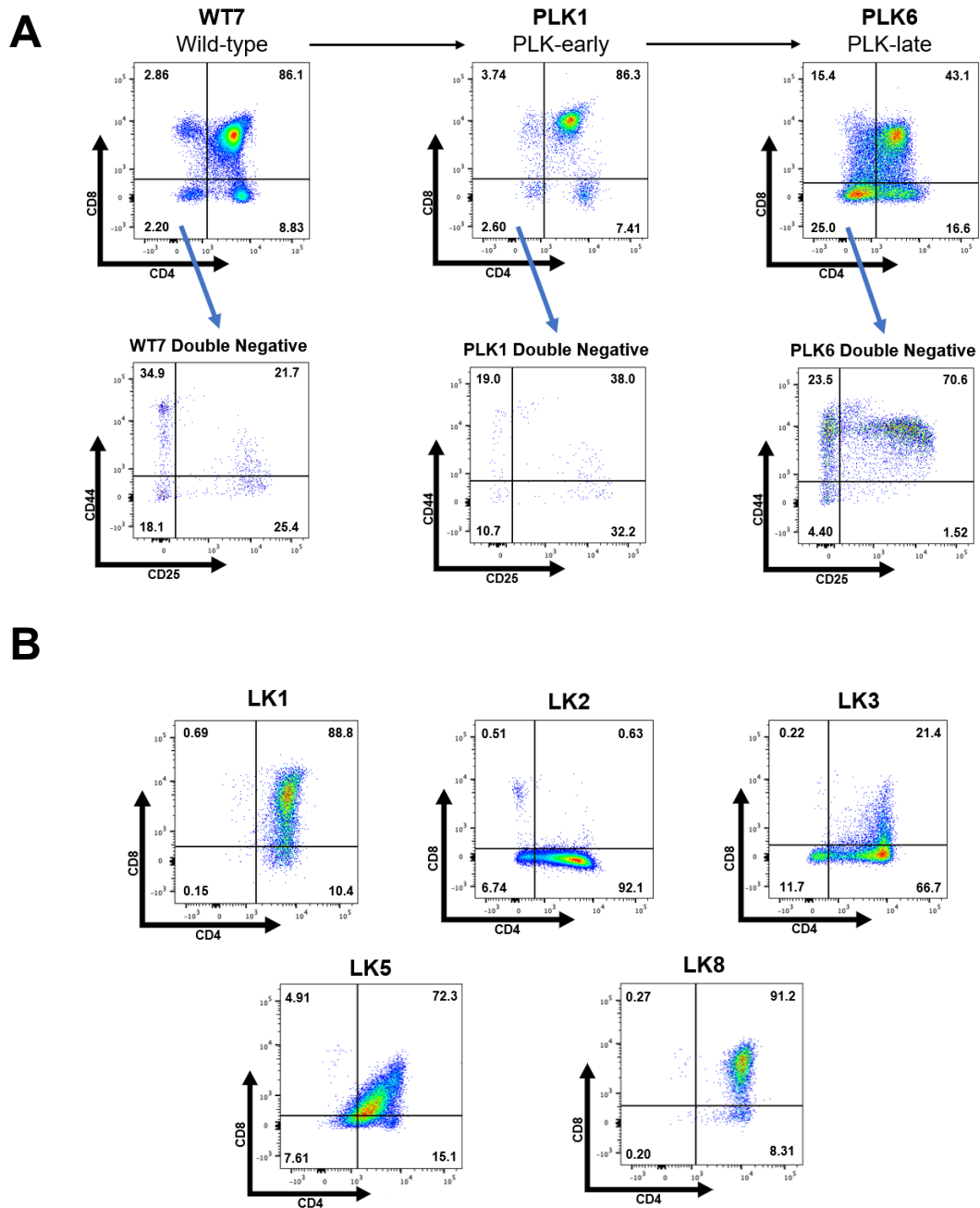


Figure 16. Flow cytometry analysis of mouse thymus across leukemia transformation.
A) Upper panels show CD4 vs CD8 gating and lower panels show DN populations for WT7, PLK1, and PLK6. Representative examples of thymocyte sub-populations in mouse thymus are shown. B) CD4 vs CD8 gating for leukemia samples used in CUT&Tag and/or RNA-seq. Relative percent of each quadrant is shown. Gating strategy described in Section 2.11.

3.12 TLX1 disrupts gene expression early in transformation.

TLX1 is a transcription factor that has been shown to affect gene expression in T-ALL by binding to promoters and enhancers. As part of our multi-omics approach, we did RNA-seq to characterize gene expression changes during leukemic transformation. For most mouse thymi we harvested, freezing cells in RNeasy lysis buffer was the first step before starting CUT&Tag and ATAC-seq experiments to ensure post-isolation gene expression changes were minimized.

PCA analysis of mouse RNA-seq shows a stepwise progression in thymocyte gene expression from WT and PLK mice, with a split into 4 very distinct leukemias (**Figure 17A**). Unexpectedly, we identified a second pre-leukemia phase, which we called “Pre-Leukemia Late.” PLK-late mice were harvested between 19 and 24 weeks, though most of the mice we harvested for the PLK condition in this age range did not show the PLK-late gene expression (3 of 14 based on RNA-seq). PLK-late is also the last cluster before the leukemia samples branch apart in their gene expression. With the survival data showing 95% penetrance of TLX1 leukemias (**Figure 15B**), PLK-late may be a critical point during transformation that most leukemias eventually pass through. **Figure 17A** also complements the differential CUT&Tag and ATAC-seq PCA plots in **Supplemental Figure 5**. This result prompted the separation of PLK mice into two groups, which reduced the number of replicates per condition for differential analyses. Normalized gene expression shows that TLX1 is not expressed in WT thymocytes and is highly expressed in PLK-early and PLK-late, with varied expression in the leukemias (**Figure 17B**). Upregulation of TLX1 in PLK-early resulted in upregulation of 591 genes and downregulation of 540, with significantly more changes in PLK-late and subsequent leukemias (**Figure 17C**). Heatmap K-means clustering of significant PLK-early and PLK-late differential genes revealed several patterns (**Figure 17D**). Cluster 1/4 showed genes that were

upregulated in PLK-early and stayed upregulated in PLK-late relative to WT. Cluster 3/5 shows genes are downregulated in PLK-early and remain or are further downregulated in PLK-late. Cluster 2 represented genes that are not downregulated until PLK-late, and Cluster 6 shows upregulation in PLK-late. Gene enrichment analysis of DEGs identified several critical processes, including regulation of T cell activation with subgroups, including differentiation and proliferation, regulation of apoptosis in leukocytes and neurons, TCR signaling, DNA replication, and ribosome biogenesis (**Figure 17E**). Notably, only DNA replication and ribosome biogenesis terms were associated to a specific cluster (C2) (**Figure 17F**), suggesting PLK-late cells undergo rapid proliferation and ribosome biogenesis hyperactivation, which is implicated in cancer¹⁷⁵. The remaining terms were enriched across clusters, suggesting that these processes are dysregulated during transformation.

When looking at all conditions, K-means clustering of the 4000 most variable genes mainly clustered according to the PLK-late and LK columns due to the more extreme changes (**Figure 18A**). Several clusters did emerge that were unique between LK2/LK5 and LK3/LK8, which likely explains the branching in Figure 17A. However, clusters 7 and 5 contain genes that are upregulated in PLK-late onwards, while cluster 4 contains genes that are downregulated in PLK-late onwards, suggesting these leukemias still share common gene expression programs. Notably, genes involved in T-cell differentiation, including CD44, NOTCH1, RAG1/2, MYC, TCF7 (encodes TCF-1), and BCL11B, had mixed expression across conditions (**Figure 18B**). Also, cell-cycle regulators CDK6 and RB1, and anti-apoptotic genes PTEN, IL17RB, and GLP1R were differentially expressed as well.

The flow cytometry data (**Figure 16**) shows WT and PLK-early had the same subpopulations. Therefore, any changes in gene expression seen should be due to direct or indirect effects of TLX1 activity as it is expressed across all thymocyte populations. Differentially

expressed genes identified in PLK-early are most likely the genes that drive transformation toward leukemia, and genes such as CDK6, PTEN, IL17RB, and GLP1R may indicate that cell cycle progression and cell survival signaling pathways are being promoted. PLK-late had notable upregulation of MYC and NOTCH1, which occurs in other T-ALLs. Their expression is also mixed in the LK mice along with TLX1, which may contribute to the mixed gene expression signatures for each. We next characterized changes in TLX1, its cofactors, and histone modifications across these stages.

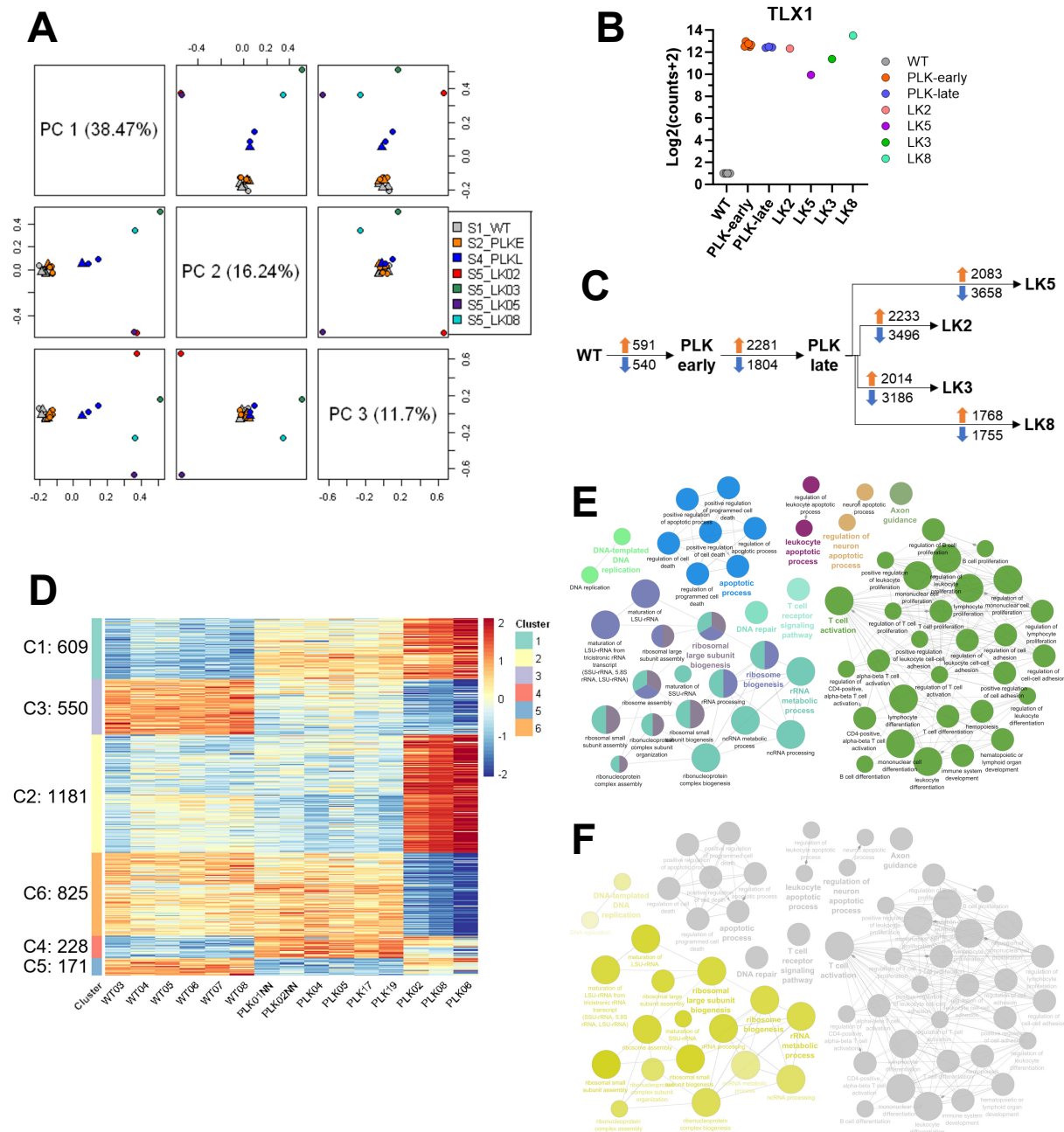


Figure 17. Mouse RNA-seq analysis shows stepwise changes in gene expression. A) PCA analysis of gene expression, groups are colored by condition, triangle represents males, circles represent females, first 3 principal components are shown. B) Normalized Log2 TLX1 gene expression. C) Differential genes identified at each stage of transformation. D) K-means clustered heatmap of all differential genes identified in PLK-early and PLK-late using $k = 6$. E) ClueGO gene enrichment analysis of k-means clusters from panel D. Groups are colored by leading term (bolded color text), not by k-means cluster. F) Panel E results colored by k-means cluster specificity by ClueGO. GO-terms not enriched for a specific k-means group (mixed enrichment) are colored grey by ClueGO. Only C2 (yellow) had specific enrichment.

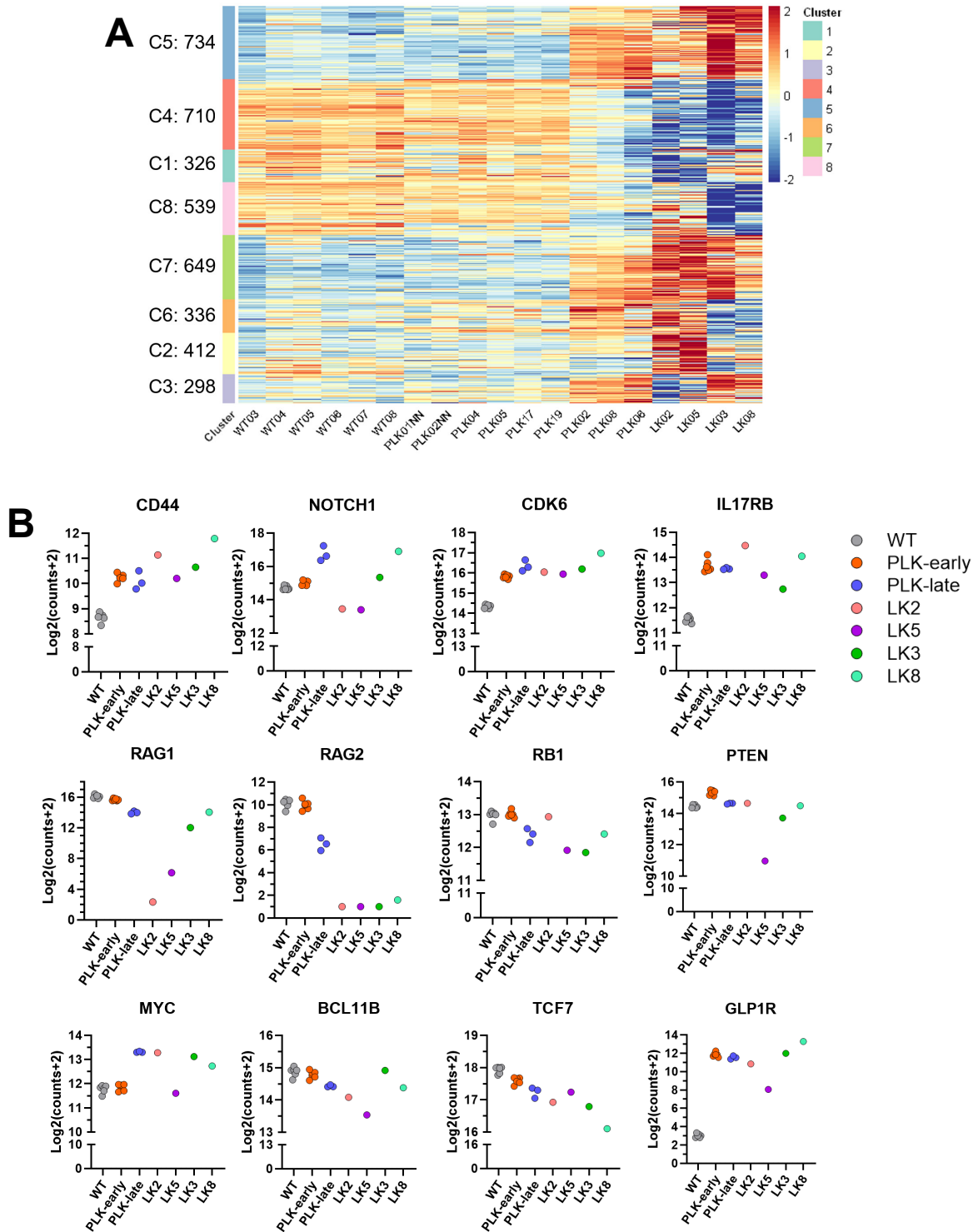


Figure 18. Leukemic Mice have distinct gene expression signatures. A) K-means clustered heatmap of top 4000 most variable genes across all conditions using $k=8$. B) Normalized gene expression counts across all conditions for genes of interest. Y-axis is adjusted to fit all data points.

3.13 CUT&Tag differential analysis shows genomic changes early in transformation.

Mouse thymus does not yield many thymocytes, especially for WT and PLK thymus. We took advantage of CUT&Tag to characterize the binding of TFs and histone marks with as few as 100k cells per tube. Similar to ALL-SIL and Patient T-ALL samples, I used CUT&Tag with mouse thymocytes to characterize the binding of TLX1, CTCF, RAD21 (cohesin), BRG1 (BAF complex), and the main epigenetic histone modifications H3K4me1, H3K4me3, H3K27ac, H3K27me3. POL2-ser2P represents actively transcribing POL2 along the gene body. I then used DiffBind¹⁶⁰ for differential analysis of CUT&Tag peaks between WT, PLK-early, PLK-late, and LK. TLX1 binding was identified at 25,850 sites in PLK-early. **Figure 19** shows early changes that occur for CTCF, RAD21, and BRG1, as well as DNA accessibility and histone marks when comparing WT to PLK-early. Additional changes occur from WT to PLK-late and WT to LK. The DiffBind PCA plots of these datasets are in **Supplemental Figure 5** and show a similar trajectory to the RNA-seq data with the available mouse samples (**Figure 17A**).

We found it appropriate to compare all conditions to WT as it is the baseline genomic distribution of targets. Differential analysis also had better statistical power with multiple replicates per condition. However, using a single PLK-late replicate (PLK6) proved difficult to show statistically significant changes between PLK-early and PLK-late and PLK-late to LK. Notably, ATAC-seq had PLK2 and PLK6 for the PLK-late condition, which also clustered together between PLK-early and the LK branches like the RNA-seq analysis (**Supplemental Figure 5**). Taken together, thymocytes that express TLX1 in PLK-early had altered DNA-accessibility and histone PTM enrichment, as well as initial changes in CTCF, cohesin, and BAF complex binding.

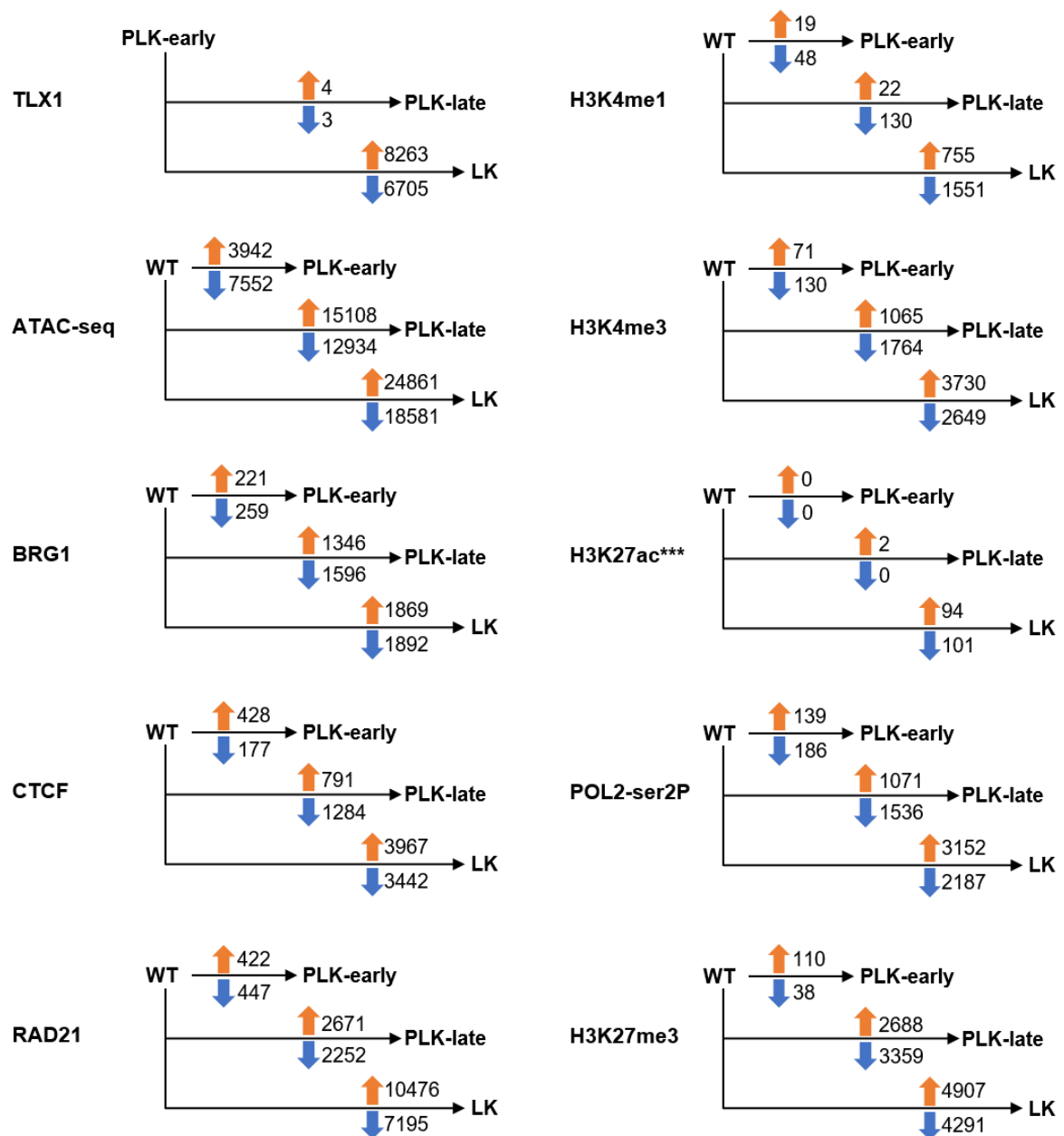


Figure 19. Differential analysis of CUT&Tag targets at WT, PLK-early, PLK-late, and LK. Total differential peaks are shown relative to WT condition. Peaks were called individually for all samples. Diffbind merges these peaks into a union set, counts were normalized and analyzed using DESeq2. Differential peaks with an FDR < 0.05 are reported. Note: H3K27ac worked well in ALL-SIL antibody optimization but the mouse data had high variability which hindered differential analysis.

3.14 TLX1 colocalization alters the binding of CTCF, cohesin, and the BAF complex.

The strong colocalization of TLX1 in ALL-SIL and the Patient T-ALL suggested an interplay between TLX1, the BAF complex, and chromatin looping. We wanted to see if TLX1 colocalization affected the binding patterns of its cofactors.

Differential analysis of WT to PLK-early showed that TLX1 binding was associated with increased and decreased BRG1, RAD21, and CTCF peak intensity (**Figure 20A**). Changes in cofactor binding suggest TLX1 can promote and disrupt the occupancy of all three individually and together. TLX1 also appears to predominantly stabilize CTCF (290+27) and RAD21(150+27) without increasing BAF complex strength, and 63 sites were strengthened together with the BAF complex. Overlap analysis by heatmap at these 63 sites also shows stronger signals in ATAC-seq as well (**Figure 20B**).

TLX1 has been described as both an activator and a repressor, and these disparate roles may be due to context-specific local interactions or additional cofactors not studied here. Chromatin looping is also a dynamic process where rates of CTCF binding and cohesin loading and unloading are dependent on several variables. TLX1 binding may enhance or disrupt CTCF binding or cohesin loop extrusion through direct local interactions on DNA. However, the precision of CUT&Tag peaks relative to the antibody target's actual binding location does not allow for high-resolution analysis of these interactions.

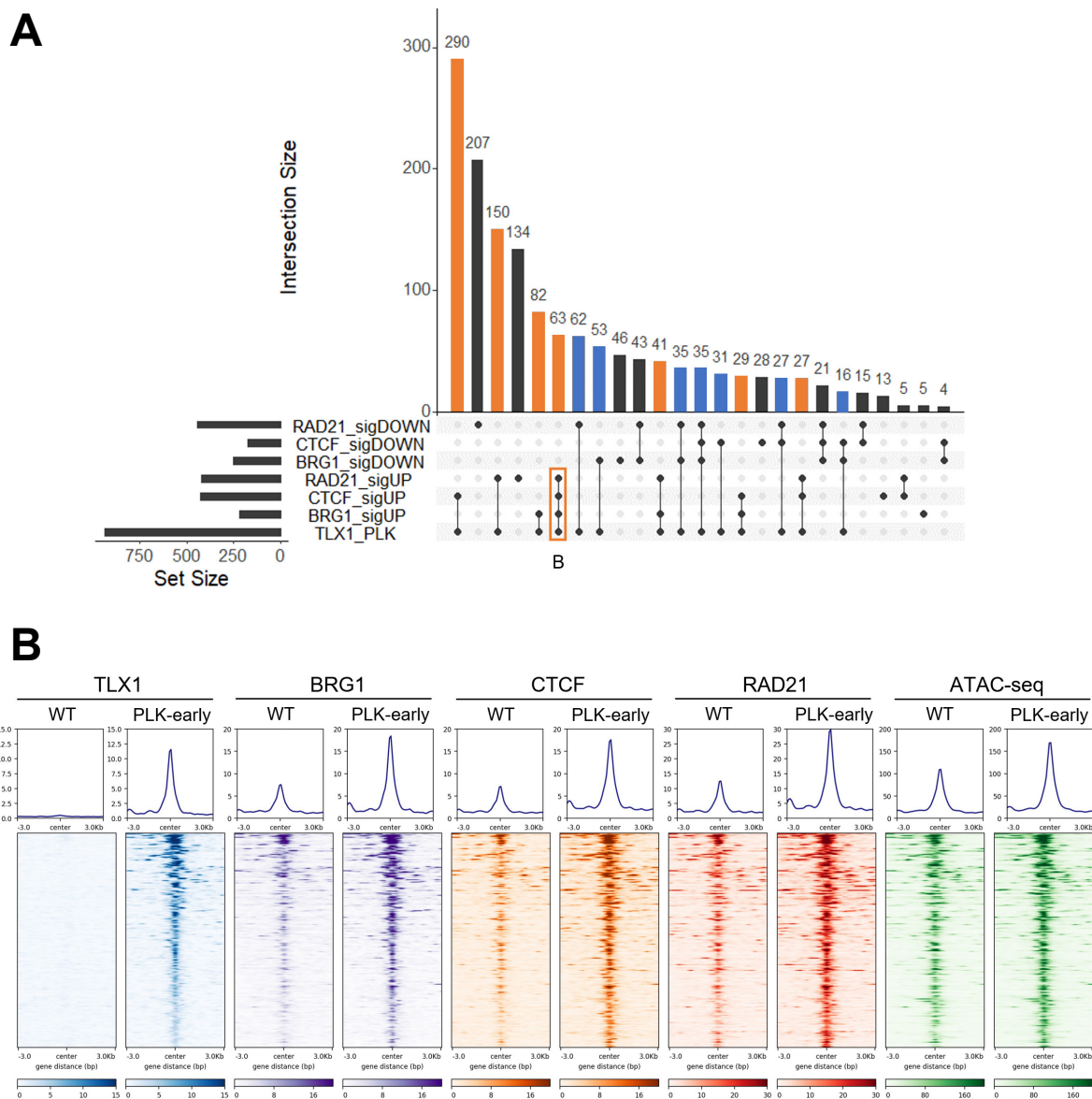


Figure 20. Differential analysis of cofactors shows TLX1 colocalization in PLK-early
A) Upset plot of differential peaks identified by Diffbind. Peaks are a subset of differential RAD21, CTCF, BRG1 peaks with or without PLK-early TLX1 peaks. Peaks are overlapped using Homer and visualized using UpsetR. Orange bars signify cofactor enrichment with TLX1 co-occupancy. Blue bars signify cofactor depletion with TLX1 co-occupancy. Black bars are differential enrichment independent of TLX1. Peak set labelled “B” were used to produce heatmap. B) Heatmap of TLX1 peaks upregulating BRG1, CTCF, RAD21. ATAC-seq signal is included. Tracks used are average normalized signal per condition.

3.15 Early pre-leukemia shows minor AB compartment changes.

HiC allows the characterization of AB compartments, TADs, and chromatin loops. The next sections unpack the changes in chromatin organization during leukemic transformation using HiC data of WT6, PLK5 (PLK-early), and LK3. As the PLK-late condition was unexpected, crosslinked cells were never prepared for HiC. Therefore, these sections focus on the changes in chromatin organization that occur in thymocytes expressing TLX1 (WT to PLK-early) and the major reorganization that occurs in one of the leukemias (LK3). I used dcHiC¹⁷³ to characterize compartmental changes, which compares the 3 conditions as a time course and identifies significant changes between each stage. In PLK-early, A compartments are much more frequently occupied by TLX1 than B compartments (**Figure 21A**). Across all conditions, 11% of all genomic bins (5357 of 48836) undergo a switch or flip across WT, PLK-early, and LK3 (**Figure 21B**). A small subset of changing bins undergo compartment switching from WT to PLK-early (9.5% of 5357), with most of those being weakA/weakB changes. However, major AB compartment switches occur in LK3 in both directions.

Generally, it does not appear that TLX1 is involved in AB compartment regulation and instead only binds in A compartments. **Figure 21C** shows an example region that shows TLX1 binds exclusively within A compartments and does not change the AB compartments in PLK-early. In LK3, TLX1 binding is lost when there is an A-to-B switch at EX1 and EX2. In addition, there are only a few examples of TAD changes being associated with AB compartment changes, and TLX1 is not found in these regions. Instead, TLX1 disappears in A-to-B transitions, signifying its binding activity can be overcome by compartment switching. Overall, AB compartment switching does not appear to be a main component of transformation, and TLX1 does not appear to be part of this level of chromatin organization.

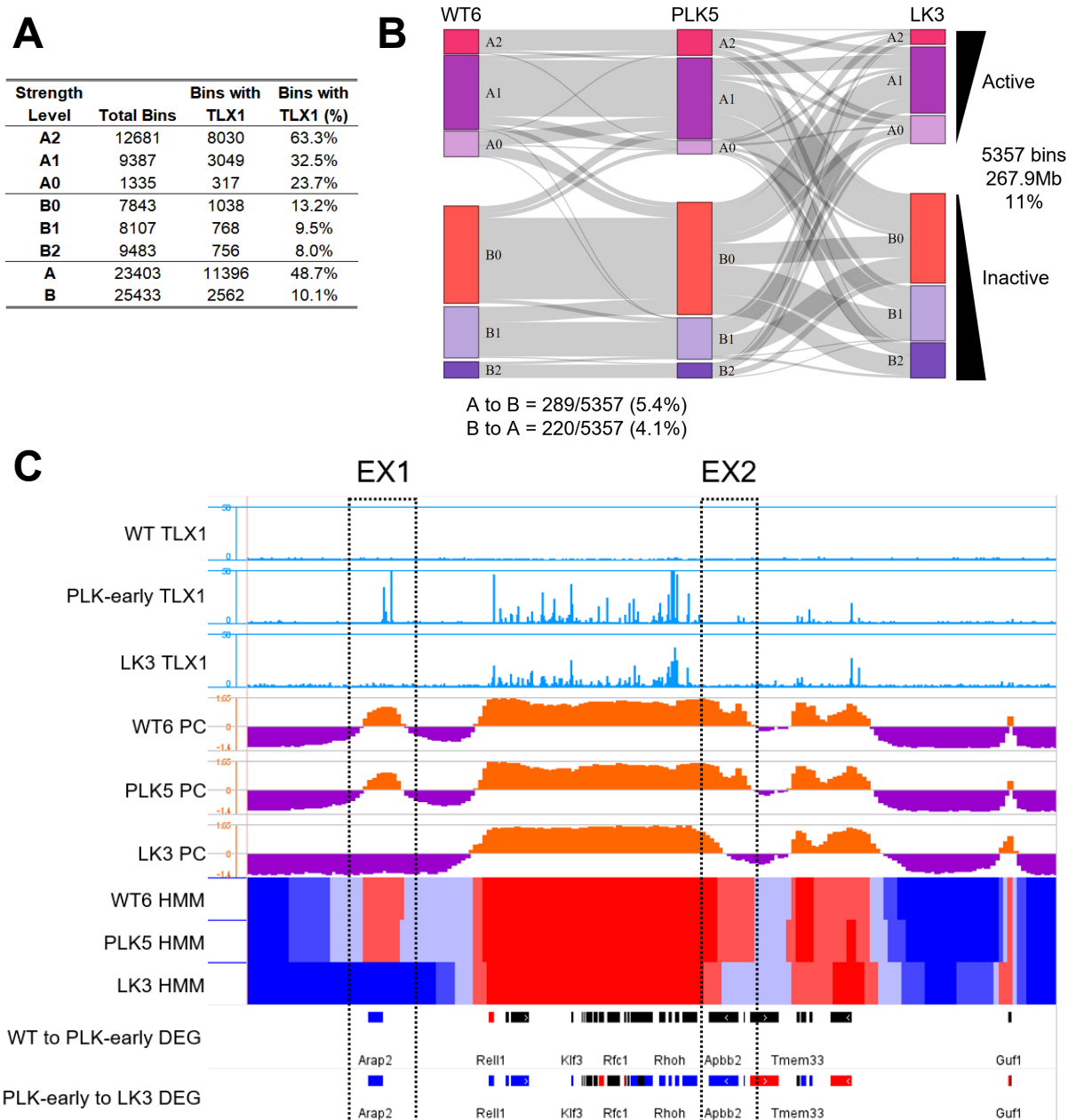


Figure 21. Mouse differential AB compartment strength analysis using dcHiC. A) PLK-early summary of AB compartment bins and percentage occupied by at least 1 TLX1 peak. B) AB compartment strength categorized into strength levels by dcHiC where $A2 > A1 > A0$ and $B0 < B1 < B2$. Note: this is not related to AB letter codes in section 1.7.1. Significant changing bins are shown (5357 of 48836 bins at 50kb). C) Example regions of LK3 AB compartment switch at EX1 and EX2. TLX1 tracks are normalized, and WT and PLK-early tracks are average bigwigs. Normalized dcHiC principal components are shown, A is colored orange and B is colored purple. dcHiC HMM categorized regions, A is colored red, and B is colored blue. Gene annotations are colored by differential expression from WT to PLK-early to LK3 where black is unchanged, red is upregulated, blue is downregulated.

3.16 TAD changes occur early in transformation.

The next levels of chromatin organization are TADs, sub-TADs, and chromatin looping. The mechanisms involved are understood to act independently of AB compartment regulation, but B-to-A switches allow for the subsequent formation of TADs, sub-TADs, and chromatin loops. B compartments are mostly devoid of these structures. I used DiffDomain¹⁷² to identify changing TADs. TAD changes are difficult to classify as they can signify a change in TAD strength or a reorganization of TADs and sub-TADs. Therefore, counting reads within a TAD does not necessarily capture what is occurring in the sub-structures of a TAD. DiffDomain identifies changing TADs and classifies them as strength, merge, loss, split, zoom (or shift), or complex, which involve overlapping structural changes¹⁷².

Significant TAD changes occur in PLK-early, suggesting TLX1 expression influences TAD organization early in transformation (**Figure 22A**). Like AB compartments, LK3 shows more TAD changes. DiffDomain can call several overlapping TADs as statistically significant, which could double-count the data, so I consolidated the regions by merging significant regions and subtracting them from non-significant TADs for a mutually exclusive comparison. This consolidated the 486 TADs and sub-TADs into 221 significant regions (9.2%) (**Figure 22B**). When looking at gene expression changes between WT and PLK-early, 67% of differential TADs had gene expression changes within them, which was more frequent than randomly sampled unchanged TADs (avg. 23%) ($p < 2.2 \times 10^{-16}$). Among all differentially expressed genes, 26% were within significantly changing TADs, which was more likely than randomly sampled unchanged TADs (avg. 6%) ($p < 2.2 \times 10^{-16}$). When normalizing for total genes in TADs, changing TADs were also more likely to have more differential genes than non-significant TADs ($p < 5.349 \times 10^{-8}$). The PTEN locus is shown as an example region where TLX1 binding in the region results in reorganizing long-range interactions while sub-TADs are

strengthened (**Figure 22C/D**). Moreover, GO-term analysis of genes that are differentially regulated within changing TADs are enriched for pathways related to T-cell differentiation, signaling pathways, cancers, and neuron developmental (**Figure 22E/F**). Taken together, TLX1 expression in thymocytes results in TAD reorganization and changes in gene expression. The genes that are within these TADs are implicated in critical cell developmental pathways that may be involved in early transformation.

3.17 TLX1 binding perturbs chromatin loop formation early in transformation.

Changes in TAD organization are largely associated with changes in chromatin looping both at TAD boundaries and within TADs. Chromatin looping is highly dynamic and is influenced by several factors, including POL2 activity, CTCF and other transcription factor binding, and DNA accessibility, among other mechanisms.

We assessed the impact that TLX1 had on chromatin looping first by identifying differential chromatin loops by mustache-diff and a stringent FDR ($1e-4$), then overlapping TLX1 binding sites with differential loops in PLK-early. Overall, 2816 of loops (15.5%) were significantly impacted between WT and PLK-early, with local TLX1 binding at 64% of loops (1791 of 2816) (**Figure 23A**). APA analysis verifies differential loop strength and shows that loop anchors affected by local TLX1 binding are both strengthened and weakened in PLK-early (**Figure 23B**). **Figure 24** shows an example region where TLX1 binding causes changes in BRG1, CTCF, and RAD21 peak intensity, which coincides with changes in chromatin loops, TAD intensity, and gene expression. Notably, not all loops that were influenced by TLX1 binding also indicated changes in BRG1, CTCF, or RAD21 binding. This could indicate that TLX1 is interacting with other cofactors at certain sites, or TLX1 binding could be changing the pausing of RAD21 during loop extrusion. The intensity of the “dot” on HiC data signifies the frequency of a chromatin loop existing at that site by paused cohesin loop extrusion. Cohesin pausing is not influenced by CTCF alone as other factors influence cohesin activity. Loop “dots” also form independent of CTCF or RAD21, but our analysis is limited to the targets we interrogated. Moreover, changes in a chromatin loop can influence the intensity of other local chromatin loops, causing a knock-on effect at other anchors within a TAD. Taken together, differential loop analysis shows that a large proportion of chromatin loops are perturbed by TLX1 binding, which can result in gene expression changes and TAD changes.

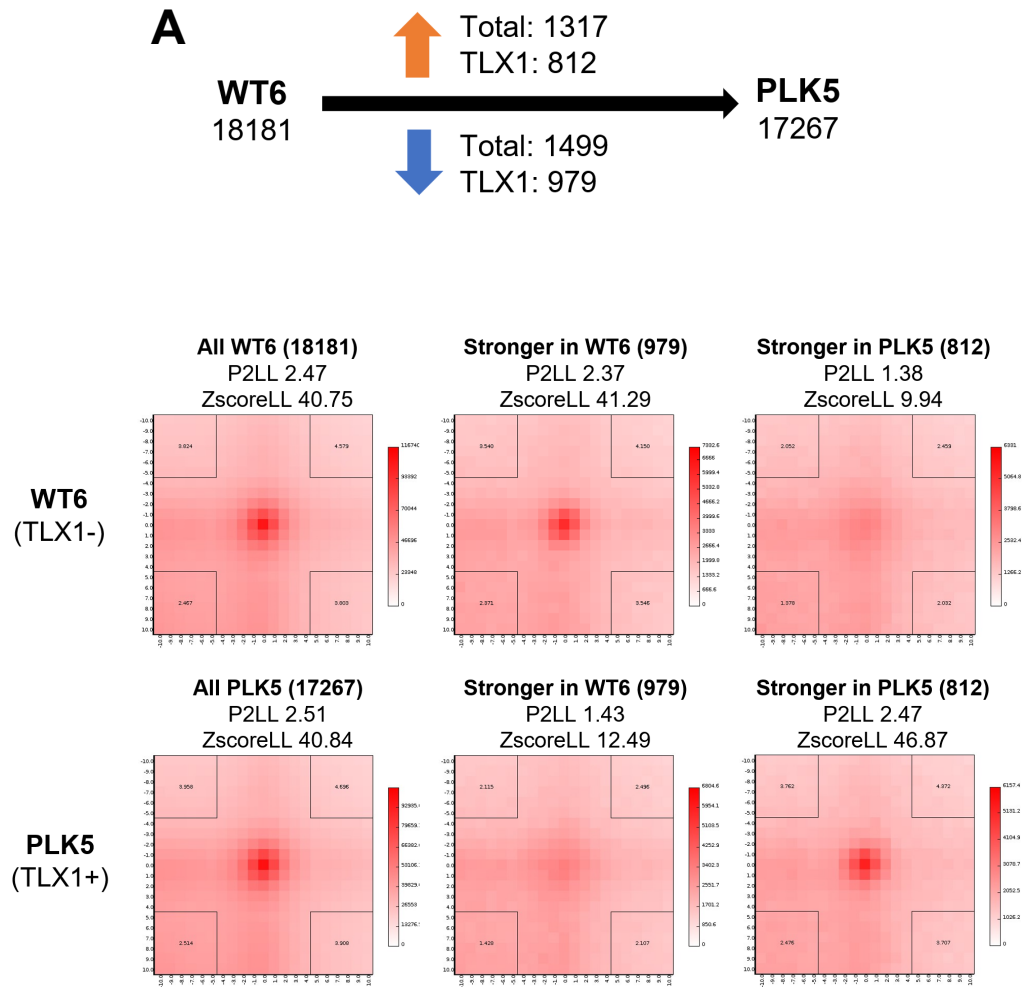


Figure 23. TLX1 binding perturbs chromatin looping in PLK-early. A) Summary of differential loops overall and overlapping TLX1. B) Aggregate peak analysis of differential loops that overlap with TLX1 at either end. P2LL is the “ratio of the central pixel to the mean of the mean of the pixels in the lower left corner”. Values significantly above 1 indicate enrichment. ZscoreLL is the Z-score of the central pixel relative to all the pixels in the lower left corner. Values significantly above zero indicate enrichment.

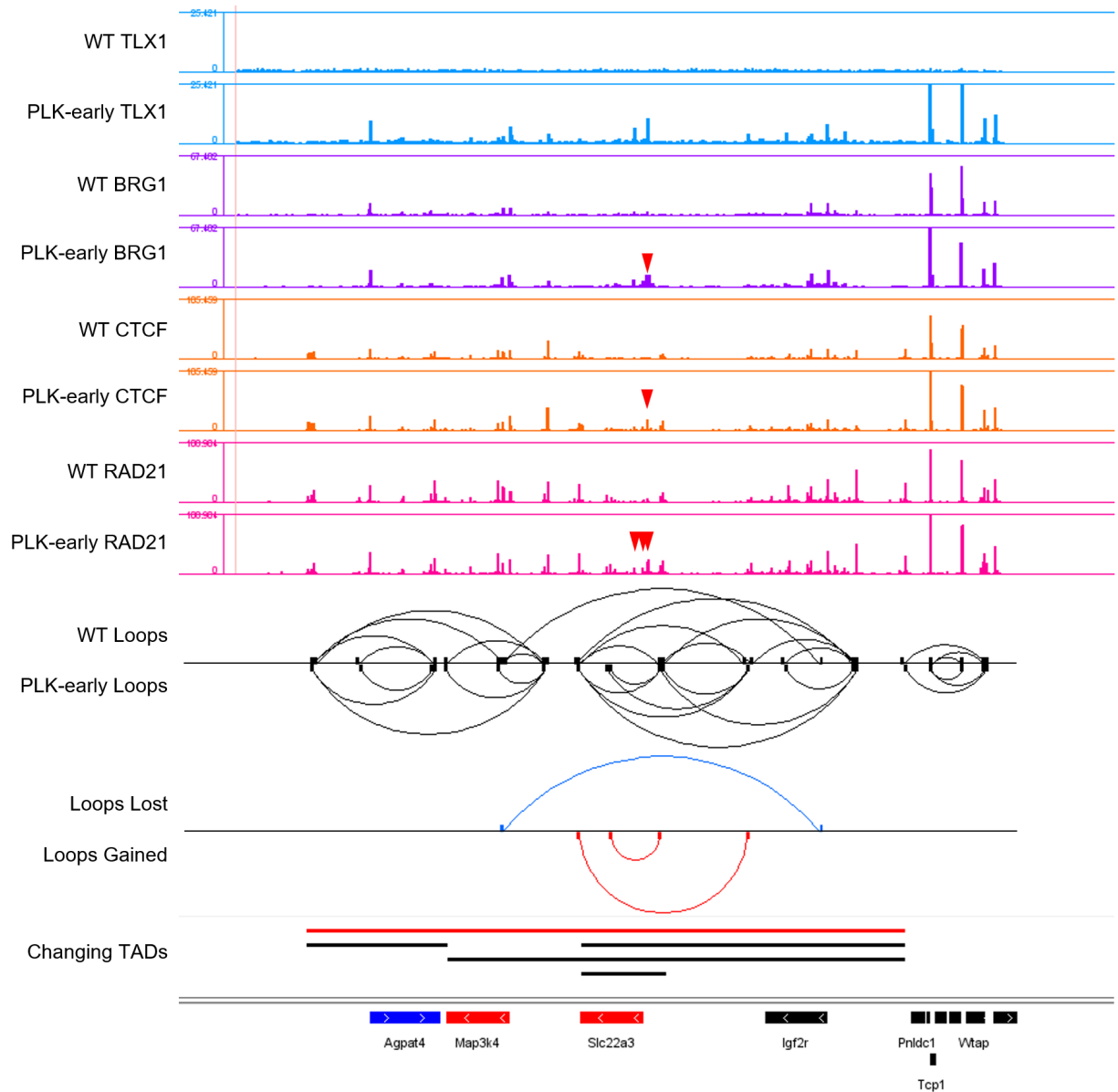


Figure 24. Example region of TLX1 binding perturbing cofactors and chromatin organization in PLK-early. CUT&Tag tracks represent average signal across WT and PLK-early replicates tracks. Red arrows within cofactor tracks represent upregulated peaks called by DiffBind. Black arcs represent loops in WT6 and PLK5. Blue and red arcs represent gained and lost differential loops. Significantly changing TADs are highlighted in red with non-changing TADs in black. For gene expression, upregulated genes are red and downregulated are blue, with non-differentially expressed active genes in black.

3.18 Ectopic expression of TLX1 results in differential loop formation.

To determine if acute ectopic expression of TLX1 could induce changes in chromatin structure, we generated stable clones of Jurkat with Dox-inducible TLX1. Jurkat is a T-ALL cell line with TAL1 as its primary oncogene. Regarding these experiments, the Day 2 HiC data and the Day 2/4 CUT&Tag were performed at different times but with the same stable clone.

Western blot of cell lysates validates the expression of TLX1 at 48 and 96 hours post-induction (**Figure 25A**). TLX1 protein levels on Day 4 were weaker than in ALL-SIL and Patient cells. Notably, differential loop calling identified 1290 loops being gained and 927 loops lost, and half of these loops were co-occupied by TLX1 (**Figure 25B**). APA analysis shows strengthening and weakening loops near TLX1 binding sites (**Figure 25C**). Taken together, this suggests TLX1 ectopic expression in a separate T-ALL cell line can acutely perturb chromatin looping.

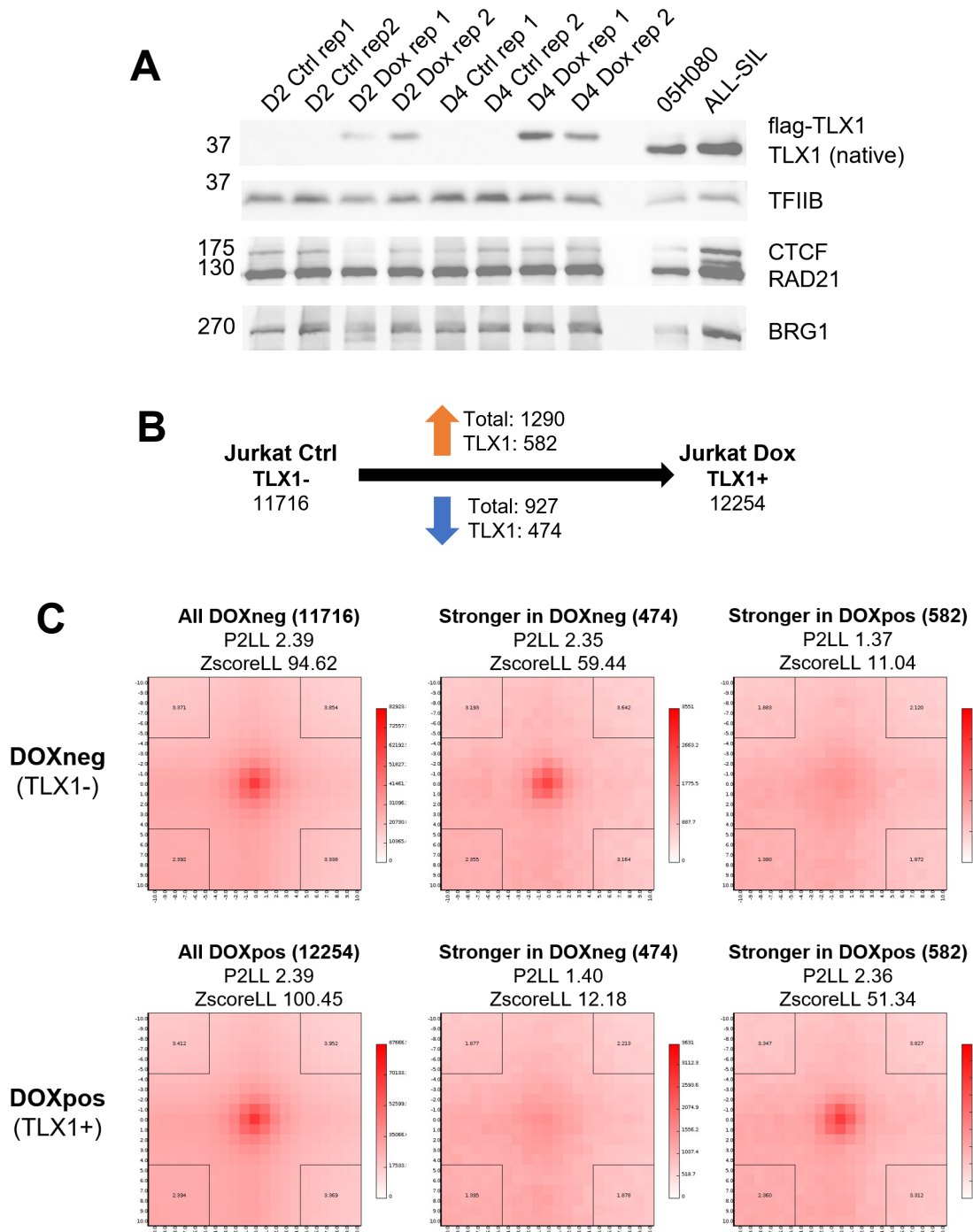


Figure 25. Expression of TLX1 results in differential loop formation. A) Western blot of Jurkat-TLX1, ALL-SIL, and Patient for comparison. Lanes equalized to 15ug whole cell lysate except for patient which was 11ug. B) Summary of differential loops overall and overlapping TLX1 peaks. C) Aggregate Peak Analysis of TLX1-overlapping loops. P2LL is the “ratio of the central pixel to the mean of the mean of the pixels in the lower left corner”. Values significantly above 1 indicate enrichment. ZscoreLL is the Z-score of the central pixel relative to all the pixels in the lower left corner. Values significantly above zero indicate enrichment.

3.19 ALL-SIL is sensitive to ACBI-1 and BRG1/BRM Inhibitor-1

Anti-cancer epigenetic drugs are an active area of research, as different types of cancers have been shown to have unique epigenetic dependencies because of their mutations. We tested whether the TLX1-expressing cell line ALL-SIL was sensitive to disruption of the BAF complex function by using a PROTAC degrader or dual BRG1/BRM ATPase inhibitor.

We used BRG1/BRM inhibitor-1, which allosterically binds to the catalytic region of both core subunits. We also used ACBI-1, a BRG1/BRM degrader PROTAC, which binds to BRG1 or BRM and marks them for proteasome degradation. Cell viability was measured using the Cell-Titer Glo assay. The addition of 200nM ACBI-1 efficiently depleted BRG1 after a single addition of the drug, and BRG1 remained depleted for the entire time course (**Figure 26A**). When assessing cell viability after 7 days, ACBI-1 had an IC₅₀ of 73.4nM, and BRG1/BRM inhibitor-1 had an IC₅₀ of 15.9nM (**Figure 26B**). ALL-SIL was also more sensitive to the inhibitor than the degrader.

Taken together, this suggests that degrading or inhibiting the core proteins of the BAF complex BRG1 and BRM could be a possible therapeutic strategy that warrants further investigation. In ALL-SIL, drug inhibition or degradation disrupts TLX1 activity that involves the BAF complex as well as activity independent of TLX1. These results show that the BAF complex is important for TLX1-expressing T-ALL cell line survival.

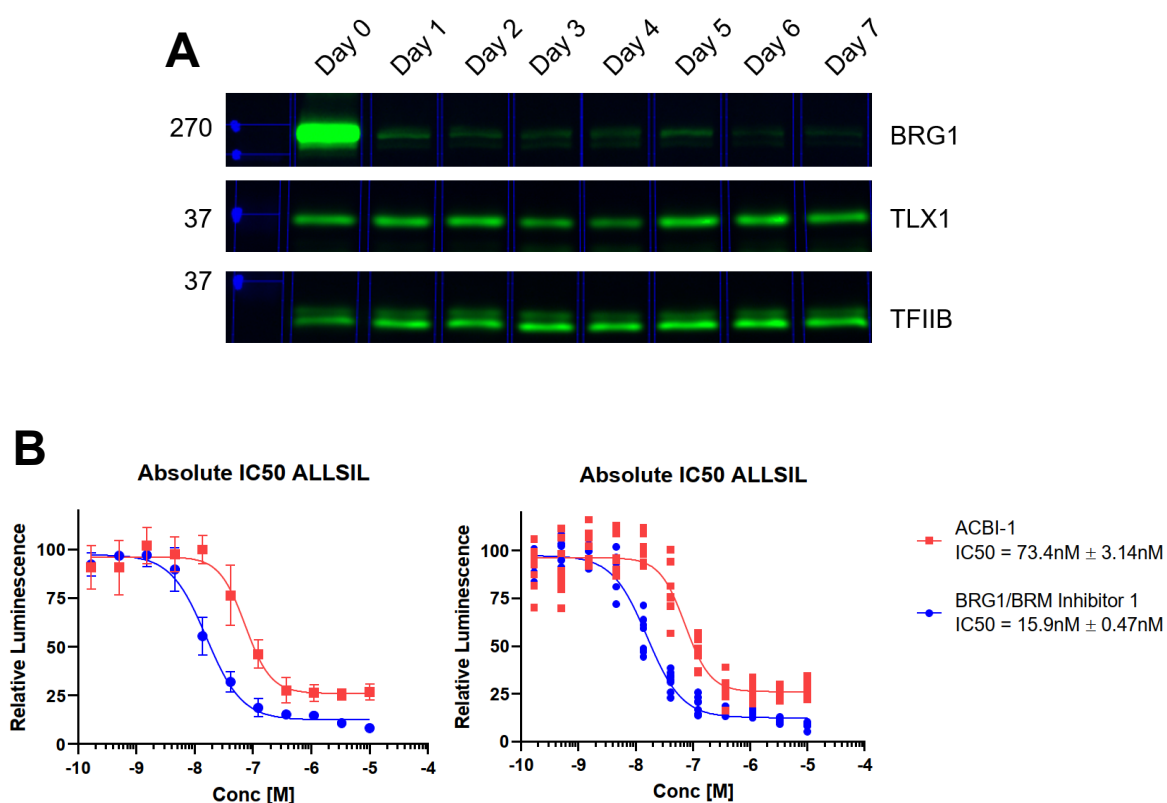


Figure 26. ALL-SIL is sensitive to ACBI-1 and BRG1/BRM inhibitor-1. A) Validation of BRG1 degradation by western blot of ALL-SIL cell lysate treated with ACBI-1 on Day 0. Each lane loaded with 10ug total protein. TFIIB is used as loading control. B) Absolute IC50 of ALL-SIL at day 7 for both drugs. Left: Average signal with standard deviation. Right: All replicates shown for mean value in left panel. Each drug curve is normalized separately from 0 to 100%. Zero line represents blank media control wells, and 100 represents the strongest mean signal point for each curve.

Chapter 4 - Discussion

4.1 A note about antibodies.

In this study, we first set out to identify possible cofactors for TLX1 in its T-ALL cell line ALL-SIL. One of the realities of research is that antibodies can be discontinued. In my case, previous research had repeatedly cited^{5,8,45,55,176,177} the same antibodies for TLX1 and TLX3 that were no longer available for purchase. After characterizing panels of antibodies for western blot (**Figure 6**), immunoprecipitation, and CUT&Tag, I found a set of antibodies that worked very well for each experiment. Namely, I found that the polyclonal rabbit TLX3 17474-1-AP antibody efficiently immunoprecipitated TLX3 and TLX1 and worked in ALL-SIL CUT&Tag for TLX1 as well. It is being addressed here that using an antibody designed for one protein to characterize another may be unconventional; this was viewed as a special case for several reasons. TLX1, TLX2, and TLX3 share high homology, and the TLX branch of HOX genes are a unique sub-group that shares very little homology with other HOX TFs. Therefore, any expression of other HOX TFs would be unlikely to cross-react, and we did not see evidence of this in western blots. In the T-ALL contexts interrogated, RNA-seq verified that TLX2 and TLX3 were not expressed in ALL-SIL and neither passed the read count filtering cutoff for the mouse analysis. TLX2 was expressed at very low read counts in Jurkat and Patient 05H080 as well. Taken together, we determined that using the TLX3 antibody was appropriate for characterizing TLX1 due to the mutually exclusive expression of possible cross-reacting proteins. If there had been clear evidence of cross-reactivity, other antibodies would have been used.

4.2 Pursuing the BAF complex and chromatin looping in TLX1 T-ALL.

Using co-immunoprecipitation of TLX1 followed by mass spectrometry, we used a semi-quantitative approach combined with GO-term analysis to identify candidate interacting cofactors (**Figure 7**). We checked for cofactors previously suggested for TLX1. Previous studies have shown that TLX1 colocalizes with RUNX1, ETS1, and PBX in the leukemia context⁶. Structurally, TLX1 contains an FPWME motif, which is a known PBX-interacting motif present in many HOX transcription factors^{55,178}. Another study had also shown TLX1 complexed with PBX2 and MEIS1¹⁷⁹. Our TLX1 IP did find peptides for RUNX1, RUNX3, and PBX2, but not ETS1 or MEIS1 (only MEIS2 was expressed in ALL-SIL) (**Supplemental Table 1**). While this corroborated the motif analysis of TLX1 (**Figure 9 and Figure 10**), our focus was identifying TLX1 interacting cofactors that could explain TLX1 activator and repressor activity. Colocalizing TFs may cooperate with TLX1 and specific complexes and that may warrant future investigation.

Our discovery-based approach initially identified the NuRD and BAF complexes as the likeliest candidates. We did find subunits of the HDAC-containing NuRD complex. However, *Dadi et al. 2012* mentioned they had seen no change in TLX1/TLX3 repressing activity when treating with HDAC inhibitor⁶. This ruled out the NuRD complex as a relevant cofactor to TLX1 function that could be exploited. They also suggested PcG complexes may be involved as with other HOX TFs, but we did not see significant enrichment for those complex subunits, and TLX1 binds almost exclusively in accessible regions⁶.

Several lines of evidence pointed to pursuing the BAF complex further as a TLX1 cofactor in relation to chromatin looping. The TLX1 ChIP-seq analysis showed motif enrichment of CTCF and strong colocalization of CTCF and RAD21 (**Figure 9**), which was seen in the CUT&Tag data as well (**Figure 10**). The BAF complex is a key regulator of

nucleosome occupancy and DNA accessibility at promoters and enhancers, which is where TLX1 is largely bound (**Figure 11**). Importantly, the BAF complex is needed for proper nucleosome occupancy at CTCF binding sites^{78,81,82,129}. It is also a critical epigenetic complex in thymocyte differentiation, and its subunits are often mutated in cancers, including leukemias^{94,180,181}. Thymocytes undergo varying degrees of chromatin reorganization at each stage of differentiation, and dysregulation is implicated in several disease contexts, including cancers^{116,182}. TLX1 has not yet been implicated in chromatin organization in T-ALL nor its normal embryonic functions, but other HOX TFs have been associated with CTCF motifs⁴¹. PBX, a previously characterized cofactor of TLX1¹⁷⁹, also interacts with the BAF complex¹⁸³. Taken together, we reasoned that there could be a relationship between TLX1, the BAF complex, and chromatin organization that warranted further investigation.

Further analysis of ALL-SIL and Patient 05H080 using ATAC-seq, CUT&Tag, and HiC supported the association of TLX1 and chromatin organization. CUT&Tag overlapping was consistent with ChIP-seq in motif analysis with enrichment of ETS, RUNX, and CTCF motifs, among others, and the strong colocalization of TLX1 and its cofactors remained (Figure 10). Notably, HiC analysis showed that TLX1 occupied a large proportion of both TAD boundaries and loop anchors in the T-ALL context (**Figure 12**). Adding to previous research studying TLX1 in ALL-SIL, CUT&Tag allowed us to analyze the epigenetic landscapes of these T-ALLs. We verified that TLX1 predominantly binds to promoters and enhancers in ALL-SIL and Patient 05H080 (**Figure 11**). Chromatin loop anchors exist at promoters and enhancers as well as distal intergenic regions, which makes colocalization with TLX1 likely. Characterization of TLX1 binding in ALL-SIL and Patient 05H080 provided validation of the initial proteomics data, though this analysis only correlated TLX1 with these

sites in the leukemia stage of the disease. The mouse model was used to characterize the changes in chromatin organization at earlier stages of leukemia transformation.

4.3 Structural variants in human TLX1 T-ALLs.

Previous studies have shown that the TLX1 translocation occurs in the early cortical stage to the TCR δ , β , or α loci⁶. Our HiC data identified that both ALL-SIL and Patient 05H080 had their breakpoints within 10kb of each other at the TCR δ locus, between TRDV2 and TRDV3 (**Figure 13**). The precise location of these breakpoints is significant as it may identify the likely moment of translocation early in TCR recombination. Previous research has shown where likely regions upstream of the TLX1 gene form G-quadruplex structures on chromosome 10. The ALL-SIL cell line had this breakpoint upstream of the TLX1 promoter, but the Patient T-ALL breakpoint was 25kb upstream, suggesting another possible G-quadruplex structure not previously identified. Contrary to previous research suggesting TLX1/3 represses TCR α -enhanceosome function⁶, our HiC data shows that the breakpoint in TCR δ completely separates the upstream and downstream portions of TCR α onto separate chromosomes, thereby blocking TCR α recombination and progression of T-cell differentiation. Regarding secondary mutations, apart from the deletion of CDKN2A and CDKN2B, the TLX1 T-ALLs had mostly unique SVs (**Supplemental Table 2,3,4,5**) and had varied aneuploidy between the two T-ALLs (**Supplemental Figure 2**), supporting previous work showing TLX1 T-ALLs have consistent and unique mutations. Many SVs were unproductive through translocations or deletions in gene-poor regions (data not shown).

The SV analysis also identified the NUP214-ABL1 episome in ALL-SIL but not Patient 05H080. This episome appears as a very strong TAD in HiC data but is really several copies of the same segment of DNA, which is also evident in the background signal of the

CUT&Tag data (**Supplemental Figure 1**). The NUP214-ABL1 oncogene is recurrently almost exclusively in TLX1 and TLX3 T-ALLs^{5,59}. This produces an episome, which is an extrachromosomal circularized segment of chromatin and can have several copies per cell⁵⁹. The NUP214-ABL1 fusion creates a constitutively active tyrosine kinase with the C-terminus of ABL1, which aberrantly phosphorylates several downstream targets, including STAT5^{5,59}. BCR-ABL1, EML1-ABL1, and DEK-NUP214 fusion genes have also been found in other leukemias^{5,59}. Vanden Bempt 2018., characterized the recurrent secondary mutation NUP214-ABL1 and how its downstream effects synergize with TLX1 activity in mice⁵. They showed that mice expressing TLX1 and NUP214-ABL1 exhibited significantly faster leukemia development than TLX1 alone. Colocalization of TLX1 and STAT5 at enhancers was also shown to synergize and drive overexpression of genes such as MYC and BCL2⁵. T-ALLs that have this mutation have mixed success with imatinib treatment, which inhibits several tyrosine kinases, including ABL1⁵. As Patient 05H080 did not have this mutation, it supports the finding that this secondary mutation can synergize with TLX1 activity but is not necessary for leukemia development⁵. Treatment with imatinib may also not be effective for this specific T-ALL.

We did not investigate further how specific genes affected by deletions, amplifications, translocations, and possible new fusion proteins contribute to TLX1 T-ALL development. However, HiC analysis of other TLX1 patient samples could identify additional SVs not seen previously and could identify additional recurring mutations that may warrant further investigation.

4.4 TLX1 mouse modeling of T-ALL progression.

The mouse model effectively modeled leukemic transformation by expression of TLX1 in developing thymocytes, and the onset of the leukemia is consistent with previous findings^{10,184}. Our results show that TLX1 is expressed at a high level in PLK-early across the thymocyte stages (where LCK is highly expressed). Previous research suggests that the initial leukemia cells arise from the DN2 stage¹⁰, and we saw this in PLK-late to varying degrees. Among WT thymocytes, the entire DN population represents a very small fraction (<2%) of thymus cells, and cell yields after sorting would not have been sufficient for the experiments used here. However, PLK-early represents thymocytes with an altered gene expression program driven by TLX1 either by direct modulation or secondary effects. They are also the pool of cells that transformed blasts eventually arise from. While the leukemia-initiating cells are likely to arise from DN2 thymocytes, that may not always be the case. Therefore, the genes that TLX1 disrupts in PLK-early are the most likely candidates that promote transformation through various possible mechanisms.

Unexpectedly, our flow cytometry, RNA-seq, and CUT&Tag data together identified the PLK-late stage, which bridged PLK-early and leukemia samples. These mice were harvested at 19, 20, and 24 weeks, but this pattern was seen in less than 20% of PLK mice harvested between 19 and 33 weeks. Moreover, the flow cytometry data showed an accumulation of DN2 cells in PLK2 and PLK6. The three mice also had mixed CD4, CD8, and DP populations. Importantly, the gene expression signatures in PLK-late strongly clustered together independent of cell sub-population distribution (**Figure 17A**), suggesting that PLK-late are rapidly proliferating transformed thymocytes that follow a consistent gene expression program. PLK-late, in fact, represents two situations occurring at the same time: the rapid proliferation of successfully transformed cells and the depletion of normal developing

thymocytes. This complicated the expression analysis as it is difficult to attribute which gene is changing in which sub-population in bulk RNA-seq of a heterogeneous population. However, the color gradient seen from PLK2 to PLK8 to PLK6 in **Figure 17D** showed that the transformed cell population is increasing, with subsets of genes being increasingly upregulated or downregulated. The changes were strong enough that they were treated as their own condition in the analysis.

Our mice cohort showed that leukemia burden has a wide window of 35 to 70 weeks (**Figure 15B**). However, the tight clustering of PLK-late gene expression suggests the transformed cells follow a consistent path that each leukemic mouse may follow before developing further into leukemias with mixed sub-populations. Heatmap clustering of differential genes in PLK-early and PLK-late highlighted a late strong upregulation of terms relating to ribosome biogenesis and DNA replication, suggesting these cells are in a state of rapid proliferation or ribosome hyperactivation (**Figure 17**). Indeed, hyperactivation of ribosome biogenesis is associated with a range of cancers, including T-ALL and AML¹⁷⁵, and this might be a key indicator of late transformation. The other GO-terms (T-cell activation, regulation of apoptosis, etc.) had genes distributed across heatmap clusters that were both upregulated and downregulated. This could mean that these processes are dysregulated, as some genes are overexpressed and others repressed, which could result in dysregulated signaling pathways or incomplete cellular mechanisms. The rate of leukemia development likely depends on various factors as well, including when secondary mutations occur and what they could be, as some may influence the speed of proliferation or enhance survivability. Many of the SVs were also seen in WT6 and PLK5, suggesting these to be artifacts from unofficial blacklisted regions or the genetic background of the mouse line itself (data not shown). Aneuploidy was a factor, however, as NeoLoopFinder identified trisomy-10 in LK3

(**Supplemental Figure 2**). The gene expression programs of the leukemia mice also indicated extensive heterogeneity between leukemias (**Figure 18A**).

4.5 Possible mechanisms of transformation promoted by TLX1.

TLX1 binds almost entirely at promoters and enhancers (**Figure 11**), and our analyses show that TLX1 also localizes to many TAD boundaries and loop anchors (**Figure 12**). The mouse model showed that TLX1 expression resulted in differentially expressed genes, with a large proportion of these genes residing within significantly changing TADs (**Figure 22**). Importantly, genes found in changing TADs in PLK-early were enriched for terms related to neuron and T-cell differentiation, suggesting these processes have common genes that are being dysregulated in early thymocytes expressing TLX1. Genes found in PLK-early differential TADs were also enriched for signaling pathways such as NOTCH and WNT signaling, as well as terms related to other cancers. While differential TAD organization is associated with changes in gene expression and changes in chromatin loop anchor sites, not all genes are necessarily affected within a changing TAD or a changing loop as there are additional levels to regulation for gene expression (**example Figure 24**). TLX1 binds to promoters and enhancers, but TLX1 binding alone does not necessarily alter gene expression, suggesting additional context-specific variables at play. A summary of the mechanisms elucidated here is summarized in **Figure 27**.

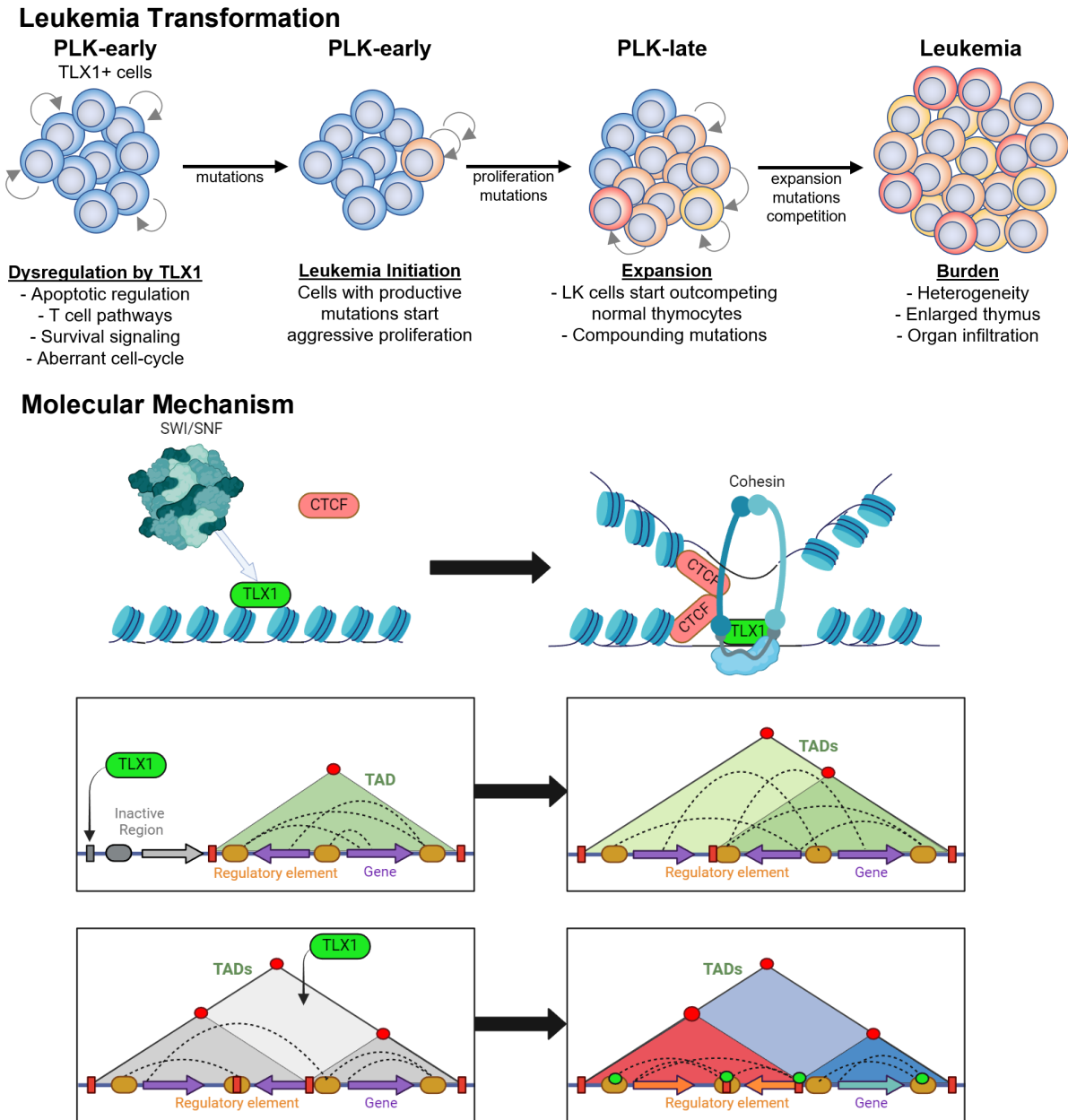


Figure 27. Model of TLX1 driven leukemia transformation. Leukemic transformation by TLX1 is characterized by aberrant gene expression resulting in dysregulation of critical processes that promote the accumulation of secondary mutations while evading apoptosis and blocking differentiation. This results in formation of leukemia cells that rapidly proliferate in the thymus while accumulating additional mutations and outcompeting normal thymocytes. Over time these cells develop into heterogeneous cell populations that enlarge the thymus and infiltrate into other organs. Lower: Part of TLX1 activity identified in this work is the interplay between TLX1, the BAF complex, and CTCF and cohesin. TLX1 has the capacity to alter chromatin accessibility at CTCF sites, resulting in TAD reorganization and activation of genes. In other cases, TLX1 can bind at chromatin looping sites and sub-TAD boundaries found at promoters and enhancers, resulting the reorientation of sub-TAD interactions and changes in gene expression. Figure partially created with BioRender.com .

Several notable genes were differentially expressed in PLK-early or PLK-late and had mixed expression in the leukemia samples. These genes were associated with local TLX1 binding with or without differential TAD changes and looping. Together with CDK4, CDK6 is involved in cell-cycle progression and entry into S-phase through partial phosphorylation of RB^{185,186}. This releases RB from transcription factor E2F1, which can then activate downstream genes for cell-cycle progression^{185,186}. CDK6 is upregulated in PLK-early and remained upregulated in all subsequent phases (**Figure 18B**). TLX1 binding at CDK6 results in changes in local chromatin looping. Upregulation of this gene may enhance the phosphorylation of RB, leading to aberrant S-phase transition and enhanced proliferation. Overexpression of CDK6 is implicated in various myeloid and lymphoid leukemias^{186,187}. CD44 is normally expressed in the DN1/DN2 stage but was upregulated 3-fold in PLK-early and remained upregulated (**Figure 18B**). Flow cytometry in PLK-early did not show a larger proportion of DN1/DN2 cells, suggesting CD44 is upregulated in DN2 cells expressing TLX1. CD44 function is associated with cell migration, proliferation, and anti-apoptotic signaling and is upregulated in other cancers¹⁸⁸. Notably, CD44, RAG1, and RAG2 shared the same differential TAD identified between WT and PLK-early, but RAG1 and RAG2 were only downregulated in PLK-late onwards. BCL11B, which is normally upregulated in DN2 and is critical for thymocyte differentiation⁸⁸, was also downregulated 1.4-fold in PLK-late (**Figure 18B**). BCL11B is a known repressed gene targeted by TLX1 in ALL-SIL and is a subunit of the BAF complex^{6,10,58}. While PLK-early did not show statistically significant downregulation (1.1-fold), the TAD for BCL11B was identified as significantly changing, which may have more pronounced effects later in transformation. Additionally, the core BAF complex subunit SMARCA2 (BRM) was downregulated 1.6-fold in PLK-early with a differential TAD. The BAF complex can either have BRM or BRG1, which may suggest more BRG1-containing

BAF complexes are present in PLK-early. Whether a change in composition influences transformation could warrant future investigation. PTEN was upregulated 1.8-fold in PLK-early with differential TAD rearrangement (**Figure 18B**), and its activity is associated with the PI3K-AKT pathway^{189–191}. PTEN is a negative regulator of the PI3K-AKT pathway, which is involved in promoting cell proliferation, cell survival, and metabolic programming, among other functions¹⁹¹. This pathway is also involved in T-cell differentiation checkpoints as well as transcription factor regulatory networks¹⁹⁰. PTEN deletion has been studied extensively in T-ALL as uncontrolled PI3K-AKT signaling is leukemogenic¹⁹¹. PTEN is a well-known tumor suppressor protein that is frequently deleted or repressed in some cancers, including T-ALLs, but is needed for cell survival and proliferation in others^{189–191}. Overexpression of PTEN in PLK-early may play a role in blocking initial differentiation. Inhibition of the PI3K/AKT pathway in developing thymocytes has been shown to block the DN-to-DP transition¹⁹². PTEN is notably downregulated in LK5 among leukemias. NOTCH1 was significantly upregulated in PLK-late (4.1-fold) (**Figure 18B**). Notably, its main TAD was significantly changing due to sub-TAD reorganization downstream and related differential gene expression in PLK-early. Aberrant NOTCH1 activity is associated mostly with activating point mutations in T-ALLs. Our results may suggest TLX1 modulates or promotes NOTCH1 upregulation indirectly through local TAD changes that result in gene expression changes later in transformation. MYC had no change in expression in PLK-early, but the TAD was called as differential, and TLX1 binding showed differential BRG1, CTCF, and RAD21 binding as well. It was strongly upregulated in PLK-late (2.8-fold), but we do not have the HiC data to verify if these initial changes prime MYC for upregulation in PLK-late. This also suggests that other transcription factors are involved in late transformation. MYC was upregulated in PLK-late, LK2, LK3, and LK8, but not LK5. NOTCH1 was also elevated PLK-late, LK3, LK8, but not LK2 or LK5.

This underscores the heterogeneity of these leukemias and suggests that different transcription factors drive the unique leukemia gene expression programs. The relevance of TLX1 at this stage of leukemia could be more relevant in some leukemias than others, as activation and repression of other transcription factors may synergize or antagonize TLX1 activity. IL-17RB is strongly upregulated in PLK-early (4.4-fold) and shares a differential TAD and loops with upregulated CHDH and CACNA1D. The IL-17RB signaling pathway has been implicated in tumorigenesis by promoting cancer cell proliferation, migration, and cell survival through activation of the ERK/NF-KB pathway¹⁹³. Elevated expression of IL-17RB is found across different cancers and is associated with poorer prognoses¹⁹³. Early upregulation of IL-17RB may be an additional signal for cell survival in TLX1-expressing thymocytes. Gene enrichment analysis of GO-terms highlighted dysregulated processes associated with differentiation, development, and apoptotic signaling. Dysregulation of these programs by TLX1 in thymocytes in combination with dysregulated signaling pathways may promote cell cycle progression and survival while inadvertently driving the accumulation of secondary mutations and transformation towards leukemia.

4.6 Targeting the BAF complex in TLX1 T-ALL.

At the writing of this thesis, a preprint was published showing that Jurkat and other T-ALL cell lines were also sensitive to treatment with drugs targeting the BAF complex^{187,194}. Our drug treatment of ALL-SIL with ACBI-1 and BRG1/BRM inhibitor-1 (**Figure 26**) adds additional evidence for T-ALL cell line sensitivity to BAF complex disruption and its importance to the leukemia gene expression program. Indeed, the BAF complex is critical to proper T-cell differentiation, and previous work has shown an important link between the BAF complex and RUNX1 binding sites^{187,195}. In the context of ALL-SIL, TLX1 interactions with the BAF complex may be critical to the survival of the cells by directing BAF to specific genes, and future studies could look at this further. As T-ALL cell lines driven by other oncogenic transcription factors have been shown to be sensitive as well, disruption of BAF complex activity likely impacts gene expression programs critical to cell proliferation and apoptosis¹⁸⁷.

The BAF complex operates in opposition to other complexes, including PcG complexes⁷⁹. BAF complex subunits have also been found to be frequently mutated across cancer types⁷⁴. BAF subunit mutations can result in dependence on certain combinations of subunits that promote altered transcriptional programs⁷⁴. Alternatively, mutations in the PRC2 complex have been shown to cause dependence on the BAF complex for cancer cell survival (synthetic lethality)⁷⁴. Further characterization of BAF complex function in T-ALL and targeted disruption of related pathways warrant further investigation.

4.7 Limitations

Our HiC analysis provided a wealth of information regarding the organization of the samples analyzed, but HiC has limitations that newer methods try to address. The resolution that HiC analysis can be analyzed at is directly related to read depth. This is partly why we opted to sequence WT6, PLK5, and LK3 at a higher read depth than sequence several samples at a lower read depth. Loop interactions exist at shorter distances than what HiC can achieve, which is about 40kb. Several protocols have been developed recently to acquire more information from HiC-like data, such as HiCAR¹⁰⁸, Hi-TrAC¹⁰⁹, or Micro-C¹⁹⁶. Some of these methods can analyze data at much higher resolution (<1kb) and excel in identifying high-fidelity chromatin loop sites. Our HiC data was able to identify up to ~20,000 chromatin loops and performed very well in this case. Newer methods improve the signal-to-noise ratio of HiC data and show that total chromatin loops can be well in upwards of 75,000 - 200,000 sites.

Using bulk-cell analysis of thymocyte populations was another possible limitation (RNA-seq, CUT&Tag, ATAC-seq, HiC). This complicated data analysis to some degree as it is not possible to distinguish whether a change in a type of data set (gene expression, peak height, TAD signal) was a major change in a sub-population or consistent change across all thymocytes. We were fortunate that the WT and PLK-early conditions had a consistent distribution of cell populations seen by flow cytometry and focused more on characterizing this transition than PLK to LK, partly for this reason. This allowed us to assume all changes occurring in PLK-early were from TLX1 expression, whether direct or indirect. There was some evidence of non-leukemia immune cell infiltration in the leukemia samples that could not be parsed out. FACS isolation of leukemic cells may have mitigated that but may have also excluded unknown sub-populations. Single-cell RNA-seq may have been useful if we were

focusing on specific differences between leukemic sub-populations, but this was not the focus of this work.

CUT&Tag is a recently developed protocol that allowed us the opportunity to do an in-depth analysis of both TFs and histone modifications across model systems. As a new protocol, we also found it required extensive optimization for the protocol itself and the data analysis. We learned that the nature of CUT&Tag, specifically sample-to-sample variability and valid peaks generally having low read counts, was suitable for identifying major changes in peak intensity. Peak calling and differential analysis tools that address these aspects of CUT&Tag are likely being developed that address these challenges. We found in our testing that MACS2 did a better job at peak calling than SEACR¹⁹⁷ or GoPeaks¹⁹⁸ for our datasets, and DiffBind with DEseq2 differential analysis worked well for our purposes as well. Higher read counts and more consistent sequencing results may have identified more changes.

Chapter 5 - Conclusion

This thesis sought to determine if TLX1 established and maintained leukemic cells by dysregulating chromatin loop formation through interactions with CTCF, the cohesin complex, and the BAF complex. Our work showed that, in addition to TLX1 binding at promoters and enhancers, TLX1 binding also perturbed chromatin structures early in the leukemic transformation process both independently and together with its cofactors, which was associated with dysregulated gene expression at critical genes. Our work also identified that TLX1 T-ALL (ALL-SIL) is sensitive to treatment with ACBI-1 and BRG1/BRM inhibitor-1, which warrants further research into T-ALL and the BAF complex. These experiments provide new insights into the mechanism of TLX1-mediated leukemia transformation and possible avenues for targeted treatment for T-ALL in the future.

References

1. Ferrando, A.A., Neuberg, D.S., Staunton, J., Loh, M.L., Huard, C., Raimondi, S.C., Behm, F.G., Pui, C.-H., Downing, J.R., Gilliland, D.G., et al. (2002). Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 1, 75–87. 10.1016/S1535-6108(02)00018-1.
2. Herranz, D., Ambesi-Impiombato, A., Palomero, T., Schnell, S.A., Belver, L., Wendorff, A.A., Xu, L., Castillo-Martin, M., Llobet-Navás, D., Cordon-Cardo, C., et al. (2014). A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. *Nat. Med.* 20, 1130–1137. 10.1038/nm.3665.
3. Tan, S.H., Bertulfo, F.C., and Sanda, T. (2017). Leukemia-initiating cells in T-cell acute lymphoblastic leukemia. *Front. Oncol.* 7. 10.3389/fonc.2017.00218.
4. Goossens, S., and Van Vlierberghe, P. (2014). Controlling Pre-leukemic Thymocyte Self-Renewal. *PLoS Genet.* 10, e1004881. 10.1371/journal.pgen.1004881.
5. Vanden Bempt, M., Demeyer, S., Broux, M., De Bie, J., Bornschein, S., Mentens, N., Vandepoel, R., Geerdens, E., Radaelli, E., Bornhauser, B.C., et al. (2018). Cooperative Enhancer Activation by TLX1 and STAT5 Drives Development of NUP214-ABL1/TLX1-Positive T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* 34, 271–285.e7. 10.1016/j.ccell.2018.07.007.
6. Dadi, S., Le Noir, S., Payet-Bornet, D., Lhermitte, L., Zacarias-Cabeza, J., Bergeron, J., Villarèse, P., Vachez, E., Dik, W.A., Millien, C., et al. (2012). TLX Homeodomain Oncogenes Mediate T Cell Maturation Arrest in T-ALL via Interaction with ETS1 and Suppression of TCR α Gene Expression. *Cancer Cell* 21, 563–576. 10.1016/j.ccr.2012.02.013.
7. Liu, Y., Easton, J., Shao, Y., Maciaszek, J., Wang, Z., Wilkinson, M.R., McCastlain, K., Edmonson, M., Pounds, S.B., Shi, L., et al. (2017). The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. *Nat. Genet.* 49, 1211–1218. 10.1038/ng.3909.
8. Durinck, K., Van Loocke, W., Van der Meulen, J., Van de Walle, I., Ongenaert, M., Rondou, P., Wallaert, A., de Bock, C.E., Van Roy, N., Poppe, B., et al. (2015). Characterization of the genome-wide TLX1 binding profile in T-cell acute lymphoblastic leukemia. *Leukemia* 29, 2317–2327. 10.1038/leu.2015.162.
9. Della Gatta, G., Palomero, T., Perez-Garcia, A., Ambesi-Impiombato, A., Bansal, M., Carpenter, Z.W., De Keersmaecker, K., Sole, X., Xu, L., Paietta, E., et al. (2012). Reverse engineering of TLX oncogenic transcriptional networks identifies RUNX1 as tumor suppressor in T-ALL. *Nat. Med.* 18, 436–440. 10.1038/nm.2610.
10. De Keersmaecker, K., Real, P.J., Gatta, G. Della, Palomero, T., Sulis, M.L., Tosello, V., Van Vlierberghe, P., Barnes, K., Castillo, M., Sole, X., et al. (2010). The TLX1 oncogene drives aneuploidy in T cell transformation. *Nat. Med.* 16, 1321–1327. 10.1038/nm.2246.
11. Nagel, S., Pommerenke, C., Scherr, M., Meyer, C., Kaufmann, M., Battmer, K., MacLeod, R.A.F., and Drexler, H.G. (2017). NKL homeobox gene activities in hematopoietic stem cells, T-cell development and T-cell leukemia. *PLoS One* 12, e0171164. 10.1371/journal.pone.0171164.
12. Rakowski, L.A., Lehotzky, E.A., and Chiang, M.Y. (2011). Transient Responses to

- NOTCH and TLX1/HOX11 Inhibition in T-Cell Acute Lymphoblastic Leukemia/Lymphoma. *PLoS One* 6, e16761. 10.1371/journal.pone.0016761.
13. Riz, I., Lee, H.J., Baxter, K.K., Behnam, R., Hawley, T.S., and Hawley, R.G. (2009). Transcriptional activation by TLX1/HOX11 involves Gro/TLE corepressors. *Biochem. Biophys. Res. Commun.* 380, 361–365. 10.1016/j.bbrc.2009.01.099.
 14. Zhang, Y., Gao, S., Xia, J., and Liu, F. (2018). Hematopoietic Hierarchy – An Updated Roadmap. *Trends Cell Biol.* 28, 976–986. 10.1016/j.tcb.2018.06.001.
 15. Buenrostro, J.D., Corces, M.R., Lareau, C.A., Wu, B., Schep, A.N., Aryee, M.J., Majeti, R., Chang, H.Y., and Greenleaf, W.J. (2018). Integrated Single-Cell Analysis Maps the Continuous Regulatory Landscape of Human Hematopoietic Differentiation. *Cell* 173, 1535–1548.e16. 10.1016/j.cell.2018.03.074.
 16. Koch, U., and Radtke, F. (2011). Mechanisms of T Cell Development and Transformation. *Annu. Rev. Cell Dev. Biol.* 27, 539–562. 10.1146/annurev-cellbio-092910-154008.
 17. Rodríguez-Caparrós, A., Álvarez-Santiago, J., Del Valle-Pastor, M.J., Suñé, C., López-Ros, J., and Hernández-Munain, C. (2020). Regulation of T-cell receptor gene expression by three-dimensional locus conformation and enhancer function. *Int. J. Mol. Sci.* 21, 1–23. 10.3390/ijms21228478.
 18. Holowiecki, J. (2005). NOTCH1 pathway: A molecular target in T-cell cancers? *Lancet* 365, 197–199. 10.1016/S0140-6736(05)70128-8.
 19. Sun, L., Su, Y., Jiao, A., Wang, X., and Zhang, B. (2023). T cells in health and disease. *Signal Transduct. Target. Ther.* 8. 10.1038/s41392-023-01471-y.
 20. Trinquand, A., dos Santos, N.R., Tran Quang, C., Rocchetti, F., Zaniboni, B., Belhocine, M., Da Costa de Jesus, C., Lhermitte, L., Tesio, M., Dussiot, M., et al. (2016). Triggering the TCR Developmental Checkpoint Activates a Therapeutically Targetable Tumor Suppressive Pathway in T-cell Leukemia. *Cancer Discov.* 6, 972–985. 10.1158/2159-8290.CD-15-0675.
 21. Dutta, A., Zhao, B., and Love, P.E. (2021). New insights into TCR β -selection. *Trends Immunol.* 42, 735–750. 10.1016/j.it.2021.06.005.
 22. Katapadi, V.K., Nambiar, M., and Raghavan, S.C. (2012). Potential G-quadruplex formation at breakpoint regions of chromosomal translocations in cancer may explain their fragility. *Genomics* 100, 72–80. 10.1016/j.ygeno.2012.05.008.
 23. Girardi, T., Vicente, C., Cools, J., and De Keersmaecker, K. (2017). The genetics and molecular biology of T-ALL. *Blood* 129, 1113–1123. 10.1182/blood-2016-10-706465.
 24. Nambiar, M., Srivastava, M., Gopalakrishnan, V., Sankaran, S.K., and Raghavan, S.C. (2013). G-Quadruplex Structures Formed at the HOX11 Breakpoint Region Contribute to Its Fragility during t(10;14) Translocation in T-Cell Leukemia. *Mol. Cell. Biol.* 33, 4266–4281. 10.1128/mcb.00540-13.
 25. Hatano, M., Roberts, C., Minden, M., Crist, W., and Korsmeyer, S. (1991). Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science* (80-.). 253, 79–82. 10.1126/science.1676542.
 26. Noronha, E.P., Marques, L.V.C., Andrade, F.G., Thuler, L.C.S., Terra-Granado, E., and Pombo-de-Oliveira, M.S. (2019). The Profile of Immunophenotype and Genotype Aberrations in Subsets of Pediatric T-Cell Acute Lymphoblastic Leukemia. *Front. Oncol.* 9, 1–10. 10.3389/fonc.2019.00316.

27. Riz, I., Hawley, T.S., Johnston, H., and Hawley, R.G. (2009). Role of TLX1 in T-cell acute lymphoblastic leukaemia pathogenesis. *Br. J. Haematol.* *145*, 140–143. 10.1111/j.1365-2141.2008.07556.x.
28. Ferrando, A.A., Neuberg, D.S., Dodge, R.K., Paietta, E., Larson, R.A., Wiernik, P.H., Rowe, J.M., Caligiuri, M.A., Bloomfield, C.D., and Look, A.T. (2004). Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. *Lancet* *363*, 535–536. 10.1016/S0140-6736(04)15542-6.
29. Kees, U.R., Heerema, N.A., Kumar, R., Watt, P.M., Baker, D.L., La, M.K., Uckun, F.M., and Sather, H.N. (2003). Expression of HOX11 in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG). *Leukemia* *17*, 887–893. 10.1038/sj.leu.2402892.
30. Hélias, C., Andrieux, J., Nguyen Khac, F., Dastugue, N., Busson, M., Berger, R., Lafage-Pochitaloff, M., Léonard, C., Speleman, F., Ballerini, P., et al. (2003). t(5;14)/HOX11L2-positive T-cell acute lymphoblastic leukemia. A collaborative study of the Groupe Français de Cytogénétique Hématologique (GFCH). *Leukemia* *17*, 1851–1857. 10.1038/sj.leu.2403061.
31. Ballerini, P., Landman-Parker, J., Cayuela, J.M., Asnafi, V., Labopin, M., Gandemer, V., Perel, Y., Michel, G., Leblanc, T., Schmitt, C., et al. (2008). Impact of genotype on survival of children with T-cell acute lymphoblastic leukemia treated according to the French protocol FRALLE-93: The effect of TLX3/HOX11L2 gene expression on outcome. *Haematologica* *93*, 1658–1665. 10.3324/haematol.13291.
32. Hu, Y., Liu, C., and Muyldermans, S. (2017). Nanobody-Based Delivery Systems for Diagnosis and Targeted Tumor Therapy. *Front. Immunol.* *8*. 10.3389/fimmu.2017.01442.
33. Simon, C., Chagraoui, J., Kros, J., Gendron, P., Wilhelm, B., Lemieux, S., Boucher, G., Chagnon, P., Drouin, S., Lambert, R., et al. (2012). A key role for EZH2 and associated genes in mouse and human adult T-cell acute leukemia. *Genes Dev.* *26*, 651–656. 10.1101/gad.186411.111.
34. Salmerón-Villalobos, J., Ramis-Zaldivar, J.E., Balagué, O., Verdú-Amorós, J., Celis, V., Sábado, C., Garrido, M., Mato, S., Uriz, J., Ortega, M.J., et al. (2022). Diverse mutations and structural variations contribute to Notch signaling deregulation in paediatric T-cell lymphoblastic lymphoma. *Pediatr. Blood Cancer* *69*. 10.1002/pbc.29926.
35. Chiang, M.Y., Xu, L., Shestova, O., Histen, G., L'Heureux, S., Romany, C., Childs, M.E., Gimotty, P.A., Aster, J.C., and Pear, W.S. (2008). Leukemia-associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. *J. Clin. Invest.* *118*, 3181–3194. 10.1172/JCI35090.
36. Zhang, Z., Yang, K., and Zhang, H. (2022). Targeting Leukemia-Initiating Cells and Leukemic Niches: The Next Therapy Station for T-Cell Acute Lymphoblastic Leukemia? *Cancers (Basel)*. *14*, 1–25. 10.3390/cancers14225655.
37. Weng, A.P., Ferrando, A.A., Lee, W., Morris IV, J.P., Silverman, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T., and Aster, J.C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science (80-.)*. *306*, 269–271. 10.1126/science.1102160.

38. Weirauch, M.T., and Hughes, T.R. (2011). A Handbook of Transcription Factors T. R. Hughes, ed. (Springer Netherlands) 10.1007/978-90-481-9069-0.
39. Paul, R., Peraldi, R., and Kmita, M. (2023). The pioneering function of the hox transcription factors. *Semin. Cell Dev. Biol.* 10.1016/j.semcdb.2022.11.013.
40. Dard, A., Reboulet, J., Jia, Y., Bleicher, F., Duffraisse, M., Vanaker, J.M., Forcet, C., and Merabet, S. (2018). Human HOX Proteins Use Diverse and Context-Dependent Motifs to Interact with TALE Class Cofactors. *Cell Rep.* 22, 3058–3071. 10.1016/j.celrep.2018.02.070.
41. Jerković, I., Ibrahim, D.M., Andrey, G., Haas, S., Hansen, P., Janetzki, C., González Navarrete, I., Robinson, P.N., Hecht, J., and Mundlos, S. (2017). Genome-Wide Binding of Posterior HOXA/D Transcription Factors Reveals Subgrouping and Association with CTCF. *PLOS Genet.* 13, e1006567. 10.1371/journal.pgen.1006567.
42. Carnesecchi, J., Pinto, P.B., and Lohmann, I. (2018). Hox transcription factors: An overview of multi-step regulators of gene expression. *Int. J. Dev. Biol.* 62, 723–732. 10.1387/ijdb.180294il.
43. Holland, P.W.H., Booth, H.A.F., and Bruford, E.A. (2007). Classification and nomenclature of all human homeobox genes. *BMC Biol.* 5, 47. 10.1186/1741-7007-5-47.
44. Rice, K., Heidari, M., Taplin, R., Kees, U., and Greene, W.K. (2009). Transcriptional regulation of the human ALDH1A1 promoter by the oncogenic homeoprotein TLX1/HOX11. *Hematol. Rep.* 1, 13. 10.4081/hr.2009.e13.
45. Lenti, E., Farinello, D., Yokoyama, K.K., Penkov, D., Castagnaro, L., Lavorgna, G., Wuputra, K., Sandell, L.L., Tjaden, N.E.B., Bernassola, F., et al. (2016). Transcription factor TLX1 controls retinoic acid signaling to ensure spleen development. *J. Clin. Invest.* 126, 2452–2464. 10.1172/JCI82956.
46. Roberts, C.W.M., Shutter, J.R., and Korsmeyer, S.J. (1994). Hox11 controls the genesis of the spleen. *Nature* 368, 747–749. 10.1038/368747a0.
47. Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P.A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P., et al. (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. *Nat. Neurosci.* 7, 510–517. 10.1038/nn1221.
48. Shimomura, A., Patel, D., Wilson, S.M., Koehler, K.R., Khanna, R., and Hashino, E. (2015). Tlx3 Promotes Glutamatergic Neuronal Subtype Specification through Direct Interactions with the Chromatin Modifier CBP. *PLoS One* 10, e0135060. 10.1371/journal.pone.0135060.
49. Patterson, K.D., and Krieg, P.A. (1999). Hox11-family genes XHox11 and XHox11L2 in *Xenopus*: XHox11L2 expression is restricted to a subset of the primary sensory neurons. *Dev. Dyn.* 214, 34–43. 10.1002/(SICI)1097-0177(199901)214:1<34::AID-DVDY4>3.0.CO;2-R.
50. Qian, Y., Shirasawa, S., Chen, C.-L.L., Cheng, L., Ma, Q., and Qiufu, M. (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes Rnx/Tlx-3 and Tlx-1. *Genes Dev.* 16, 1220–1233. 10.1101/gad.982802.
51. Borghini, S., Di Duca, M., Santamaria, G., Vargiolu, M., Bachetti, T., Cargnin, F., Pini Prato, A., De Giorgio, R., Lerone, M., Stanghellini, V., et al. (2007). Transcriptional

- regulation of TLX2 and impaired intestinal innervation: Possible role of the PHOX2A and PHOX2B genes. *Eur. J. Hum. Genet.* *15*, 848–855. 10.1038/sj.ejhg.5201852.
52. Pujato, M., Kieken, F., Skiles, A.A., Tapinos, N., and Fiser, A. (2014). Prediction of DNA binding motifs from 3D models of transcription factors; identifying TLX3 regulated genes. *Nucleic Acids Res.* *42*, 13500–13512. 10.1093/nar/gku1228.
 53. Heidari, M., Rice, K.L., Phillips, J.K., Kees, U.R., and Greene, W.K. (2006). The nuclear oncoprotein TLX1/HOX11 associates with pericentromeric satellite 2 DNA in leukemic T-cells. *Leukemia* *20*, 304–312. 10.1038/sj.leu.2404071.
 54. Rice, K.L., Izon, D.J., Ford, J., Boodhoo, A., Kees, U.R., and Greene, W.K. (2008). Overexpression of stem cell associated ALDH1A1, a target of the leukemogenic transcription factor TLX1/HOX11, inhibits lymphopoiesis and promotes myelopoiesis in murine hematopoietic progenitors. *Leuk. Res.* *32*, 873–883. 10.1016/j.leukres.2007.11.001.
 55. Owens, B.M., Zhu, Y.-X., Suen, T.-C., Wang, P.-X., Greenblatt, J.F., Goss, P.E., and Hawley, R.G. (2003). Specific homeodomain-DNA interactions are required for HOX11-mediated transformation. *Blood* *101*, 4966–4974. 10.1182/blood-2002-09-2857.
 56. Kagan, J., Finger, L.R., Letofsky, J., Finan, J., Nowell, P.C., and Croce, C.M. (1989). Clustering of breakpoints on chromosome 10 in acute T-cell leukemias with the t(10;14) chromosome translocation. *Proc. Natl. Acad. Sci. U. S. A.* *86*, 4161–4165. 10.1073/pnas.86.11.4161.
 57. Bernard, O.A., Busson-LeConiat, M., Ballerini, P., Mauchauffé, M., Della Valle, V., Monni, R., Khac, F.N., Mercher, T., Penard-Lacronique, V., Pasturaud, P., et al. (2001). A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. *Leukemia* *15*, 1495–1504. 10.1038/sj.leu.2402249.
 58. Zweier-Renn, L.A., Riz, I., Hawley, T.S., and Hawley, R.G. (2013). The DN2 Myeloid-T (DN2mt) Progenitor is a Target Cell for Leukemic Transformation by the TLX1 Oncogene. *J. bone marrow Res.* *1*, 1–12. 10.4172/2329-8820.1000105.
 59. Graux, C., Cools, J., Melotte, C., Quentmeier, H., Ferrando, A., Levine, R., Vermeesch, J.R., Stul, M., Dutta, B., Boeckx, N., et al. (2004). Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. *Nat. Genet.* *36*, 1084–1089. 10.1038/ng1425.
 60. Knoechel, B., Roderick, J.E., Williamson, K.E., Zhu, J., Lohr, J.G., Cotton, M.J., Gillespie, S.M., Fernandez, D., Ku, M., Wang, H., et al. (2014). An epigenetic mechanism of resistance to targeted therapy in T cell acute lymphoblastic leukemia. *Nat. Genet.* *46*, 364–370. 10.1038/ng.2913.
 61. Verboom, K., Van Looke, W., Volders, P.-J., Decaestecker, B., Cobos, F.A., Bornschein, S., de Bock, C.E., Atak, Z.K., Clappier, E., Aerts, S., et al. (2018). A comprehensive inventory of TLX1 controlled long non-coding RNAs in T-cell acute lymphoblastic leukemia through polyA+ and total RNA sequencing. *Haematologica* *103*, e585–e589. 10.3324/haematol.2018.190587.
 62. Centore, R.C., Sandoval, G.J., Soares, L.M.M., Kadoch, C., and Chan, H.M. (2020). Mammalian SWI/SNF Chromatin Remodeling Complexes: Emerging Mechanisms and Therapeutic Strategies. *Trends Genet.* *36*, 936–950. 10.1016/j.tig.2020.07.011.

63. Krasteva, V., Crabtree, G.R., and Lessard, J.A. (2017). The BAF45a/PHF10 subunit of SWI/SNF-like chromatin remodeling complexes is essential for hematopoietic stem cell maintenance. *Exp. Hematol.* *48*, 58-71.e15. 10.1016/j.exphem.2016.11.008.
64. Lessard, J.A., and Crabtree, G.R. (2010). Chromatin regulatory mechanisms in pluripotency. *Annu. Rev. Cell Dev. Biol.* *26*, 503–532. 10.1146/annurev-cellbio-051809-102012.
65. Ho, L., Ronan, J.L., Wu, J., Staahl, B.T., Chen, L., Kuo, A., Lessard, J., Nesvizhskii, A.I., Ranish, J., and Crabtree, G.R. (2009). An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency. *Proc. Natl. Acad. Sci. U. S. A.* *106*, 5181–5186. 10.1073/pnas.0812889106.
66. Gatchalian, J., Liao, J., Maxwell, M.B., and Hargreaves, D.C. (2020). Control of Stimulus-Dependent Responses in Macrophages by SWI/SNF Chromatin Remodeling Complexes. *Trends Immunol.* *41*, 126–140. 10.1016/j.it.2019.12.002.
67. Wu, J.I., Lessard, J., Olave, I.A., Qiu, Z., Ghosh, A., Graef, I.A., and Crabtree, G.R. (2007). Regulation of Dendritic Development by Neuron-Specific Chromatin Remodeling Complexes. *Neuron* *56*, 94–108. 10.1016/j.neuron.2007.08.021.
68. Euskirchen, G., Auerbach, R.K., and Snyder, M. (2012). SWI/SNF Chromatin-remodeling Factors: Multiscale Analyses and Diverse Functions. *J. Biol. Chem.* *287*, 30897–30905. 10.1074/jbc.R111.309302.
69. Clapier, C.R., Iwasa, J., Cairns, B.R., and Peterson, C.L. (2017). Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nat. Rev. Mol. Cell Biol.* *18*, 407–422. 10.1038/nrm.2017.26.
70. Hohmann, A.F., and Vakoc, C.R. (2014). A rationale to target the SWI/SNF complex for cancer therapy. *Trends Genet.* *30*, 356–363. 10.1016/j.tig.2014.05.001.
71. Ho, P.J., Lloyd, S.M., and Bao, X. (2019). Unwinding chromatin at the right places: How BAF is targeted to specific genomic locations during development. *Dev.* *146*, 1–14. 10.1242/dev.178780.
72. Kadam, S., and Emerson, B.M. (2003). Transcriptional Specificity of Human SWI/SNF BRG1 and BRM Chromatin Remodeling Complexes. *Mol. Cell* *11*, 377–389. 10.1016/S1097-2765(03)00034-0.
73. Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P.A., Biggar, S.R., Muchardt, C., Kalpana, G. V, Goff, S.P., Yaniv, M., et al. (1996). Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *EMBO J.* *15*, 5370–5382.
74. Lu, C., and Allis, C.D. (2017). SWI/SNF complex in cancer. *Nat. Genet.* *49*, 178–179. 10.1038/ng.3779.
75. Laurent, A., Bihan, R., Omilli, F., Deschamps, S., and Pellerin, I. (2008). PBX proteins: Much more than Hox cofactors. *Int. J. Dev. Biol.* *52*, 9–20. 10.1387/ijdb.072304al.
76. Samartzis, E., Noske, A., Dedes, K., Fink, D., and Imesch, P. (2013). ARID1A Mutations and PI3K/AKT Pathway Alterations in Endometriosis and Endometriosis-Associated Ovarian Carcinomas. *Int. J. Mol. Sci.* *14*, 18824–18849. 10.3390/ijms140918824.
77. Nakayama, R.T., Pulice, J.L., Valencia, A.M., McBride, M.J., McKenzie, Z.M., Gillespie, M.A., Ku, W.L., Teng, M., Cui, K., Williams, R.T., et al. (2017). SMARCB1 is required for widespread BAF complex-mediated activation of enhancers and bivalent

- promoters. *Nat. Genet.* *49*, 1613–1623. 10.1038/ng.3958.
78. Alver, B.H., Kim, K.H., Lu, P., Wang, X., Manchester, H.E., Wang, W., Haswell, J.R., Park, P.J., and Roberts, C.W.M. (2017). The SWI/SNF chromatin remodelling complex is required for maintenance of lineage specific enhancers. *Nat. Commun.* *8*, 14648. 10.1038/ncomms14648.
 79. Hargreaves, D.C., and Crabtree, G.R. (2011). ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. *Cell Res.* *21*, 396–420. 10.1038/cr.2011.32.
 80. Stanton, B.Z., Hodges, C., Calarco, J.P., Braun, S.M.G., Ku, W.L., Kadoch, C., Zhao, K., and Crabtree, G.R. (2017). Smarca4 ATPase mutations disrupt direct eviction of PRC1 from chromatin. *Nat. Genet.* *49*, 282–288. 10.1038/ng.3735.
 81. Barutcu, A.R., Lian, J.B., Stein, J.L., Stein, G.S., and Imbalzano, A.N. (2017). The connection between BRG1, CTCF and topoisomerases at TAD boundaries. *Nucleus* *8*, 150–155. 10.1080/19491034.2016.1276145.
 82. Barutcu, A.R., Lajoie, B.R., Fritz, A.J., McCord, R.P., Nickerson, J.A., van Wijnen, A.J., Lian, J.B., Stein, J.L., Dekker, J., Stein, G.S., et al. (2016). SMARCA4 regulates gene expression and higher-order chromatin structure in proliferating mammary epithelial cells. *Genome Res.* *26*, 1188–1201. 10.1101/gr.201624.115.
 83. Michel, B.C., D’Avino, A.R., Cassel, S.H., Mashtalir, N., McKenzie, Z.M., McBride, M.J., Valencia, A.M., Zhou, Q., Bocker, M., Soares, L.M.M., et al. (2018). A non-canonical SWI/SNF complex is a synthetic lethal target in cancers driven by BAF complex perturbation. *Nat. Cell Biol.* *20*, 1410–1420. 10.1038/s41556-018-0221-1.
 84. Priam, P., Krasteva, V., Rousseau, P., D’Angelo, G., Gaboury, L., Sauvageau, G., and Lessard, J.A. (2017). SMARCD2 subunit of SWI/SNF chromatin-remodeling complexes mediates granulopoiesis through a CEBP ϵ dependent mechanism. *Nat. Genet.* *49*, 753–764. 10.1038/ng.3812.
 85. Spaeth, J.M., Liu, J.H., Peters, D., Guo, M., Osipovich, A.B., Mohammadi, F., Roy, N., Bhushan, A., Magnuson, M.A., Hebrok, M., et al. (2019). The Pdx1-bound Swi/Snf chromatin remodeling complex regulates pancreatic progenitor cell proliferation and mature islet β -cell function. *Diabetes* *68*, 1806–1818. 10.2337/db19-0349.
 86. Bakshi, R., Hassan, M.Q., Pratap, J., Lian, J.B., Montecino, M.A., van Wijnen, A.J., Stein, J.L., Imbalzano, A.N., and Stein, G.S. (2010). The human SWI/SNF complex associates with RUNX1 to control transcription of hematopoietic target genes. *J. Cell. Physiol.* *225*, 569–576. 10.1002/jcp.22240.
 87. Li, P., and Leonard, W.J. (2018). Chromatin Accessibility and Interactions in the Transcriptional Regulation of T Cells. *Front. Immunol.* *9*, 1–8. 10.3389/fimmu.2018.02738.
 88. Wakabayashi, Y., Watanabe, H., Inoue, J., Takeda, N., Sakata, J., Mishima, Y., Hitomi, J., Yamamoto, T., Utsuyama, M., Niwa, O., et al. (2003). Bcl11b is required for differentiation and survival of $\alpha\beta$ T lymphocytes. *Nat. Immunol.* *4*, 533–539. 10.1038/ni927.
 89. Li, L., Leid, M., and Rothenberg, E. V. (2010). An Early T Cell Lineage Commitment Checkpoint Dependent on the Transcription Factor Bcl11b. *Science* (80-.). *329*, 89–93. 10.1126/science.1188989.
 90. Hu, G., Cui, K., Fang, D., Hirose, S., Wang, X., Wangsa, D., Jin, W., Ried, T., Liu, P., Zhu, J., et al. (2018). Transformation of Accessible Chromatin and 3D Nucleome

- Underlies Lineage Commitment of Early T Cells. *Immunity* 48, 227–242.e8. 10.1016/j.immuni.2018.01.013.
91. Gebuhr, T.C., Kovalev, G.I., Bultman, S., Godfrey, V., Su, L., and Magnuson, T. (2003). The Role of Brg1, a Catalytic Subunit of Mammalian Chromatin-remodeling Complexes, in T Cell Development. *J. Exp. Med.* 198, 1937–1949. 10.1084/jem.20030714.
 92. Roberts, C.W.M., Leroux, M.M., Fleming, M.D., and Orkin, S.H. (2002). Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene *Snf5*. *Cancer Cell* 2, 415–425. 10.1016/S1535-6108(02)00185-X.
 93. Mittal, P., and Roberts, C.W.M. (2020). The SWI/SNF complex in cancer — biology, biomarkers and therapy. *Nat. Rev. Clin. Oncol.* 17, 435–448. 10.1038/s41571-020-0357-3.
 94. Morel, D., Almouzni, G., Soria, J.-C., and Postel-Vinay, S. (2016). Targeting chromatin defects in selected solid tumors based on oncogene addiction, synthetic lethality and epigenetic antagonism. *Ann. Oncol.* 28, mdw552. 10.1093/annonc/mdw552.
 95. Bitler, B.G., Aird, K.M., Garipov, A., Li, H., Amatangelo, M., Kossenkov, A. V., Schultz, D.C., Liu, Q., Shih, I.-M., Conejo-Garcia, J.R., et al. (2015). Synthetic lethality by targeting EZH2 methyltransferase activity in ARID1A-mutated cancers. *Nat. Med.* 21, 231–238. 10.1038/nm.3799.
 96. Sandoval, G.J., Pulice, J.L., Pakula, H., Schenone, M., Takeda, D.Y., Pop, M., Boulay, G., Williamson, K.E., McBride, M.J., Pan, J., et al. (2018). Binding of TMPRSS2-ERG to BAF Chromatin Remodeling Complexes Mediates Prostate Oncogenesis. *Mol. Cell* 71, 554–566.e7. 10.1016/j.molcel.2018.06.040.
 97. Rowley, M.J., and Corces, V.G. (2018). Organizational principles of 3D genome architecture. *Nat. Rev. Genet.* 19. 10.1038/s41576-018-0060-8.
 98. Hansen, A.S., Cattoglio, C., Darzacq, X., and Tjian, R. (2018). Recent evidence that TADs and chromatin loops are dynamic structures. *Nucleus* 9, 20–32. 10.1080/19491034.2017.1389365.
 99. Cremer, T., and Cremer, C. (2001). Chromosome territories, nuclear architecture and gene regulation in mammalian cells. *Nat. Rev. Genet.* 2, 292–301. 10.1038/35066075.
 100. Lieberman-Aiden, E., van Berkum, N.L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., Amit, I., Lajoie, B.R., Sabo, P.J., Dorschner, M.O., et al. (2009). Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome. *Science* (80-.). 326, 289–293. 10.1126/science.1181369.
 101. Rao, S.S.P., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., et al. (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell* 159, 1665–1680. 10.1016/j.cell.2014.11.021.
 102. Robson, M.I., Ringel, A.R., and Mundlos, S. (2019). Regulatory Landscaping: How Enhancer-Promoter Communication Is Sculpted in 3D. *Mol. Cell* 74, 1110–1122. 10.1016/j.molcel.2019.05.032.
 103. Muñoz, S., Minamino, M., Casas-Delucchi, C.S., Patel, H., and Uhlmann, F. (2019). A Role for Chromatin Remodeling in Cohesin Loading onto Chromosomes. *Mol. Cell* 74, 664–673.e5. 10.1016/j.molcel.2019.02.027.
 104. Wang, X., Xu, J., Zhang, B., Hou, Y., Song, F., Lyu, H., and Yue, F. (2021). Genome-

- wide detection of enhancer-hijacking events from chromatin interaction data in rearranged genomes. *Nat. Methods* 18, 661–668. 10.1038/s41592-021-01164-w.
105. Wang, X., Luan, Y., and Yue, F. (2022). EagleC: A deep-learning framework for detecting a full range of structural variations from bulk and single-cell contact maps. *Sci. Adv.* 8. 10.1126/sciadv.abn9215.
 106. Marchal, C., Singh, N., Corso-Díaz, X., and Swaroop, A. (2022). HiCRes: a computational method to estimate and predict the genomic resolution of Hi-C libraries. *Nucleic Acids Res.* 50, E35. 10.1093/nar/gkab1235.
 107. Hsieh, T.H.S., Weiner, A., Lajoie, B., Dekker, J., Friedman, N., and Rando, O.J. (2015). Mapping Nucleosome Resolution Chromosome Folding in Yeast by Micro-C. *Cell* 162, 108–119. 10.1016/j.cell.2015.05.048.
 108. Wei, X., Xiang, Y., Peters, D.T., Li, W., Southerland, K.W., Diao, Y., Wei, X., Xiang, Y., Peters, D.T., Marius, C., et al. (2022). Technology HiCAR is a robust and sensitive method to analyze open-chromatin-associated genome organization Technology HiCAR is a robust and sensitive method to analyze open-chromatin-associated genome organization. *Mol. Cell*, 1–14. 10.1016/j.molcel.2022.01.023.
 109. Liu, S., Cao, Y., Cui, K., Tang, Q., and Zhao, K. (2022). Hi-TrAC reveals division of labor of transcription factors in organizing chromatin loops. *Nat. Commun.* 13. 10.1038/s41467-022-34276-8.
 110. Pelham-Webb, B., Murphy, D., and Apostolou, E. (2020). Dynamic 3D Chromatin Reorganization during Establishment and Maintenance of Pluripotency. *Stem Cell Reports* 15, 1176–1195. 10.1016/j.stemcr.2020.10.012.
 111. Wang, J., Lawry, S.T., Cohen, A.L., and Jia, S. (2014). Chromosome boundary elements and regulation of heterochromatin spreading. *Cell. Mol. Life Sci.* 71, 4841–4852. 10.1007/s00018-014-1725-x.
 112. Hanssen, L.L.P., Kassouf, M.T., Oudelaar, A.M., Biggs, D., Preece, C., Downes, D.J., Gosden, M., Sharpe, J.A., Sloane-Stanley, J.A., Hughes, J.R., et al. (2017). Tissue-specific CTCF–cohesin-mediated chromatin architecture delimits enhancer interactions and function in vivo. *Nat. Cell Biol.* 19, 952–961. 10.1038/ncb3573.
 113. Hua, P., Badat, M., Hanssen, L.L.P., Hentges, L.D., Crump, N., Downes, D.J., Jeziorska, D.M., Oudelaar, A.M., Schwessinger, R., Taylor, S., et al. (2021). Defining genome architecture at base-pair resolution. *Nature*. 10.1038/s41586-021-03639-4.
 114. Fang, C., Wang, Z., Han, C., Safgren, S., Helmin, K., Adelman, E., Eagen, K., Gaspar-Maia, A., Figueroa, M., Singer, B., et al. (2020). Cancer-specific CTCF binding facilitates oncogenic transcriptional dysregulation. 1–30. 10.1101/2020.01.17.910687.
 115. Cuartero, S., Weiss, F.D., Dharmalingam, G., Guo, Y., Ing-Simmons, E., Masella, S., Robles-Rebollo, I., Xiao, X., Wang, Y.F., Barozzi, I., et al. (2018). Control of inducible gene expression links cohesin to hematopoietic progenitor self-renewal and differentiation. *Nat. Immunol.* 19, 932–941. 10.1038/s41590-018-0184-1.
 116. Kloetgen, A., Thandapani, P., Ntziachristos, P., Ghebrechristos, Y., Nomikou, S., Lazaris, C., Chen, X., Hu, H., Bakogianni, S., Wang, J., et al. (2020). Three-dimensional chromatin landscapes in T cell acute lymphoblastic leukemia. *Nat. Genet.* 52, 388–400. 10.1038/s41588-020-0602-9.
 117. Hildebrand, E.M., and Dekker, J. (2020). Mechanisms and Functions of Chromosome Compartmentalization. *Trends Biochem. Sci.* 45, 385–396. 10.1016/j.tibs.2020.01.002.

118. Mir, M., Bickmore, W., Furlong, E.E.M., and Narlikar, G. (2019). Chromatin topology, condensates and gene regulation: Shifting paradigms or just a phase? *Dev.* *146*, 1–6. 10.1242/dev.182766.
119. Nuebler, J., Fudenberg, G., Imakaev, M., Abdennur, N., and Mirny, L.A. (2018). Chromatin organization by an interplay of loop extrusion and compartmental segregation. *Proc. Natl. Acad. Sci. U. S. A.* *115*, E6697–E6706. 10.1073/pnas.1717730115.
120. Kim, S., and Shendure, J. (2019). Mechanisms of Interplay between Transcription Factors and the 3D Genome. *Mol. Cell* *76*, 306–319. 10.1016/j.molcel.2019.08.010.
121. Liang, Z., Zickler, D., Prentiss, M., Chang, F.S., Witz, G., Maeshima, K., and Kleckner, N. (2015). Chromosomes progress to metaphase in multiple discrete steps via global compaction/expansion cycles. *Cell* *161*, 1124–1137. 10.1016/j.cell.2015.04.030.
122. Nagano, T., Lubling, Y., Várnai, C., Dudley, C., Leung, W., Baran, Y., Mendelson Cohen, N., Wingett, S., Fraser, P., and Tanay, A. (2017). Cell-cycle dynamics of chromosomal organization at single-cell resolution. *Nature* *547*, 61–67. 10.1038/nature23001.
123. Fudenberg, G., Imakaev, M., Lu, C., Goloborodko, A., Abdennur, N., and Mirny, L.A. (2016). Formation of Chromosomal Domains by Loop Extrusion. *Cell Rep.* *15*, 2038–2049. 10.1016/j.celrep.2016.04.085.
124. Hafner, A., and Boettiger, A. (2023). The spatial organization of transcriptional control. *Nat. Rev. Genet.* *24*, 53–68. 10.1038/s41576-022-00526-0.
125. Nagy, G., Czipa, E., Steiner, L., Nagy, T., Pongor, S., Nagy, L., and Barta, E. (2016). Motif oriented high-resolution analysis of ChIP-seq data reveals the topological order of CTCF and cohesin proteins on DNA. *BMC Genomics* *17*. 10.1186/s12864-016-2940-7.
126. Tang, Z., Luo, O.J., Li, X., Zheng, M., Zhu, J.J., Szalaj, P., Trzaskoma, P., Magalska, A., Wlodarczyk, J., Ruszczycki, B., et al. (2015). CTCF-Mediated Human 3D Genome Architecture Reveals Chromatin Topology for Transcription. *Cell* *163*, 1611–1627. 10.1016/j.cell.2015.11.024.
127. Rao, S.S.P., Huang, S.-C., Glenn St Hilaire, B., Engreitz, J.M., Perez, E.M., Kieffer-Kwon, K.-R., Sanborn, A.L., Johnstone, S.E., Bascom, G.D., Bochkov, I.D., et al. (2017). Cohesin Loss Eliminates All Loop Domains. *Cell* *171*, 305–320.e24. 10.1016/j.cell.2017.09.026.
128. Horsfield, J.A. (2023). Full circle: a brief history of cohesin and the regulation of gene expression. *FEBS J.* *290*, 1670–1687. 10.1111/febs.16362.
129. Valletta, M., Russo, R., Baglivo, I., Russo, V., Ragucci, S., Sandomenico, A., Iaccarino, E., Ruvo, M., De Feis, I., Angelini, C., et al. (2020). Exploring the Interaction between the SWI/SNF Chromatin Remodeling Complex and the Zinc Finger Factor CTCF. *Int. J. Mol. Sci.* *21*, 8950. 10.3390/ijms21238950.
130. Klemm, S.L., Shipony, Z., and Greenleaf, W.J. (2019). Chromatin accessibility and the regulatory epigenome. *Nat. Rev. Genet.* *20*, 207–220. 10.1038/s41576-018-0089-8.
131. Hansen, A.S. (2020). CTCF as a boundary factor for cohesin-mediated loop extrusion: evidence for a multi-step mechanism. *Nucleus* *11*, 132–148. 10.1080/19491034.2020.1782024.
132. Haarhuis, J.H.I., van der Weide, R.H., Blomen, V.A., Yáñez-Cuna, J.O., Amendola, M.,

- van Ruiten, M.S., Krijger, P.H.L., Teunissen, H., Medema, R.H., van Steensel, B., et al. (2017). The Cohesin Release Factor WAPL Restricts Chromatin Loop Extension. *Cell* 169, 693–707.e14. 10.1016/j.cell.2017.04.013.
133. Hsieh, T.-H.S., Cattoglio, C., Slobodyanyuk, E., Hansen, A.S., Darzacq, X., and Tjian, R. (2022). Enhancer–promoter interactions and transcription are largely maintained upon acute loss of CTCF, cohesin, WAPL or YY1. *Nat. Genet.* 54, 1919–1932. 10.1038/s41588-022-01223-8.
134. Wendt, K.S., Yoshida, K., Itoh, T., Bando, M., Koch, B., Schirghuber, E., Tsutsumi, S., Nagae, G., Ishihara, K., Mishiro, T., et al. (2008). Cohesin mediates transcriptional insulation by CCCTC-binding factor. *Nature* 451, 796–801. 10.1038/nature06634.
135. Liu, N.Q., Maresca, M., van den Brand, T., Braccioli, L., Schijns, M.M.G.A., Teunissen, H., Bruneau, B.G., Nora, E. èP, and de Wit, E. (2021). WAPL maintains a cohesin loading cycle to preserve cell-type-specific distal gene regulation. *Nat. Genet.* 53, 100–109. 10.1038/s41588-020-00744-4.
136. Nora, E.P., Goloborodko, A., Valton, A.L., Gibcus, J.H., Uebersohn, A., Abdennur, N., Dekker, J., Mirny, L.A., and Bruneau, B.G. (2017). Targeted Degradation of CTCF Decouples Local Insulation of Chromosome Domains from Genomic Compartmentalization. *Cell* 169, 930–944.e22. 10.1016/j.cell.2017.05.004.
137. Wutz, G., Várnai, C., Nagasaka, K., Cisneros, D.A., Stocsits, R.R., Tang, W., Schoenfelder, S., Jessberger, G., Muhar, M., Hossain, M.J., et al. (2017). Topologically associating domains and chromatin loops depend on cohesin and are regulated by CTCF, WAPL, and PDS5 proteins. *EMBO J.* 36, 3573–3599. 10.15252/embj.201798004.
138. Banigan, E.J., Tang, W., van den Berg, A.A., Stocsits, R.R., Wutz, G., Brandão, H.B., Busslinger, G.A., Peters, J.-M.M., and Mirny, L.A. (2023). Transcription shapes 3D chromatin organization by interacting with loop extrusion. *Proc. Natl. Acad. Sci.* 120, 2017. 10.1073/pnas.2210480120.
139. Bond, M.L., Davis, E.S., Quiroga, I.Y., Dey, A., Kiran, M., Love, M.I., Won, H., and Phanstiel, D.H. (2023). Chromatin loop dynamics during cellular differentiation are associated with changes to both anchor and internal regulatory features. *Genome Res.* 33, 1258–1268. 10.1101/gr.277397.122.
140. Kloetgen, A., Thandapani, P., Tsirigos, A., and Aifantis, I. (2019). 3D Chromosomal Landscapes in Hematopoiesis and Immunity. *Trends Immunol.* 40, 809–824. 10.1016/j.it.2019.07.003.
141. Weintraub, A.S., Li, C.H., Zamudio, A. V., Sigova, A.A., Hannett, N.M., Day, D.S., Abraham, B.J., Cohen, M.A., Nabet, B., Buckley, D.L., et al. (2017). YY1 Is a Structural Regulator of Enhancer-Promoter Loops. *Cell* 171, 1573–1588.e28. 10.1016/j.cell.2017.11.008.
142. Monahan, K., Horta, A., and Lomvardas, S. (2019). LHX2- and LDB1-mediated trans interactions regulate olfactory receptor choice. *Nature* 565, 448–453. 10.1038/s41586-018-0845-0.
143. Gentile, C., Berlivet, S., Mayran, A., Paquette, D., Guerard-Millet, F., Bajon, E., Dostie, J., and Kmita, M. (2019). PRC2-Associated Chromatin Contacts in the Developing Limb Reveal a Possible Mechanism for the Atypical Role of PRC2 in HoxA Gene Expression. *Dev. Cell* 50, 184–196.e4. 10.1016/j.devcel.2019.05.021.

144. Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., Van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., et al. (2010). Mediator and cohesin connect gene expression and chromatin architecture. *Nature* *467*, 430–435. 10.1038/nature09380.
145. Mellacheruvu, D., Wright, Z., Couzens, A.L., Lambert, J.P., St-Denis, N.A., Li, T., Miteva, Y. V., Hauri, S., Sardi, M.E., Low, T.Y., et al. (2013). The CRAPome: A contaminant repository for affinity purification-mass spectrometry data. *Nat. Methods* *10*, 730–736. 10.1038/nmeth.2557.
146. Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.-H., Pagès, F., Trajanoski, Z., and Galon, J. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* *25*, 1091–1093. 10.1093/bioinformatics/btp101.
147. Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* *13*, 2498–2504. 10.1101/gr.1239303.
148. Bernstein, B.E., Brown, M., Johnson, D.S., Liu, X.S., Nussbaum, C., Myers, R.M., Liu, T., Li, W., Meyer, C.A., Eeckhoutte, J., et al. (2008). Model-based Analysis of ChIP-Seq (MACS). *Genome Biol.* *9*, R137. 10.1186/gb-2008-9-9-r137.
149. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Mol. Cell* *38*, 576–589. 10.1016/j.molcel.2010.05.004.
150. Bailey, T.L., Johnson, J., Grant, C.E., and Noble, W.S. (2015). The MEME Suite. *Nucleic Acids Res.* *43*, W39–W49. 10.1093/nar/gkv416.
151. Ramírez, F., Dündar, F., Diehl, S., Grüning, B.A., and Manke, T. (2014). DeepTools: A flexible platform for exploring deep-sequencing data. *Nucleic Acids Res.* *42*, 187–191. 10.1093/nar/gku365.
152. Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* *14*, 417–419. 10.1038/nmeth.4197.
153. Risso, D., Schwartz, K., Sherlock, G., and Dudoit, S. (2011). GC-Content Normalization for RNA-Seq Data.
154. Risso, D., Ngai, J., Speed, T.P., and Dudoit, S. (2014). Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat. Biotechnol.* *32*, 896–902. 10.1038/nbt.2931.
155. Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* *26*, 139–140. 10.1093/bioinformatics/btp616.
156. Sheikh, A.A., and Blais, A. (2022). Improved sensitivity and resolution of ATAC-seq differential DNA accessibility analysis. *bioRxiv*, 2022.03.16.484118.
157. Kaya-Okur, H.S., Wu, S.J., Codomo, C.A., Pledger, E.S., Bryson, T.D., Henikoff, J.G., Ahmad, K., and Henikoff, S. (2019). CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nat. Commun.* *10*, 1–10. 10.1038/s41467-019-09982-5.
158. Li, Y., Nakka, K., Olender, T., Gingras-Gelinas, P., Wong, M.M.K., Robinson, D.C.L.,

- Bandukwala, H., Palii, C.G., Neyret, O., Brand, M., et al. (2021). Chromatin and transcription factor profiling in rare stem cell populations using CUT&Tag. *STAR Protoc.* 2, 100751. 10.1016/j.xpro.2021.100751.
159. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17, 10. 10.14806/ej.17.1.200.
 160. Stark, R., and Brown, G. (2022). DiffBind: differential binding analysis of ChIP-Seq peak data. <http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf>.
 161. Ross-Innes, C.S., Stark, R., Teschendorff, A.E., Holmes, K.A., Ali, H.R., Dunning, M.J., Brown, G.D., Gojis, O., Ellis, I.O., Green, A.R., et al. (2012). Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature* 481, 389–393. 10.1038/nature10730.
 162. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. 10.1186/s13059-014-0550-8.
 163. Conway, J.R., Lex, A., and Gehlenborg, N. (2017). UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics* 33, 2938–2940. 10.1093/bioinformatics/btx364.
 164. Lihua J Zhu, Claude Gazin, Nathan D Lawson, Hervé Pagès, Simon M Lin, David S Lapointe, and Michael R Green (2010). ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data. *BMC Bioinformatics* 11, 237–247.
 165. Zerbino, D.R., Johnson, N., Juettemann, T., Wilder, S.P., and Flicek, P. (2014). WiggleTools: Parallel processing of large collections of genome-wide datasets for visualization and statistical analysis. *Bioinformatics* 30, 1008–1009. 10.1093/bioinformatics/btt737.
 166. Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. *Nat. Biotechnol.* 29, 24–26. 10.1038/nbt.1754.
 167. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. 10.1093/bioinformatics/btp352.
 168. Durand, N.C., Shamim, M.S., Machol, I., Rao, S.S.P., Huntley, M.H., Lander, E.S., and Aiden, E.L. (2016). Juicer Provides a One-Click System for Analyzing Loop-Resolution Hi-C Experiments. *Cell Syst.* 3, 95–98. 10.1016/j.cels.2016.07.002.
 169. van der Weide, R.H., van den Brand, T., Haarhuis, J.H.I., Teunissen, H., Rowland, B.D., and de Wit, E. (2021). Hi-C analyses with GENOVA: a case study with cohesin variants. *NAR Genomics Bioinforma.* 3, 2021.01.22.427620. 10.1093/nargab/lqab040.
 170. Roayaei Ardakany, A., Gezer, H.T., Lonardi, S., and Ay, F. (2020). Mustache: multi-scale detection of chromatin loops from Hi-C and Micro-C maps using scale-space representation. *Genome Biol.* 21, 256. 10.1186/s13059-020-02167-0.
 171. An, L., Yang, T., Yang, J., Nuebler, J., Xiang, G., Hardison, R.C., Li, Q., and Zhang, Y. (2019). OnTAD: Hierarchical domain structure reveals the divergence of activity among TADs and boundaries. *Genome Biol.* 20, 1–16. 10.1186/s13059-019-1893-y.
 172. Hua, D., Gu, M., Zhang, X., Du, Y., Xie, H., Qi, L., Du, X., Bai, Z., Zhu, X., and Tian, D. (2024). DiffDomain enables identification of structurally reorganized topologically

- associating domains. *Nat. Commun.* *15*. 10.1038/s41467-024-44782-6.
173. Chakraborty, A., Wang, J.G., and Ay, F. (2022). dcHiC detects differential compartments across multiple Hi-C datasets. *Nat. Commun.* *13*. 10.1038/s41467-022-34626-6.
 174. Skene, P.J., and Henikoff, S. (2017). An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. *Elife* *6*, 1–35. 10.7554/elife.21856.
 175. Elhamamsy, A.R., Metge, B.J., Alsheikh, H.A., Shevde, L.A., and Samant, R.S. (2022). Ribosome Biogenesis: A Central Player in Cancer Metastasis and Therapeutic Resistance. *Cancer Res.* *82*, 2344–2353. 10.1158/0008-5472.CAN-21-4087.
 176. Owens, B.M., Hawley, T.S., Spain, L.M., Kerkel, K.A., and Hawley, R.G. (2006). TLX1/HOX11-mediated disruption of primary thymocyte differentiation prior to the CD4⁺ CD8⁺ double-positive stage. *Br. J. Haematol.* *132*, 216–229. 10.1111/j.1365-2141.2005.05850.x.
 177. Zweier-Renn, L.A., Hawley, T.S., Burkett, S., Ramezani, A., Riz, I., Adler, R.L., Hickstein, D.D., and Hawley, R.G. (2009). Hematopoietic immortalizing function of the NKL-subclass homeobox gene TLX1. *Genes, Chromosom. Cancer* *396*, NA-NA. 10.1002/gcc.20725.
 178. Phelan, M.L., and Featherstone, M.S. (1997). Distinct HOX N-terminal arm residues are responsible for specificity of DNA recognition by HOX monomers and HOX·PBX heterodimers. *J. Biol. Chem.* *272*, 8635–8643. 10.1074/jbc.272.13.8635.
 179. Allen, T.D., Zhlp, Y.-X., Hawley, T.S., and Hawley, R.G. (2000). TALE Homeoproteins as HOX11-Interacting Partners in T-cell Leukemia. *Leuk. Lymphoma* *39*, 241–256. 10.3109/10428190009065824.
 180. Kadoch, C., Hargreaves, D.C., Hodges, C., Elias, L., Ho, L., Ranish, J., and Crabtree, G.R. (2013). Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. *Nat. Genet.* *45*, 592–601. 10.1038/ng.2628.
 181. Han, L., Madan, V., Mayakonda, A., Dakle, P., Woon, T.W., Shyamsunder, P., Nordin, H.B.M., Cao, Z., Sundaresan, J., Lei, I., et al. (2019). Chromatin remodeling mediated by ARID1A is indispensable for normal hematopoiesis in mice. *Leukemia* *33*, 2291–2305. 10.1038/s41375-019-0438-4.
 182. Fang, C., Wang, Z., Han, C., Safgren, S.L., Helmin, K.A., Adelman, E.R., Eagen, K.P., Gaspar-Maia, A., Figueroa, M.E., Singer, B.D., et al. (2020). Cancer-specific CTCF binding facilitates oncogenic transcriptional dysregulation. *Genome Biol.* *21*, 1–30. 10.1186/s13059-020-02152-7.
 183. Debril, M.B., Gelman, L., Fayard, E., Annicotte, J.S., Rocchi, S., and Auwerx, J. (2004). Transcription Factors and Nuclear Receptors Interact with the SWI/SNF Complex through the BAF60c Subunit. *J. Biol. Chem.* *279*, 16677–16686. 10.1074/jbc.M312288200.
 184. De Keersmaecker, K., and Ferrando, A.A. (2011). TLX1-induced T-cell acute lymphoblastic leukemia. *Clin. Cancer Res.* *17*, 6381–6386. 10.1158/1078-0432.CCR-10-3037.
 185. Follini, E., Marchesini, M., and Roti, G. (2019). Strategies to Overcome Resistance Mechanisms in T-Cell Acute Lymphoblastic Leukemia. *Int. J. Mol. Sci.* *20*, 3021. 10.3390/ijms20123021.

186. Nebenfuhr, S., Kollmann, K., and Sexl, V. (2020). The role of CDK6 in cancer. *Int. J. Cancer* *147*, 2988–2995. 10.1002/ijc.33054.
187. Aoki, K., Hyuga, M., Tarumoto, Y., Nishibuchi, G., Ueda, A., Ochi, Y., Sugino, S., Mikami, T., Kobushi, H., Kato, I., et al. (2024). Canonical BAF complex regulates the oncogenic program in human T-cell acute lymphoblastic leukemia. *Blood* *143*, 604–618. 10.1182/blood.2023020857.
188. Senbanjo, L.T., and Chellaiah, M.A. (2017). CD44: A multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. *Front. Cell Dev. Biol.* *5*. 10.3389/fcell.2017.00018.
189. Shin, J., Kim, S.-H., and Yoon, J. (2021). PTEN downregulation induces apoptosis and cell cycle arrest in uterine cervical cancer cells. *Exp. Ther. Med.* *22*, 1–7. 10.3892/etm.2021.10534.
190. Martelli, A.M., Paganelli, F., Fazio, A., Bazzichetto, C., Conciatori, F., and McCubrey, J.A. (2019). The key roles of PTEN in T-cell acute lymphoblastic leukemia development, progression, and therapeutic response. *Cancers (Basel)*. *11*. 10.3390/cancers11050629.
191. Wu, Y., Zhu, H., and Wu, H. (2020). Pten in regulating hematopoiesis and leukemogenesis. *Cold Spring Harb. Perspect. Med.* *10*, 1–16. 10.1101/cshperspect.a036244.
192. Fayard, E., Moncayo, G., Hemmings, B.A., and Holländer, G.A. (2010). Phosphatidylinositol 3-kinase signaling in thymocytes: The need for stringent control. *Sci. Signal.* *3*, 1–13. 10.1126/scisignal.3135re5.
193. Bastid, J., Dejou, C., Docquier, A., and Bonnefoy, N. (2020). The Emerging Role of the IL-17B/IL-17RB Pathway in Cancer. *Front. Immunol.* *11*, 1–7. 10.3389/fimmu.2020.00718.
194. Chen, B., and Abdel-Wahab, O. (2024). Targeting the cBAF complex in T-ALL. *Blood* *143*, 566–567. 10.1182/blood.2023023040.
195. Ho, L., and Crabtree, G.R. (2010). Chromatin remodelling during development. *Nature* *463*, 474–484. 10.1038/nature08911.
196. Hsieh, T.H.S., Fudenberg, G., Goloborodko, A., and Rando, O.J. (2016). Micro-C XL: Assaying chromosome conformation from the nucleosome to the entire genome. *Nat. Methods* *13*, 1009–1011. 10.1038/nmeth.4025.
197. Meers, M.P., Tenenbaum, D., and Henikoff, S. (2019). Peak calling by Sparse Enrichment Analysis for CUT&RUN chromatin profiling. *Epigenetics and Chromatin* *12*, 1–11. 10.1186/s13072-019-0287-4.
198. Yashar, W.M., Kong, G., VanCampen, J., Curtiss, B.M., Coleman, D.J., Carbone, L., Yardimci, G.G., Maxson, J.E., and Braun, T.P. (2022). GoPeaks: histone modification peak calling for CUT&Tag. *Genome Biol.* *23*, 1–21. 10.1186/s13059-022-02707-w.

Appendix

Ottawa Health Science Network Research Ethics Board Approval



Ottawa Health Science Network Research Ethics Board (OHSN-REB) / Conseil d'éthique de la recherche du réseau de science de la santé d'Ottawa (CÉR-RSSO)

Date: October 13, 2022
To: Dr. Marjorie Brand, TOH/OHRI
Protocol ID: 2007804-01H
Post Form ID: 2479
Study Title: Ex Vivo Differentiation of Human Hematopoietic Stem Cells for Genomics and Proteomics Analysis
Application Type: Continuing Review
Review Type: Delegated
Approval Date: October 13, 2022
Study Approval Expiry Date: October 13, 2023

Dear Dr. Brand,

Thank you for submitting the Continuing Review Form to the Ottawa Health Science Network Research Ethics Board (OHSN-REB). The OHSN-REB has reviewed the study and granted approval as of the date noted above.

Ethical approval lapsed August 5-October 13, 2022.

Protocol dated December 20, 2013 is currently approved by the OHSN-REB.

The **OHSN-REB ethics approval** is applicable only for The Ottawa Hospital.

No deviations from, or changes to, the protocol should be initiated without prior written approval of an appropriate amendment from the OHSN-REB, except when necessary to eliminate immediate hazard(s) to study participants.

REB members involved in the research project do not participate in the review, discussion or decision.

If the study is to continue beyond the expiry date noted above, a Continuing Review Form must be received by the OHSN-REB 40-50 days prior to the study expiry date. If the study has been completed by the expiry noted above, a Study Closure Form must be received by the OHSN-REB.

The OHSN-REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; Integrated Addendum to ICH E6 (R1); Guideline for Good Clinical Practice E6 (R2); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. OHSN-REB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Please do not hesitate to contact us if you have any questions.

Sincerely,

Raphael Saginur, M.D.
Chairperson
Ottawa Health Science Network Research Ethics Board

/dw

Ethical Acceptance of Animal Research: Report of the Animal Care Committee



uOttawa

L'Université canadienne
Canada's university

ETHICAL ACCEPTANCE OF ANIMAL RESEARCH: REPORT OF THE ANIMAL CARE COMMITTEE/ CONFORMITE DEONTOLOGIQUE DE LA RECHERCHE SUR LES ANIMAUX : RAPPORT DU COMITE DE PROTECTION DES ANIMAUX

Principal Investigator/Chercheur(e) principal(e) **MARJORIE BRAND**
Department/Département : **Ottawa Hospital Research Institute**
Title of Protocol(s) submitted to ACC/ Titre du protocole de recherche soumis au CPA :

OHRIe-3413: STUDY OF MOLECULAR REGULATOR OF HUMAN LEUKEMIA CELL THE TYPE LYMPHOID (T-ALL) AND MYELOID (AML,CML) BY USING IMMUNOCOMPROMISED (NSG) MICE [Replacement of OHRI-2420]

This protocol is in good standing with the Animal Care Committee.

Date of Approval: August 17, 2020

Date of Expiry: July 31, 2024

Date of Renewal: July 31, 2021

The Animal Care Committee has examined the experimental procedures proposed. It certifies, with the applicant, that the care and treatment of animals used will be in accordance with the standards of the Canadian Council on Animal Care and with the Animals for Research Act, R.S.O. 1990, c.22, s. 17(1-3).

Further, all matters arising from this proposal that relate to animal care and treatment, and all experimental procedures proposed for use with animals have been reviewed and approved by the Animal Care Committee before they are initiated or undertaken by the applicant. This protocol will be reviewed on a regular basis during the entire period in which the research is done.

Le Comité de protection des animaux a examiné les méthodes d'expérimentation proposées. Le Comité atteste, conjointement avec le candidat ou la candidate, que les animaux utilisés aux fins de ces travaux seront traités conformément aux normes du Conseil canadien de protection des animaux et à la Loi sur les animaux destinés à la recherche, L.R.O. 1990, c.22, s.17(1-3).

En outre, le Comité de protection des animaux a examiné et approuvé les points de la proposition qui ont trait au soin et au traitement des animaux, ainsi que les méthodes d'expérimentation proposées avant qu'elles ne soient entreprises par le candidat ou la candidate. Cet examen se répétera régulièrement au cours de la période entière des travaux de recherche.

Signature:

A black rectangular box redacting the signature of the Chair of the Animal Care Committee.

Chair of the Animal Care Committee/
Président du Comité de protection des animaux

Date: August 27, 2020

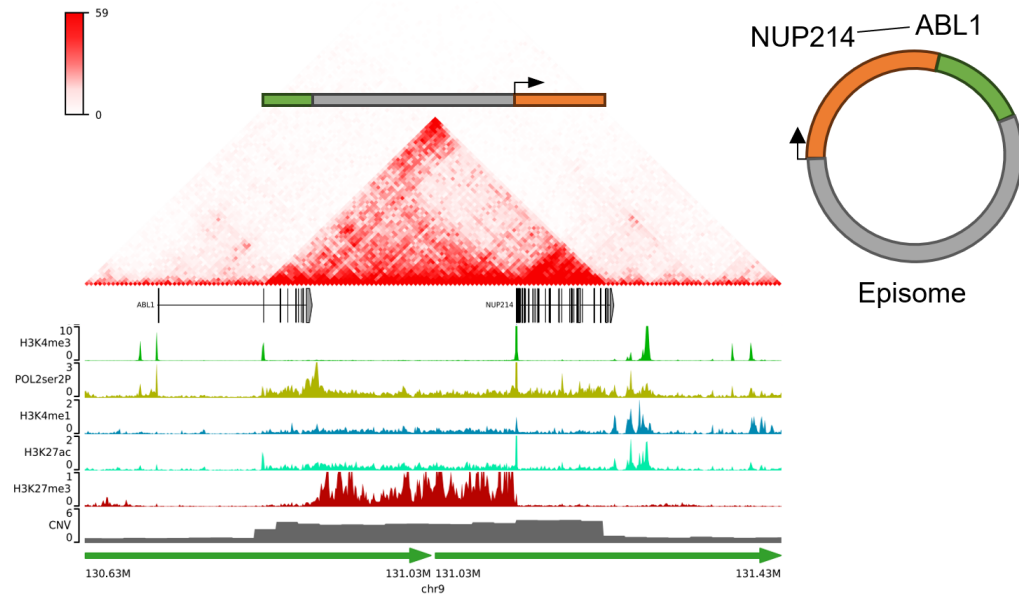
Supplemental Data

Supplemental Table 1. TLX1 IP mass spectrometry total spectra for proteins of interest.

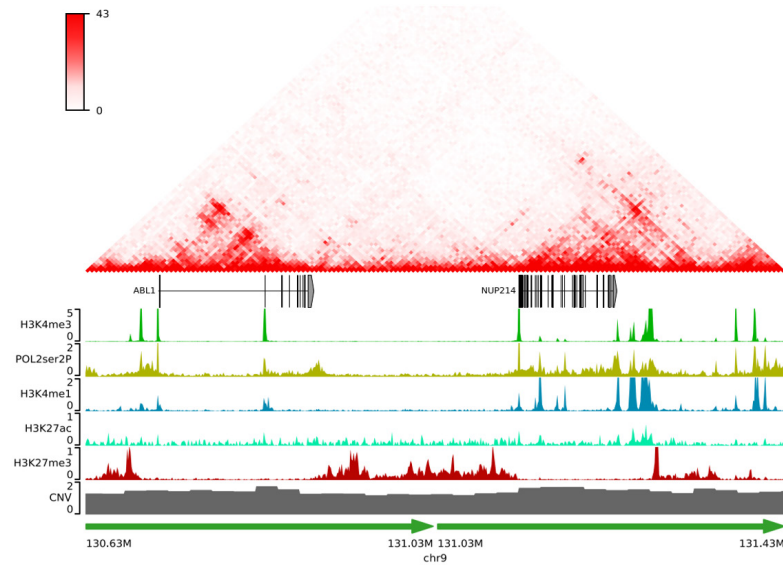
Total spectra counts for proteins identified in TLX1 IP in Figure 7, as well as additional proteins related to previous research for TLX1 interactions and other proteins implicated in chromatin looping. The BAF complex has several alternative names, which are included as an additional column. Proteins not identified in IP samples are not included.

Protein Name	Alternate Names	Relationship	IgG IP Total Spectra	TLX1 IP Total Spectra
TLX1	HOX11	IP Protein	2	28
PBX2	-	TF	0	41
RUNX1	-	TF	0	36
RUNX3	-	TF	0	25
ACTB	-	BAF	74	396
ACTL6A	BAF53A	BAF	35	232
ARID1A	BAF250A, SMARCF1	BAF	58	475
ARID1B	BAF250B, SMARCF2	BAF	0	76
ARID2	BAF200, SMARCF3	BAF	7	42
BCL11B	CTIP2, SMARCM2	BAF	4	117
BCL7A	SMARCF1	BAF	0	44
BCL7B	SMARCF2	BAF	0	14
BICRA	GLTSCR1	BAF	0	29
BICRAL	GLTSCR1L	BAF	0	18
BRD7	SMARCF1	BAF	0	36
BRD9	SMARCF2	BAF	0	12
DPF1	BAF45B	BAF	0	9
DPF2	BAF45D,REQU	BAF	24	233
DPF3	BAF45C	BAF	0	32
PBRM1	BAF180, SMARCF1	BAF	8	83
PHF10	BAF45A, SMARCG4	BAF	0	11
SMARCA2	BRM	BAF	0	121
SMARCA4	BRG1	BAF	18	199
SMARCB1	BAF47	BAF	14	109
SMARCC1	BAF155	BAF	17	293
SMARCC2	BAF170	BAF	26	224
SMARCC2	BAF170	BAF	25	212
SMARCD1	BAF60A	BAF	13	162
SMARCD2	BAF60B	BAF	15	178
SMARCE1	BAF57	BAF	11	228
SS18	SMARCL1	BAF	0	31
CTCF	-	Looping TF	0	4
HCFC1	-	Looping TF	0	75
MAU2	-	Cohesin Loading	0	4
NIPBL	-	Cohesin Loading	0	11
PDS5A	-	Cohesin Unloading	0	3
RAD21	-	Cohesin Complex	0	26
SMC1A	-	Cohesin Complex	0	28
SMC3	-	Cohesin Complex	2	22
WIZ	-	Looping TF	0	3
YY1	-	Looping TF	0	16
ZNF143	-	Looping TF	2	17

ALL-SIL

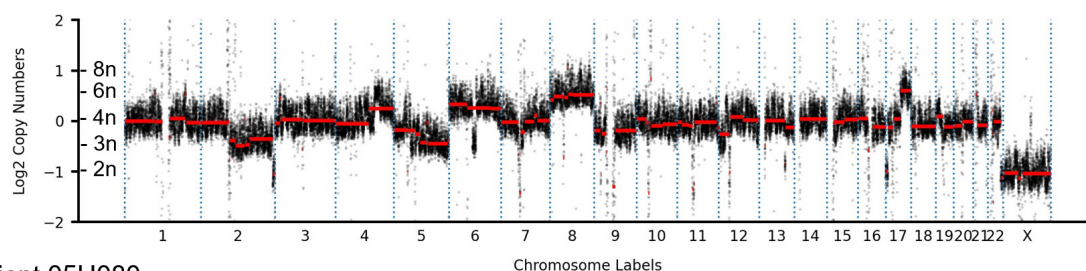


Patient 05H080

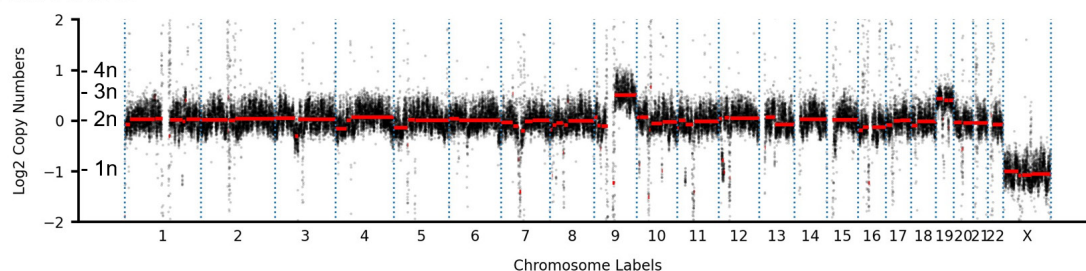


Supplemental Figure 1. NUP214-ABL1 mutation in ALL-SIL but not Patient T-ALL. HiC pyramids using raw counts at the ABL1 NUP214 locus. This region forms episomal copies in ALL-SIL that express the fusion oncogene NUP214-ABL1. Diagram depicts regions on canonical genome and how it recombines into an episome. Figures were generated using NeoLoopFinder with CPM-normalized histone mark tracks and copy number tracks. Genes between ABL1 and NUP214 are not shown for clarity.

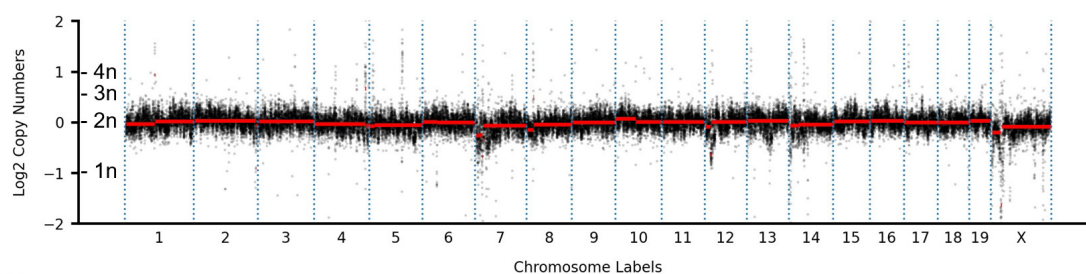
ALL-SIL (hyper-tetraploid karyotype)



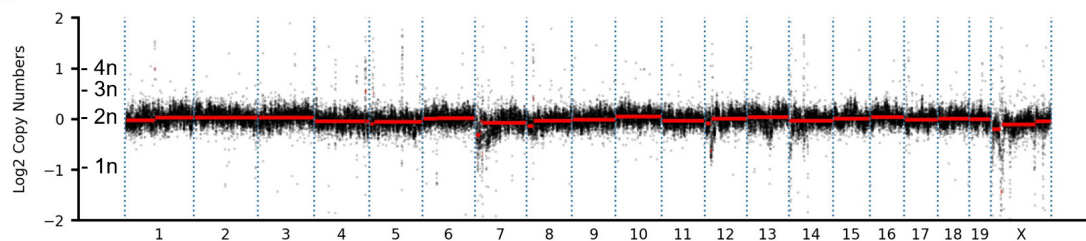
Patient 05H080



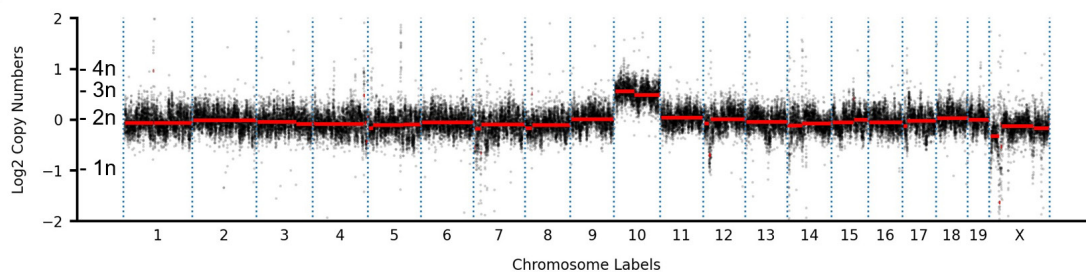
WT6



PLK5



LK3



Supplemental Figure 2. Copy number variation calculated by NeoLoopFinder. Copy number results per chromosome for human ALL-SIL (male), Patient 05H080 (male), and female mice WT6, PLK5, and LK3 are shown. Log2(copy number) is normalized against the diploid genome (default settings) at 50kb resolution, ploidy indicated on y-axis. Note: ALL-SIL is known hyper-tetraploid (90-95 chromosomes).

Supplemental Table 2. Fusion-gene analysis of Patient 05H080. Possible gene fusions created by SVs identified by NeoLoopFinder. A window within 10kb of each breakpoint outputs all possible gene fusions with overlapping genes. These genes are not necessarily expressed or functional.

Breakpoint #1		Breakpoint #2		Possible Fusions (+/- 10kb)
chr10	50015000	chr10	50065000	TIMM23B-AGAP6-WASHC2A AGAP6-WASHC2A
chr12	10155000	chr12	14690000	OLR1-GUCY2C
chr17	28715000	chr17	28950000	RPL23A-SEZ6 RPL23A-PHF12 RPL23A-PIPOX TLCD1-SEZ6 TLCD1-PHF12 TLCD1-PIPOX PROCA1-SEZ6 PROCA1-PHF12 PROCA1-PIPOX RAB34-SEZ6 RAB34-PHF12 RAB34-PIPOX
chr19	645000	chr19	2225000	RNF126-DOT1L RNF126-PLEKHJ1 FGF22-DOT1L FGF22-PLEKHJ1
chr19	41225000	chr19	43470000	AXL-LYPD3 AXL-PHLDB3
chr1	25255000	chr1	25330000	RSRP1-RHD RSRP1-TMEM50A

Supplemental Table 3 Patient T-ALL genes affected by deletions and duplications.

Verified SVs were annotated with an estimated degree of deletion or duplication and coding gene annotations.

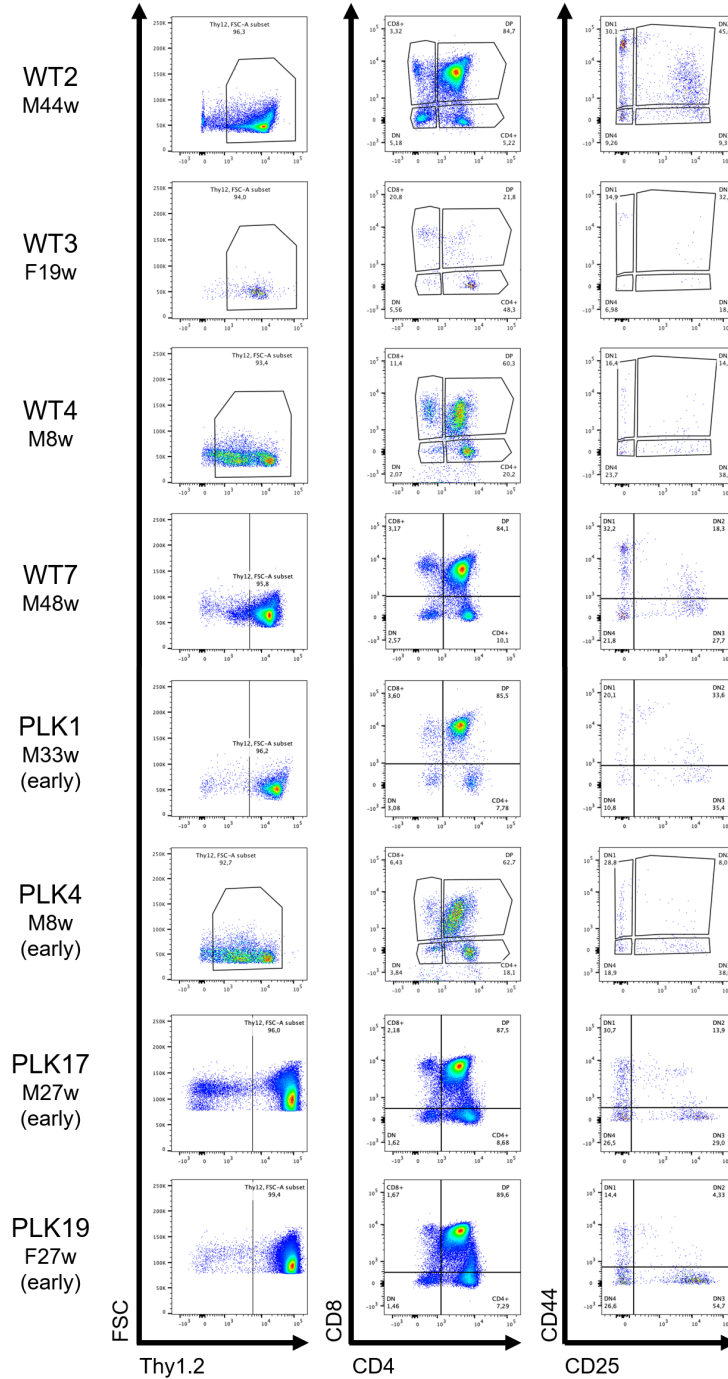
Chrom	Start	End	Interpretation	Genes within SV
chr1	25255000	25330000	complete deletion	RHD, RSRP1
chr11	25975000	27370000	partial deletion 0.5x	ANO3, BBOX1, CCDC34, FIBIN, LGR4, MUC15, SLC5A12
chr12	10155000	14690000	partial deletion 0.5x	APOLD1, ATF7IP, BCL2L14, BORCS5, CDKN1B, CREBL2, DDX47, DUSP16, EIF2S3B, EMP1, ENSG00000255641, ENSG00000275778, ETV6, FAM234B, GABARAPL1, GPR19, GPRC5A, GPRC5D, GRIN2B, GSG1, GUCY2C, HEBP1, KLRC1, KLRC2, KLRC3, KLRC4, KLRC4-KLRK1, KLRD1, KLRK1, LRP6, MAGOHB, MANSC1, OLR1, PLBD1, PRB1, PRB2, PRB3, PRB4, PRH1, PRH2, PRR4, SMIM10L1, STYK1, TAS2R10, TAS2R13, TAS2R14, TAS2R19, TAS2R20, TAS2R30, TAS2R31, TAS2R42, TAS2R43, TAS2R46, TAS2R50, TAS2R7, TAS2R8, TAS2R9, TMEM52B, YBX3
chr14	106390000	106760000	complete deletion	IGHV1-45, IGHV1-46, IGHV1-58, IGHV1-69, IGHV1-69-2, IGHV2-70D, IGHV3-38, IGHV3-43, IGHV3-48, IGHV3-49, IGHV3-53, IGHV3-64, IGHV3-66, IGHV4-39, IGHV4-59, IGHV4-61, IGHV5-51, IGHV8-51-1
chr16	16605000	18750000	partial deletion 0.5x	NOMO2, NPIPA8, NPIPA9, XYLT1
chr17	28715000	28950000	duplication 2.0x	DHRS13, ERAL1, FAM222B, FLOT2, NEK8, PHF12, RAB34, RPL23A, TLCD1, TRAF4
chr18	12775000	12890000	complete deletion	PTPN2
chr19	645000	2225000	partial deletion 0.5x	ABCA7, ABHD17A, ADAMTSL5, ADAT3, AP3D1, APC2, ARHGAP45, ARID3A, ATP5F1D, ATP8B3, AZU1, BTBD2, C19orf25, CBARP, CFD, CIRBP, CNN2, CSNK1G2, DAZAP1, DOT1L, EFNA2, ELANE, ENSG00000267059, FAM174C, FSTL3, GAMT, GPX4, GRIN3B, IZUMO4, KISS1R, KLF16, MBD3, MED16, MEX3D, MIDN, MISP, MKNK2, MOB3A, NDUFS7, ONECUT3, PALM, PCSK4, PLK5, PLPPR3, POLR2E, PRSS57, PRTN3, PTBP1, PWWP3A, R3HDM4, REEP6, REXO1, RNF126, RPS15, SBNO2, SCAMP4, STK11, TCF3, TMEM259, UQCR11, WDR18
chr3	65305000	76670000	partial deletion 0.5x	ARL6IP5, CNTN3, EBLN2, EIF4E3, ENSG00000285708, EOGT, FOXP1, FRG2C, FRMD4B, GPR27, GXYLT2, KBTBD8, LMOD3, LRIG1, MAGI1, MDFIC2, MITF, PDZRN3, PPP4R2, PROK2, ROBO2, RYBP, SHQ1, SLC25A26, SUCLG2, TAFA1, TAFA4, TMF1, UBA3, ZNF717
chr5	585000	685000	duplication 2.0x	CEP72, TPPP
chr7	38255000	38330000	complete deletion	TRGC1, TRGJ1, TRGJP, TRGJP1, TRGJP2, TRGV10, TRGV11, TRGV9
chr7	142460000	142800000	complete deletion 0x	MTRNR2L6, PRSS1, PRSS2, TRBC1, TRBD1, TRBJ1-1, TRBJ1-2, TRBJ1-3, TRBJ1-4, TRBJ1-5, TRBJ1-6, TRBJ2-1, TRBJ2-2, TRBJ2-2P, TRBJ2-3, TRBJ2-4, TRBJ2-5, TRBJ2-6, TRBJ2-7, TRBV10-3, TRBV11-3, TRBV12-3, TRBV12-4, TRBV12-5, TRBV13, TRBV14, TRBV15, TRBV16, TRBV17, TRBV18, TRBV19, TRBV20-1, TRBV23-1, TRBV24-1, TRBV25-1, TRBV27, TRBV28, TRBV29-1, TRBV5-4, TRBV5-5, TRBV5-6, TRBV5-7, TRBV6-6, TRBV6-7, TRBV6-8, TRBV7-6, TRBV7-7, TRBV7-9
chr8	134685000	134890000	duplication 2.0x	ZFAT
chr9	20370000	22950000	complete deletion	CDKN2A, CDKN2B, DMRTA1, ENSG00000264545, FOCAD, HACD4, IFNA1, IFNA10, IFNA13, IFNA14, IFNA16, IFNA17, IFNA2, IFNA21, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNB1, IFNE, IFNW1, KLHL9, MLLT3, MTAP

Supplemental Table 4. Fusion-gene analysis of ALL-SIL Possible gene fusions created by SVs identified by NeoLoopFinder. A window within 10kb of each breakpoint outputs all possible gene fusions with overlapping genes. These genes are not necessarily expressed or functional.

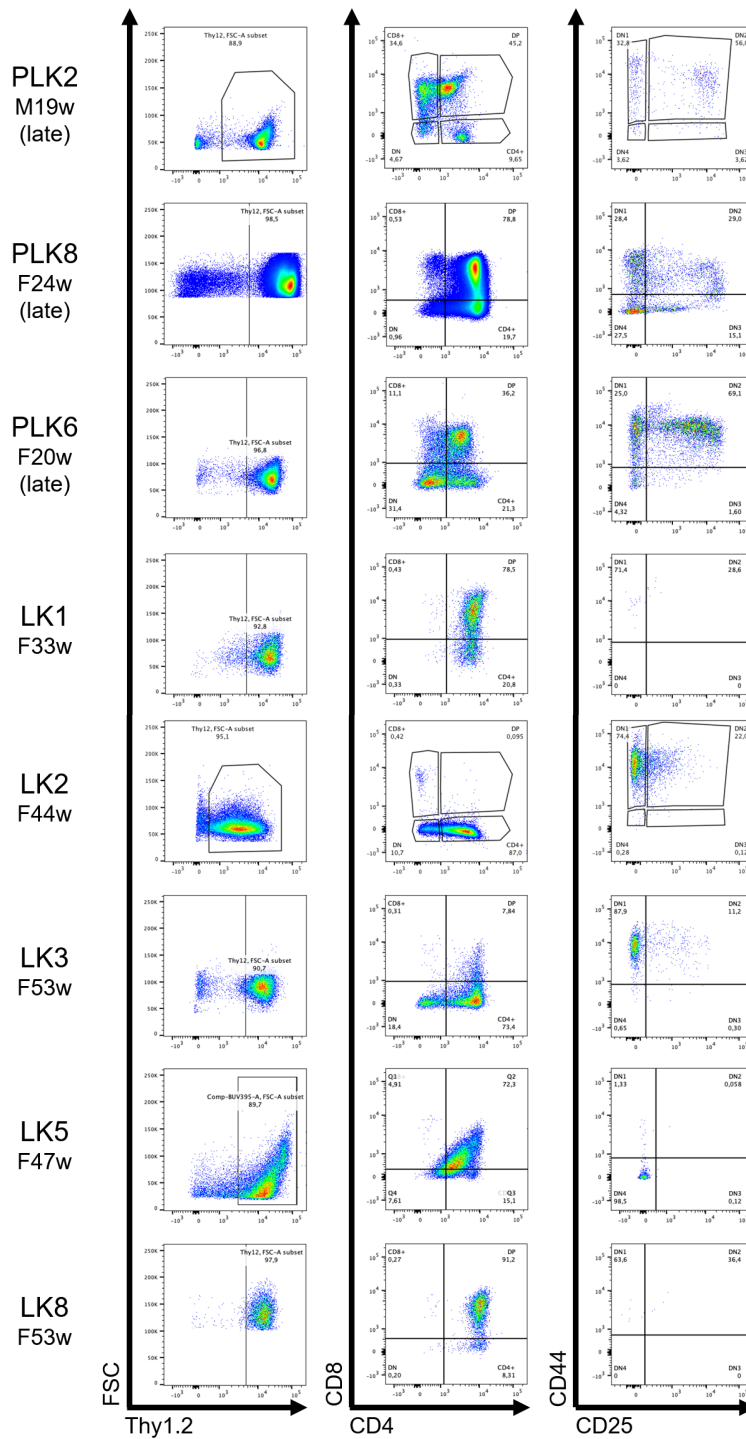
Breakpoint #1		Breakpoint #2		Possible Fusions (+/- 10kb)
chr13	48405000	chr13	48495000	RB1-RCBTB2 LPAR6-RB1 LPAR6-RCBTB2
chr3	136140000	chr3	136970000	PPP2R3A-IL20RB MSL2-IL20RB
chr7	32205000	chr7	32485000	PDE1C-LSM5
chr8	103240000	chr8	103410000	BAALC-SLC25A32 BAALC-AC012213.5, BAALC-DCAF13
chr9	130835000	chr9	131225000	ABL1-NUP214
chr9	127580000	chr9	127785000	NIBAN2-CDK9 NIBAN2-FPGS NIBAN2-SH2D3C STXBP1-CDK9 STXBP1-FPGS STXBP1-SH2D3C

Supplemental Table 5. ALL-SIL genes affected by deletions and duplications. Verified SVs were annotated with an estimated degree of deletion or duplication and coding gene annotations.

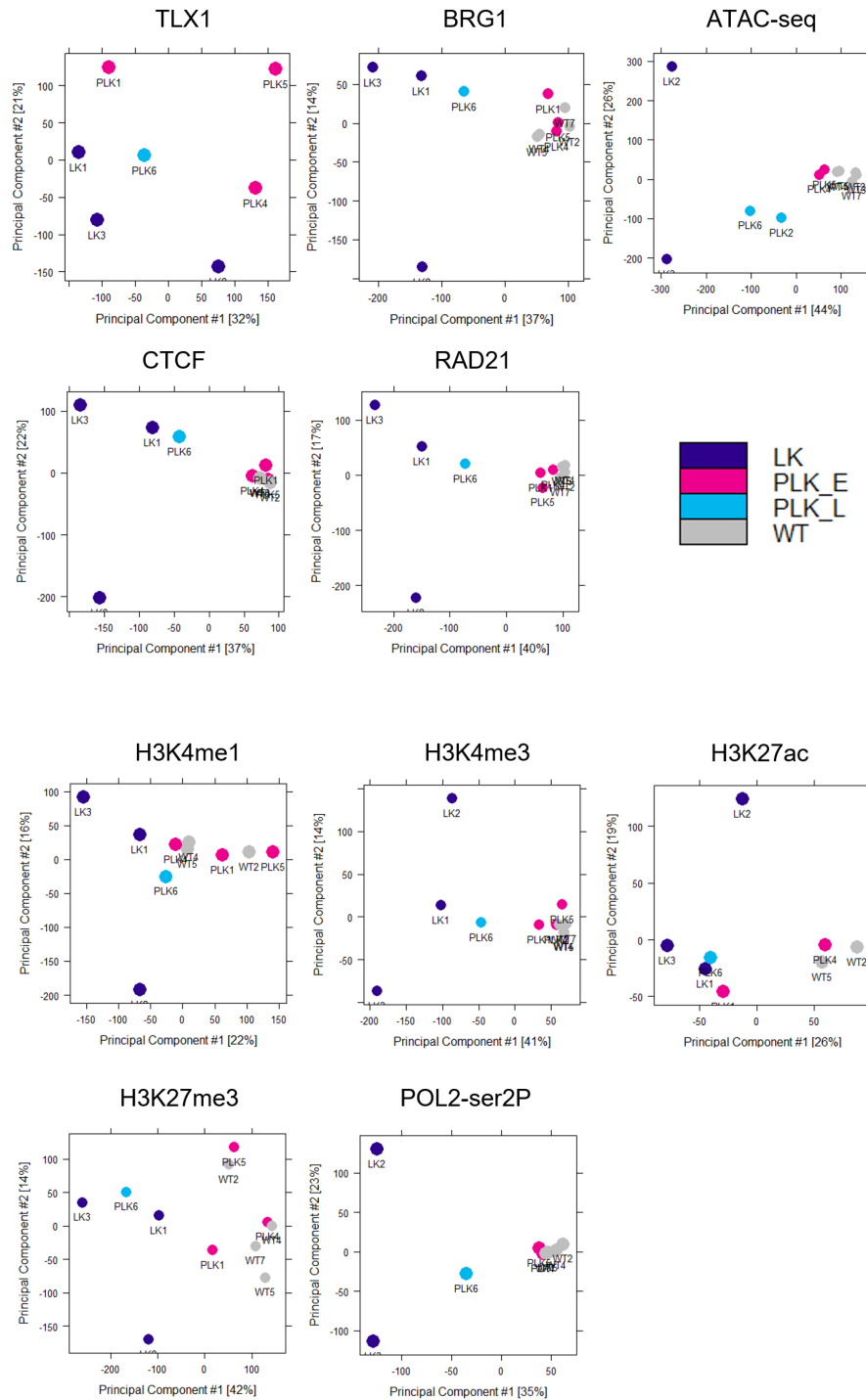
Chrom	Start	End	Interpretation	Genes within SV
chr13	48405000	48495000	total_deletion	LPAR6, RB1, RCBTB2
chr3	136140000	136970000	partial_duplication 1.5x	IL20RB, MSL2, NCK1, PCCB, PPP2R3A, SLC35G2, STAG1
chr4	68505000	68620000	partial_deletion 0.5x	UGT2B17
chr5	81745000	81855000	partial_duplication 1.5x	SSBP2
chr5	177995000	178050000	total_deletion	PROP1
chr6	77220000	89045000	partial_deletion 0.5x	AKIRIN2, BCKDHB, C6orf163, CEP162, CFAP206, CGA, CNR1, CYB5R4, DOP1A, ELOVL4, ENSG00000213204, ENSG00000271793, GJB7, HMGN3, HTR1B, HTR1E, IBTK, IRAK1BP1, LCA5, ME1, MEI4, MRAP2, NT5E, ORC3, PGM3, PHIP, PRSS35, RARS2, RIPPLY2, RNGTT, RWDD2A, SH3BGR2, SLC35A1, SMIM8, SNAP91, SNX14, SPACA1, SYNCRIP, TBX18, TENT5A, TPBG, TTK, UBE3D, ZNF292
chr6	135170000	135360000	multi_fold_duplications	AHI1, MYB
chr7	142610000	142805000	total_deletion	MTRNR2L6, PRSS1, PRSS2, TRBC1, TRBC2, TRBD1, TRBJ1-1, TRBJ1-2, TRBJ1-3, TRBJ1-4, TRBJ1-5, TRBJ1-6, TRBJ2-1, TRBJ2-2, TRBJ2-2P, TRBJ2-3, TRBJ2-4, TRBJ2-5, TRBJ2-6, TRBJ2-7, TRBV18, TRBV19, TRBV20-1, TRBV23-1, TRBV24-1, TRBV25-1, TRBV27, TRBV28, TRBV29-1
chr8	103240000	103410000	partial_deletion 0.5x	CTHRC1, ENSG00000285982, FZD6, SLC25A32
chr9	21495000	22635000	compound partial_deletion_0.5x with total_deletion_0x	CDKN2A, CDKN2B, DMRTA1, ENSG00000264545, MTAP
chr9	127580000	127785000	partial_duplication 1.5x	CFAP157, PTRH1, SH2D3C, STXBP1, TOR2A, TTC16
chr9	130835000	131225000	multi_fold_duplications (episome copies)	ABL1, AIF1L, FIBCD1, LAMC3, NUP214, QRFP



Supplemental Figure 3. Additional flow cytometry plots for WT and PLK-early mice. Representative flow cytometry of wild-type and PLK-early mice for gating strategy described in section 2.11. Mice shown were used in CUT&Tag and/or RNA-seq. There is no FACS for PLK5 or WT6 because it was prioritized for HiC and other experiments.



Supplemental Figure 4. Additional flow cytometry data for PLK-late and LK mice. Flow cytometry plots for all PLK-late and all LK mice used in CUT&Tag and/or RNA-seq.



Supplemental Figure 5. Diffbind PCA analysis of CUT&Tag targets in tgTLX1 mice. Diffbind PCA plots of targets at their respective peak sets. Samples are colored by condition. PC1 versus PC2 is shown.

Supplemental Table 6. Additional Mouse Information. The mouse name used in the thesis is provided, along with the conditions for the data analyses, mouse ID, sex, age in weeks, and the weight of thymus and spleen in grams. NR indicates that the weight was not recorded at the time of the experiment.

Mouse Name	Condition	Mouse ID	Sex	Age	Thymus Weight (g)	Spleen Weight (g)
WT2	WT	20MBS085_EN3	Female	44	0.16	0.11
WT3	WT	21MBS018_EN2	Male	19	0.07	0.09
WT4	WT	21MBS036_EN1	Male	8	0.06	0.07
WT5	WT	21MBS036_EN2	Male	8	0.06	0.07
WT6	WT	21MBS023_EN1	Female	19	NR	NR
WT7	WT	20MBS113_EN2	Male	48	0.06	0.07
PLK1	PLK-early	20MBS073_EN2	Male	33	0.05	0.15
PLK01NN	PLK-early	21MBS026_EN3	Female	9	0.05	0.09
PLK02NN	PLK-early	21MBS026_EN4	Female	9	0.06	0.08
PLK4	PLK-early	21MBS036_EN3	Male	8	0.06	0.06
PLK5	PLK-early	21MBS023_EN4	Female	19	NR	NR
PLK17	PLK-early	22MBS014_EN1	Female	27	0.11	0.14
PLK18	PLK-early	22MBS014_EN2	Female	27	0.09	0.11
PLK2	PLK-late	21MBS018_EN3	Male	19	0.07	0.09
PLK6	PLK-late	21MBS023_EN5	Female	20	0.06	0.06
PLK8	PLK-late	22MBS018_EN1	Female	24	0.13	0.13
LK1	LK	20MBS072_EN1	Female	33	0.23	0.13
LK2	LK	20MBS085_EN1	Female	44	0.56	1.72
LK3	LK	20MBS085_EN2	Female	53	0.41	0.15
LK5	LK	21MBS057_EN5	Female	47	0.70	0.16
LK8	LK	21MBS065_EN5	Female	47	0.66	0.48

Curriculum Vitae

Research Experience

Doctoral Candidate, Lab of Dr. Marjorie Brand Ottawa, Ontario
Sprott Center for Stem Cell Research, Ottawa Hospital Research Institute 2017/09 - Present

For my thesis, I was tasked with identifying the function of the oncogenic transcription factor TLX1, which drives the transformation of a T cell acute lymphoblastic leukemia subtype. The project started by identifying candidate TLX1-interacting complexes in leukemia using immunoprecipitation and mass spectrometry analysis. Following this, I used a combined multi-omics approach in T-ALL cell lines, patient cells, and transgenic mouse models to characterize the interactions of TLX1 and its cofactors on the genome and how this disrupted chromatin accessibility, 3D chromatin structure, epigenetics, and transcription to drive the transformation of differentiating thymocytes into leukemic cells. I am currently in the process of finishing my thesis. These findings are currently being synthesized with work done by my colleagues in the Brand Lab and collaborators for publication.

Research Assistant, Lab of Dr. Marjorie Brand Ottawa, Ontario
Sprott Center for Stem Cell Research, Ottawa Hospital Research Institute 2017/05 - 2017/08

Before starting my thesis in Dr. Marjorie Brand's lab, I was initially hired as a research assistant. This included learning the basics of wet lab experiments, such as Western blot, immunoprecipitation, and cell culturing. My progress resulted in an offer to start a master's thesis, which was expanded into my Ph.D. thesis.

Education

Doctorate in Philosophy (in progress) Ottawa, Ontario
Ph.D., Cellular and Molecular Medicine, University of Ottawa 2017/09 – Present

Bachelor's Ottawa, Ontario
Honours Bachelor of Science in Biochemistry, University of Ottawa 2012/9 – 2017/5

Skills

- Experiments intended for sequencing: ChIP-seq, CUT&Tag, CUTAC, ATAC-seq, RNA-seq, HiC, HiCAR, and follow-up integrated analysis of these experiments.
- Highly proficient in writing and implementing code for analyses of genomic data for use in Linux-based cluster computing (Digital Research Alliance of Canada) and moderately skilled in R.
- Co-immunoprecipitation, sample preparation for mass spectrometry, and proteomics analysis.
- E. coli protein expression and purification (ex., several versions of Tn5 transposase, tagged fusion-proteins for in vitro assays)
- Plasmid cloning; insert design and sub-cloning, bacteria transformation, plasmid validation, maxiprep kits.
- Generating stable cell lines; insert design, sub-cloning of lentiviral vectors, production of lentivirus in HEK293T, and purification, transduction, and selection of stable cells.
- Gel-type assays: Western-blot, coomassie staining, silver staining, DNA agarose gel.

- PCR and qPCR; primer design, sequencing library preparation, validation of target enrichment
- Biosafety level 2 cell culture handling, particularly cell lines, in vitro drug assays, and time-course knockdown experiments using electroporated siRNA.
- Some experience with fluorescence microscopy.
- Highly adept at experiment troubleshooting, data analysis optimization, and student training.

Publications

Jung, M.M., Shen, S., Botten, G.A., Olender, T., Katsumura, K.R., Johnson, K.D., Soukup, A.A., Liu, P., Zhang, Q., Jensvold, Z.D., et al. (2023). *Pathogenic human variant that dislocates GATA2 zinc fingers disrupts hematopoietic gene expression and signaling networks*. J. Clin. Invest. 133. 10.1172/JCI162685.

Li, Y., Nakka, K., Olender, T., Gingras-Gelinas, P., Wong, M.M.K., Robinson, D.C.L., Bandukwala, H., Palii, C.G., Neyret, O., Brand, M., et al. (2021). *Chromatin and transcription factor profiling in rare stem cell populations using CUT&Tag*. STAR Protoc. 2, 100751. 10.1016/j.xpro.2021.100751.

Balakrishnan, A; Adnani, L; Chinchalongporn, V; Vasan, L; Prokopchuk, O; Chen, MJ; El-Sehemy, A; Olender, T; Touahri, Y; Ahmad, ST; Islam, R; Sujanthan, S; Zinyk, D; Comanita, LC; Kan, B; Fleming, T; Leong, HS; Morshead, CM; Brand, M; Wallace, VA; Chan, JS; Schuurmans, C. (2020). *SMPD3-mediated extracellular vesicle biogenesis inhibits oligodendroglioma growth*. bioRxiv. 10.1101/2020.07.14.202200 (recently submitted to Communications Biology for final review)

Han, S., Dennis, D.J., Balakrishnan, A., Dixit, R., Britz, O., Zinyk, D., Touahri, Y., Olender, T., Brand, M., Guillemot, F., et al. (2018). *A non-canonical role for the proneural gene Neurog1 as a negative regulator of neocortical neurogenesis*. Development 145, dev157719. 10.1242/dev.157719.

Conference Publications (Abstracts)

Olender, T., Tur, S., Jalan, M., Sarakbi, R., Fallahi, M., Kopp, A., and Brand, M. (2022). *2015 – Disruption of Ctf Binding By the Oncogenic Transcription Factor Tlx1 Alters the Genomic Topology in T Cell Acute Lymphoblastic Leukemia (T-ALL)*. Exp. Hematol. 111, S39. 10.1016/j.exphem.2022.07.047.

Olender, T., and Brand, M. (2018). *3128 - Characterizing the Role of TLX1 in T-Cell Acute Lymphoblastic Leukemia*. Exp. Hematol. 64, S91. 10.1016/j.exphem.2018.06.110.

Conferences, Research Days, and Seminar Presentations

Conferences (*oral presentations)

* Olender, T., Tur, S., Jalan, M., Sarakbi, R., Fallahi, M., Kopp, A., and Brand, M. (2022). *Disruption of CTCF Binding By the Oncogenic Transcription Factor TLX1 Alters the Genomic Topology in T Cell Acute Lymphoblastic Leukemia (T-ALL)*, International Society for Experimental Hematology 51st Annual Scientific Meeting, Edinburgh, Scotland

Olender, T., Tur, S., Jalan, M., Sarakbi, R., Kopp, A., and Brand, M. (2022). *Characterizing the Role of TLX1 and TLX3 in T-cell Acute Lymphoblastic Leukemia*. Ottawa Institute for Systems Biology 2022 Scientific Meeting, Cornwall, Canada

Olender T, Jalan M, Brand M. (2019). *Characterizing the Role of TLX1 and TLX3 in T-cell Acute Lymphoblastic Leukemia*. Ottawa Institute for Systems Biology 2019 Scientific Meeting, Cornwall, Canada

Olender T, Brand M. (2018). *Characterizing the Role of TLX1 in T-cell Acute Lymphoblastic Leukemia*. Till & McCulloch Meetings 2018, Ottawa, Canada

Olender T, Brand M. (2018). *Characterizing the Role of TLX1 in T-cell Acute Lymphoblastic Leukemia*, International Society for Experimental Hematology 47th Annual Scientific Meeting, Los Angeles, United States

Research Days and Seminars

Olender T, Jalan M, Brand M. (2020). *Characterizing the Role of TLX1 and TLX3 in T-cell Acute Lymphoblastic Leukemia*. University of Ottawa CMM Poster Day 2020, Ottawa, Canada

Olender T, Jalan M, Brand M. (2019). *Characterizing the Role of TLX1 and TLX3 in T-cell Acute Lymphoblastic Leukemia*. University of Ottawa CMM/NSC Research Day 2019, Ottawa, Canada

Olender T, Jalan M, Brand M. (2019). *Characterizing the Role of TLX1 and TLX3 in T-cell Acute Lymphoblastic Leukemia*. Ottawa Hospital Research Institute Research Day 2019, Ottawa, Canada

Olender T, Brand M. (2018). *Characterizing the Role of TLX1 in T-cell Acute Lymphoblastic Leukemia*. Ottawa Hospital Research Institute Research Day 2018, Ottawa, Canada

Olender T, Brand M. (2018). *Characterizing the Role of TLX1 in T-cell Acute Lymphoblastic Leukemia*. University of Ottawa CMM/NSC Research Day, Ottawa, Canada

Scholarships

Tuition Fee Scholarship – tuition coverage for 1-year	2023 – 2024
Queen Elizabeth II Graduate Scholarship in Science and Technology – \$15,000	2022 – 2023
University of Ottawa Excellence Scholarship – tuition coverage for 1-year	2022 – 2023
Queen Elizabeth II Graduate Scholarship in Science and Technology – \$15,000	2021 – 2022
University of Ottawa Excellence Scholarship – tuition coverage for 1-year	2021 – 2022
Ontario Graduate Scholarship – \$15,000	2020 – 2021
University of Ottawa Excellence Scholarship – up to \$10,000 / year	2020 – 2021
Walter Faigan Kiwanis Club Fellowship in Lymphoma Research - \$15,000	2018 – 2019

Awards

T. Ray Bradley Award - ISEH New Investigator PhD Student Award - \$500 2022

For ISEH conferences, submitted abstracts are ranked by a panel of judges, and the six highest-ranking abstracts are selected for oral presentations (3 PhDs and 3 Postdocs). The T. Ray Bradley Award is one of three Ph.D. student awards and recognizes outstanding promise and excelling work using cutting-edge experimental techniques.

Second Place - Ph.D. Poster Competition - uOttawa CMM Poster Day - \$200 2020

First Place - Master's Student Poster Competition - OHRI Research Day - \$500 2018

Other Employment

Interlock Installer and Laborer Ottawa, Ontario
Ottawa Home and Garden - Full-time 2016/05 - 2016/08

Interlock Installer and Landscaper Ottawa, Ontario
Creative Designs Landscape - Full-time 2015/05 - 2015/08

Landscaper and Laborer Kanata, Ontario
Matheson Landscaping Services Ltd - Full-time 2014/05 - 2014/08

Landscaper and Laborer Kanata, Ontario
Matheson Landscaping Services Ltd - Full-time 2013/05 - 2013/08

Veterinary Assistant Kanata, Ontario
March Road Veterinary Hospital - Part-time 2009/03 - 2012/08