The Role of the YEATS Epigenetic Reader in MLL-r Fusion Proteins

by

Hsiangyu Hu

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Doctoral Committee:

Associate Professor Andrew G. Muntean, Chair
Professor Yali Dou, University of Southern California
Professor James Douglas Engel
Professor Zaneta Nikolovska-Coleska
Assistant Professor Joshua Welch

Hsiangyu Hu

dhhu@umich.edu

ORCID iD: 0000-0001-7881-3608

Dedication

To my grandmothers, who passed away from cancer in 2009 and 2019. You showed me resilience, humility, and more importantly what it means to be a good person.

May you rest in peace.

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Abstract

MLL (KMT2a) translocations are found in ~10% of acute leukemia patients, giving rise to an aggressive subset of leukemias. The majority of MLL fusion partners are members of epigenetic and transcriptional regulatory complexes, such as the Super Elongation Complex (SEC) and the DOT1L Complex (DotComm). In MLL-rearranged (MLL-r) leukemias, these protein complexes are hijacked to deregulate transcription and the epigenetic landscape of pro-leukemic genes. Thus, identifying druggable targets within these transcriptional and epigenetic complexes has become an attractive area of research for developing future therapeutics. Two common MLL translocation partners, ENL and AF9, are homologous proteins of the YEATS domain family. Both proteins contain a highly conserved N-terminal YEATS epigenetic reader domain that binds to histone acylation and interacts with the PAF1c, an epigenetic regulator protein complex essential for MLL-fusion leukemia. Recent work demonstrated the importance of wild-type ENL, and specifically its YEATS domain, in acute myeloid leukemia (AML) growth. In contrast, AF9 is dispensable for AML proliferation. This pointed to the ENL YEATS domain as a potential AML therapeutic target but did not address whether the YEATS domain impacts MLL-ENL fusion protein oncogenesis. Thus, we sought to investigate the YEATS epigenetic reader domain in the context of the MLL-ENL fusion proteins to uncover potential therapeutic opportunities.

In my thesis work, we found that the YEATS domain is retained in the majority (84.1%) of MLL-ENL fusions in t(11;19)(q23;p13.3) patients (N=302) and excluded in almost all MLL-AF9 fusions in t(9;11)(p22;q23) patients (N=449). This striking difference in YEATS domain inclusion between the MLL-ENL and MLL-AF9 fusion proteins prompted us to investigate the significance of the YEATS domain on MLL-ENL fusion proteins. Through our biochemical and biological studies, we show that the ENL YEATS epigenetic reader function critically modulates MLL-ENL fusion protein mediated leukemic stem cell (LSC) frequency. Indeed, global transcriptomic analyses confirmed that the YEATS domain impacts MLL-ENL target genes involved in LSCs. Genetic perturbation of the MLL-ENL YEATS epigenetic reader function significantly extends leukemia latency, while absence of the YEATS domain and downstream sequence abrogated leukemogenesis *in vivo*. Mechanistically, YEATS domain deletion impaired the MLL-ENL fusion protein and PAF1c binding at a subset of MLL-ENL targets. Mutations in the MLL-ENL YEATS reader pocket led to depletion of active histone marks and compromised expression of a subset of MLL-ENL targets, including the transcriptional factor *Eya1*. Therapeutically, the YEATS domain sensitizes MLL-ENL fusion driven leukemic cells to YEATS inhibitor treatment.

Our results demonstrated an oncogenic role for an epigenetic reader presented on a subset of MLL-r fusion proteins. We functionally linked the YEATS epigenetic reader with leukemic stem cell (LSC) frequency in MLL-ENL leukemias and showed its values as a therapeutic target. Potentially, YEATS inhibitor could be utilized either as a single agent or in part of a combination therapy in treating MLL-ENL leukemia and/or drug-resistant AML. Our data further contributes to the greater understanding of ENL

versus AF9, in how different parts of these proteins (i.e., the YEATS domain, the internally disordered region (IDR), the ANC-1 homology domain (AHD)) contribute to their roles as key epigenetic and transcriptional regulators in normal and/or malignant hematopoiesis.

Chapter 1 : Introduction

1.1 An Overview on Hematopoiesis and Acute Myeloid Leukemia

Normal development of the hematopoietic system is an essential part of our body and is mediated by an intricate network of signaling pathways, transcriptional factors, and epigenetics regulations. Disruption of these processes can produce pathogenic conditions, such as hematopoietic malignancies. In this section, I will provide an overview of the hematopoietic system, as well as introducing acute myeloid leukemia (AML), a blood malignancy that arises from the hematopoietic system.

1.1.1 The Hematopoietic System

The blood system constitutes a network of specialized cells essential for a myriad of biological functions, such as gas transport, immunity, wound healing and clotting[252]. It is composed of approximately 55% of plasma and 45% of blood cells. The hematopoietic system is responsible for generating all specialized cells in the blood throughout the entire lifespan of vertebrates[232]. Within the hematopoietic system, the hematopoietic stem cells (HSCs) are multipotent stem cells able to give rise to all the blood cells and self-renew through a process called hematopoiesis[233,252]. Hematopoiesis happens as early as the yolk sac in mammalian species through a process called primitive hematopoiesis, with the primary purpose of generating erythroid cells to the rapidly growing embryo[52,233,252]. As the fetus develops, primitive hematopoiesis is soon replaced by definitive hematopoiesis in the aorta-gonad

mesonephros (AGM) area, the fetal liver, thymus, spleen and finally the bone marrow[233,252]. In adult mammals, the HSCs reside inside the bone marrow where hematopoiesis occurs, although hematopoiesis can happen outside of the bone marrow under unusual circumstances such extramedullary hematopoiesis[52,149,252]. The HSC compartment could be further divided into long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells (ST-HSC) and multipotent progenitors (MPP)[41,78]. These cells are characterized by their self-renewal ability and capacity to differentiate into any cell type within the hematopoietic system[41]. Further down the hematopoietic lineage, the MPPs can give rise to the committed progenitors such as the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLP)[41,78]. The CMPs can further give rise to myeloid and erythroid cells, such as monocyte, macrophage, neutrophil, basophil, thrombocyte/platelets, erythrocyte, and mast cells[41,78,233,252]. The CLPs on the other hand can differentiate into lymphoid lineage cells such as the natural killer cells, B lymphocytes and T lymphocytes[41,78,233,252]. For simplistic purposes, the overview here doesn't recapitulate the complexity of the hematopoietic system and present all different cell types in it. However, it is important to note that the stem and hematopoietic progenitor cells gradually lose their self-renewal ability and become committed into specific lineages and cell types upon differentiation. The process is often observed by the expression of cell-surface markers on different cell types within the hematopoietic system, and an overview of some of these cell surface markers can be found in **Figure 1-1**.

Figure 1-1: An Overview of the Hematopoietic System

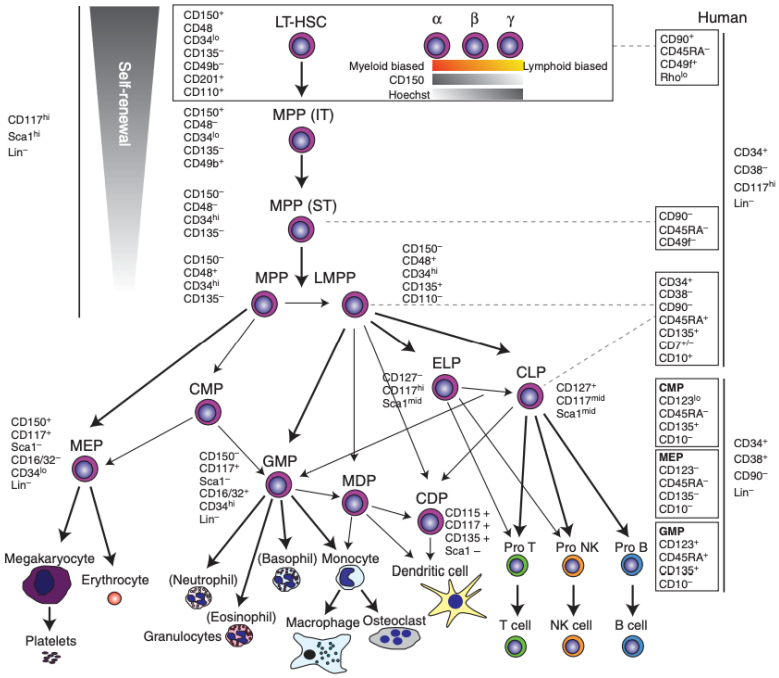


Figure delineates the hematopoietic hierarchy from the primitive to differentiated populations. Specifically, cell surface markers used to distinguish each of the distinct populations were listed in the figure. Figure is taken from Rieger and Schroeder, Cold Spring Harbor Laboratory Press, 2012.[252]

Hematopoietic stem and progenitor cell maintenance and differentiation are mediated in large by factors such as cytokines[253]. Cytokines are secreted signaling factors, such as interleukins (ILs), interferons, colony stimulating factors (CSFs), thrombopoietin (TPO) and erythropoietin (TPO), which exert their effect by binding to different cell surface receptors[233,253]. Among many cytokines, the stem cell factor (SCF) is one of the more well studied and binds to cKIT, a receptor tyrosine kinase (RTK) expressed on all HSCs[349]. cKIT-SCF signaling is important in maintaining proper HSC population and function. For instance, SCF has an anti-apoptotic effect on HSCs and drives its proliferation[72,122]. Consequentially, cKIT defects are linked with decreased numbers and function of HSCs[273,349]. Other cytokines, such as EPO, TPO, CSFs, and ILs are critical in promoting the expansion and differentiation of HSCs into platelets,

megakaryocytes, erythrocytes, granulocytes, and monocytes. The functions and properties of these cytokines were originally characterized at least through knockout studies and *ex vivo* colony formation assays, correlating their presence or absence with production or perturbation of certain cell types within the hematopoietic system[110,176,198]. An overview of how different cytokines drive the expansion of different hematopoietic cell types is illustrated in **Figure 1-2**.

Figure 1-2: Cytokines and Hematopoietic Transcriptional Factors in Hematopoietic Lineage Specifications

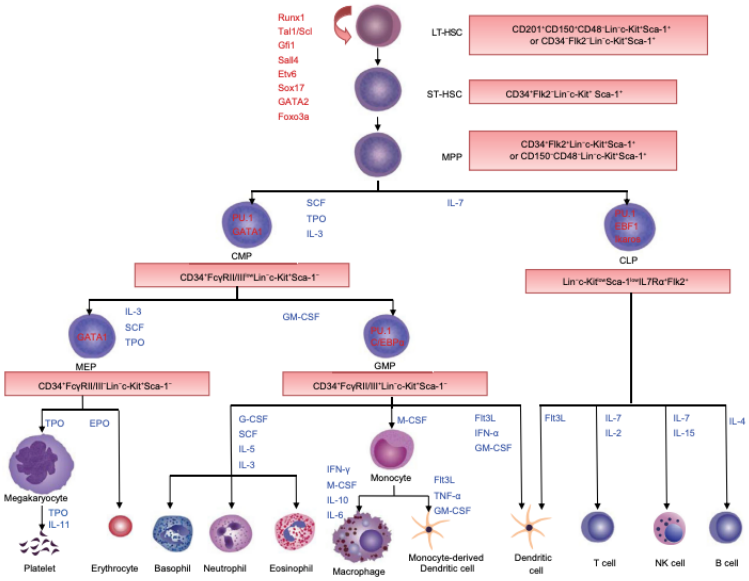


Figure delineates the hematopoietic hierarchy labeled with specific hematopoietic transcriptional factors and cytokines required for the differentiation into or maintenance of different hematopoietic populations. Figure is taken from Cheng et al., Protein & Cell, 2020.[41]

Many transcriptional factors have also been shown to be important in maintaining proper HSC functions and drive lineage specification and cell fate in the hematopoietic system. A few examples of how transcriptional factors contribute to lineage specification is illustrated in **Figure 1-2**. Among many, transcriptional factors such as the GATA proteins, the RUNX family, certain ETS family members have been shown to contribute to the proper HSC functions and hematopoiesis[2,83]. For example, RUNX1 has been

shown to be important for fetal hematopoiesis and myeloid/lymphoid/megakaryocytic maturation[25]. The ETS family member PU.1 and the GATA family member GATA1 interact with each other and act both antagonistically and synergistically to determine myeloid, lymphoid or erythroid cell fates[26,218,353]. These two proteins can interact with other transcriptional factors and epigenetic complexes to modulate the transcription and epigenetic landscape of their targets[26]. Interestingly, transcriptional factors such as PU.1 have been shown to regulate the expression of several CSFs and cell surface markers, providing an example of linkage between cytokine signaling and transcriptional factor in hematopoietic lineage decisions[103].

The homeobox (HOX) transcriptional factors are a subset of highly conserved homeobox proteins[238]. They are important in regulating body segment identity along the anterior-posterior axis during embryogenesis and contribute to the development and maintenance of many tissues, including the hematopoietic system[238,272]. In the mammalian system, there are a total of 39 Hox genes that exist in 4 different clusters: *Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*[4,272]. Within each cluster, Hox gene expression is coordinated in a phenomenon called temporal colinearity, where the temporal expression is correlated with the relative position within each of the clusters[106]. Together, Hox genes work in concert to regulate a wide range of cellular activities, such as proliferation, differentiation, adhesion, migration, and apoptosis[4,272].

The importance of Hox genes have been implicated in the hematopoietic system. Hox genes are most highly expressed in primitive HSCs and progenitor cells, and the expression is diminished in differentiated populations such as those marked with CD34-[242,265]. Several Hox genes, such as Hoxa9, Hoxa10, Hoxb4, and Hoxb6, have been

linked with HSCs expansion and differentiation blocks through overexpression and gain of function studies[4]. Loss of function studies displayed less pronounced effects on HSC function for certain Hox genes. For example, Hoxa9 knockout led to decreases in population and defective repopulating capacity of HSCs and several progenitors[163]. Together, this evidence suggests the importance of Hox genes and their proper expression in maintaining a properly functional hematopoietic system.

1.1.2 Acute Myeloid Leukemia

Failure for HSCs and progenitors to properly differentiate result in pathological conditions such as leukemia. Leukemia is a collective group of hematopoietic malignancies characterized by the accumulation of transformed and undifferentiated cells with growth advantages that eventually outpopulate cells in the normal hematopoietic system. Leukemias can be further divided into chronic or acute and myeloid or lymphoid leukemias depending on the disease latency and the lineage.

For instance, acute myeloid leukemia (AML) is characterized by the accumulation of >20% undifferentiated and blast-like cells with myeloid phenotype in the peripheral blood and the bone marrow[56,71,264]. The French-American-British (FAB) classification was the first attempt to categorize AMLs using morphology, cytochemical, and immune-phenotypes[56,264]. This method of classification was replaced by the AML classification by the World Health Organization first published in 2008, with AML with recurrent genetic abnormalities as a major category[56,71,264]. AMLs accounts for about 1% of all cancer cases and it is estimated that over 20,000 new cases will be diagnosed in 2022 with over 11,000 deaths in the United States (American Cancer Society). The current standard of care for most AMLs involves intensive induction therapy involving

cytarabine and anthracycline, intensive consolidation therapy, and/or hematopoietic cell transplantation (HCT)[70], suggesting the need of personalized therapeutics for such a heterogeneous group of diseases.

Figure 1-3: Recurrent Mutations and Translocations in Acute Myeloid Leukemia

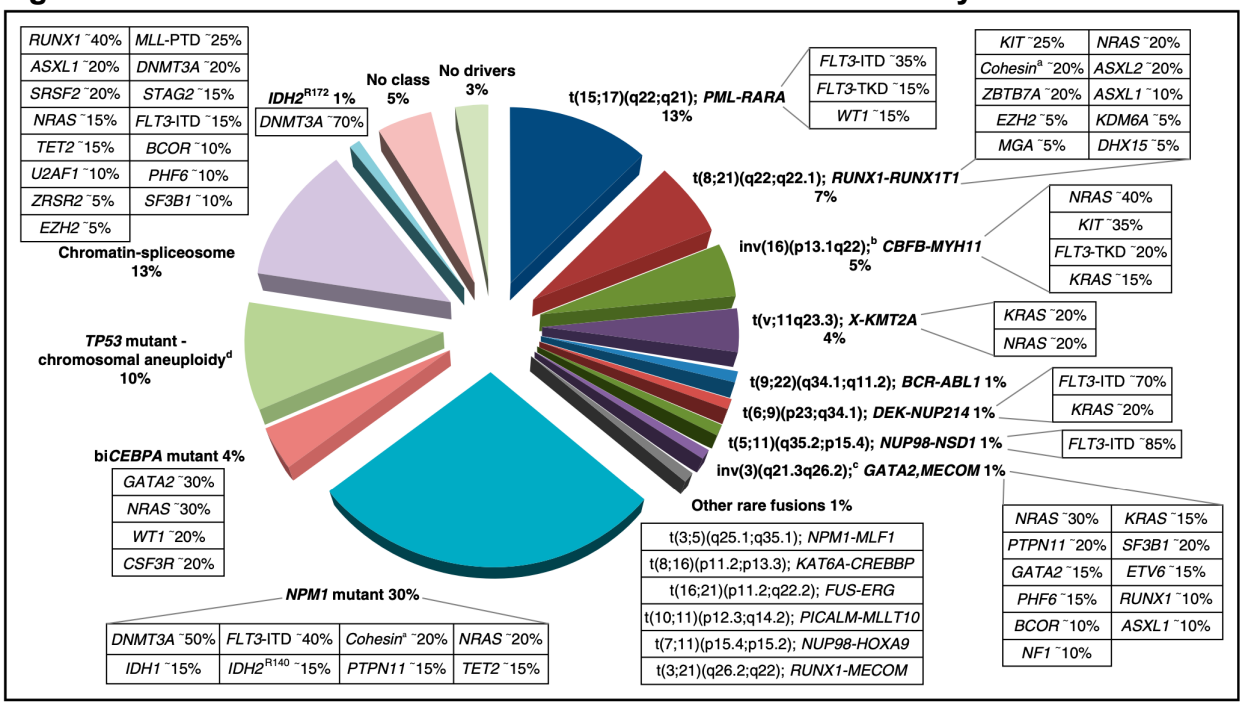


Figure shows common recurrent mutations and chromosomal translocations found in a total of 1540 acute myeloid leukemia patients. Analysis is based on Papaemmanuil et al., the New England Journal of Medicine, 2016[234]. Figure is taken from Döhner et al., Blood, 2017.[70]

AMLs are driven by recurrent mutations and chromosomal translocations, and an example of AML mutation landscape is shown in **Figure 1-3**[70,114]. A significant portion of AMLs are characterized with chromosomal abnormalities. Approximately one-third of AMLs harbors balanced chromosomal translocations, and these chromosomal translocations give rise to oncogenic fusion proteins driving leukemogenesis[70,114]. About 10% of AMLs have mutations in TP53[70,114]. TP53 mutant AMLs are associated with complex or monosomal karyotypes and poor prognosis[114,259]. Another one-third of AMLs

have relatively normal karyotype but harbor recurrent somatic mutations in NPM1 or CEBPA[70,114].

Cytokine and MAPK signaling are commonly mutated in AMLs[70,114]. FLT3, a RTK receptor, is over expressed in 70-100% of AMLs and mutated (most commonly FLT3 internal tandem duplications or FLT3 ITD) in about one-third of AMLs[108]. Another RTK receptor cKIT is mutated in about 17% of AML patients[184]. Mutations in different components of the MAPK pathway resulting in MAPK signaling activation (including NRAS, KRAS, PTPN11, and NF1) occur in 10-15% of AML patients[67]. In addition to cytokine and MAPK signaling, hematopoietic transcriptional factors are commonly misregulated in AMLs[70,114]. Hematopoietic transcriptional factors are involved frequently in fusion proteins, such as through core binding factor (CBF) AMLs, which are AMLs harboring the RUNX1/RUNX1T1 or the CBFB/MYH11 fusion proteins[113,114], and in PML-RARA AMLs[114]. Additionally, somatic mutations are found in hematopoietic transcriptional factors in a subset of AMLs, with the most common examples of RUNX1 and CEBPA somatic mutations happening in ~8-16% and ~10% of AMLs respectively[111,114,197]. Another category of genes commonly mutated in AMLs is genes involved in epigenetic and chromatin regulation. Genes that are involved in DNA methylation and demethylation, such as TET2, IDH1, IDH2 and WT1, as well as genes involved in chromatin regulation, such as ASXL1, ASXL2, PHF6, BCOR, and EZH2, are commonly mutated in AMLs[114]. Additionally, the protein MLL, and epigenetic modifier, is involved in chromosomal translocation in about 10% of AMLs.

Together, the mutational landscape of AMLs provides a comprehensive picture of somatic mutations and chromosomal translocations that drive dysregulated cytokine

signaling, hematopoietic transcriptional factors, epigenetic pathways, and many others in AMLs. The continued understanding on how these processes are hijacked in AML is imperative in the development of targeted therapeutics. One focused area of research includes epigenetics, as mutations in several epigenetic and chromatin regulators are associated with adverse to poor prognoses in AML, demanding our attention to address the underlying molecular mechanism. In the next section, I will provide an overview on the topic of epigenetics, and how it is linked to AMLs in the context of my thesis project.

1.2 A Brief Overview on Epigenetics and MLL

Approximately 3 million base pairs of DNA in our genome are organized into higher order structures through proteins and RNAs interactions. The field of epigenetic focuses on how DNA and protein chemical modifications alter the higher order structures and produce phenotypes at the cellular and organism level. Epigenetics plays a significant role in facilitating different normal developmental stages and is also critical to cellular responses to different external stimuli and stress conditions. At the same time, epigenetic dysregulation is linked to hematopoietic malignancies such as AMLs[30]. In this section of my introduction, I will provide an overview of key epigenetic concepts pertinent to my thesis.

1.2.1 Nucleosome Organization: The Basics of Epigenetics

Approximately 147 base pairs of DNA are wrapped around the histone octamer, and this basic unit of DNA organization is named the nucleosome[7]. The histone octamer is composed of 2 copies of each of the 4 histone proteins: H2A, H2B, H3 and H4[7,304]. Histones are relatively small proteins, and the high content of basic amino acids such as

lysines and arginine confers the positive charge and allows tight binding with the negatively charged double-stranded DNA[107]. It is worth noting that the composition of the histone octamer can change under certain cases. For instance, the histone H3.3 variants have been observed at active promoters as well as pericentromeric heterochromatin and telomeres[276], and the histone H2A.Z variant is observed to be incorporated into the histone octamer in regions of DNA double strand breaks[331].

The N-terminal part of all four histones as well as the C-terminal part of H2A protrude outward from the core nucleosome and are largely unstructured[180]. These regions, termed “histone tails” can be chemically modified and play an important role in epigenetic regulation. To date, over 128 residues across the 4 histones have been found to be post-translationally modified[133]. A summary of these chemical modifications on the four different histones can be found in **Figure 1-4**. Over 15 different chemical modifications have been identified on histones[133] with the advent of mass spectrometry-based proteomics studies[291], with methylation and acetylation amongst the most well-studied.

Figure 1-4: Histone Chemical Modifications

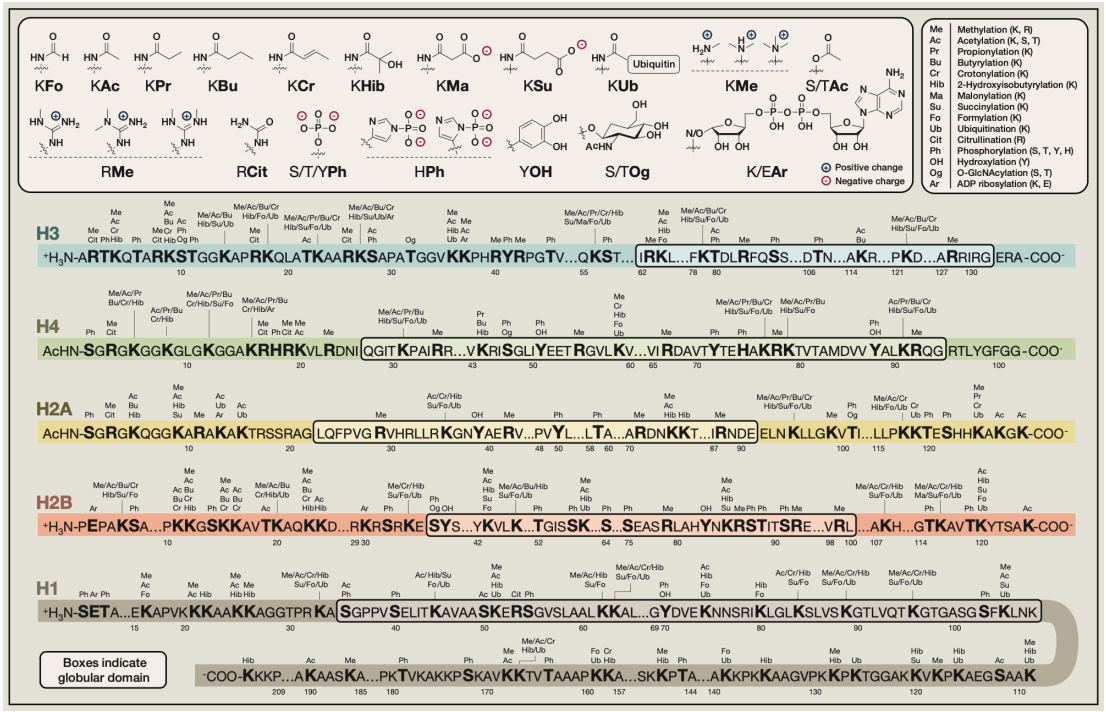


Figure lists out the known histone chemical modifications and the histone protein residues for these modifications. Figure is taken from Huang et al., Cell, 2014.[70]

These histone modifications can be regulated by a diverse pool of proteins, which either deposit (writers), remove (erasers), or bind (readers) to these chemical modifications to elicit chromatin and transcriptional changes[1,2]. Studying the network of histone modifications and understanding how these chemical modifications are deposited, read, and removed therefore provided a mechanistic basis linking epigenetics with a wide range of biological phenotypes.

1.2.2 Epigenetic Readers, Writers, and Erasers

Figure 1-5: Histone Readers, Writers, and Erasers

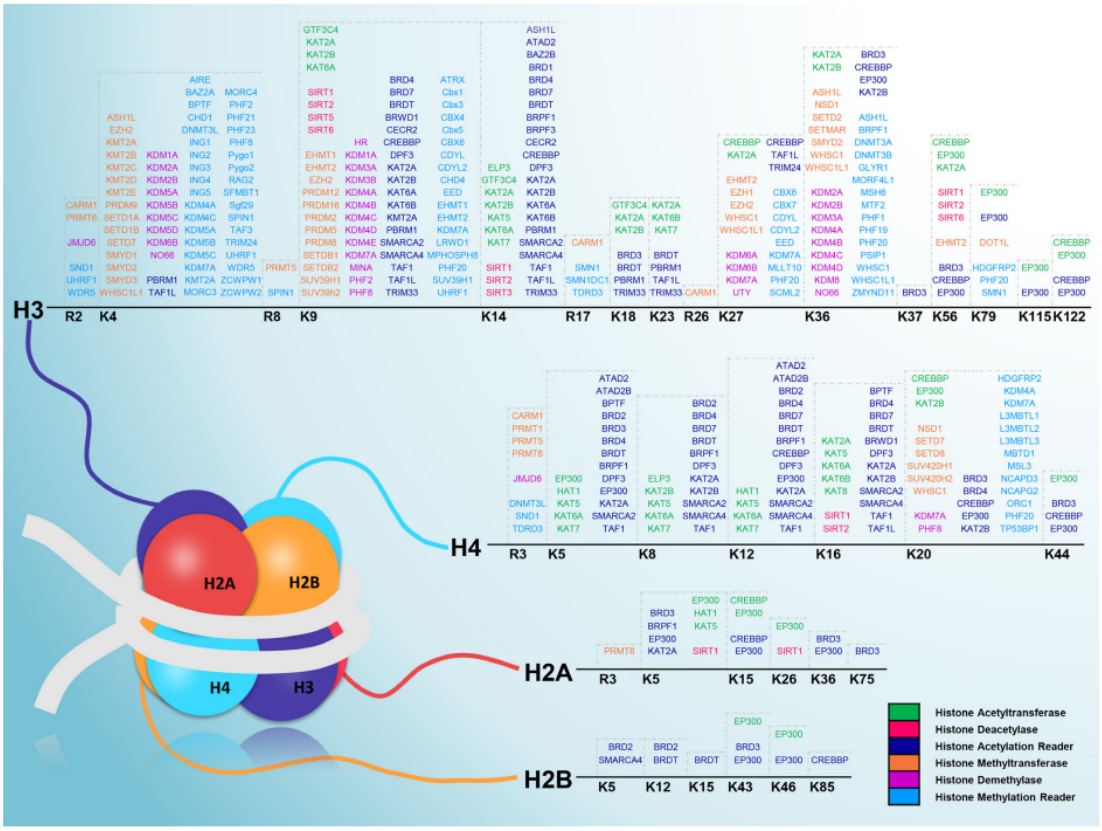


Figure lists the epigenetic factors, including readers, writers, and erasers, that catalyze or bind to the different chemical modifications on the histone proteins. Figure is taken from Xu et al., Nucleic Acids Research, 2017.[332]

Epigenetic writers and erasers are modifying enzymes that deposit or remove chemical modifications onto histones. They are often named after the chemical groups that they covalently bind or remove from the histones. For instance, methyltransferases or demethylase can add or remove methyl group(s), while acetyltransferases or deacetylases are able to attach or remove an acetyl group. These reactions often require different co-factors. For instance, most methyltransferases require the co-factor s-adenosyl methionine (SAM) to facilitate histone methylation, after which SAM is converted into s-adenosyl homocysteine (SAH)[94]. Histone demethylases such as LSD1 require flavin adenine dinucleotide (FAD) as a co-factor[98], while the jumonji family of

demethylases use α-ketoglutarate and converts it to succinate during demethylation reactions[1]. Protein domains have been identified and associated with epigenetic writer or eraser functions. For instance, all lysine methyltransferases identified to date except DOT1L in humans contain the SET methyltransferase domain and belong to the larger SET-domain protein super family[66]. The Jumonji C domain (JmjC) is a major protein domain associated with histone demethylation activity[1]. Epigenetic readers describe proteins that can recognize and bind to epigenetic marks on the histones. Like epigenetic writer and eraser domains, epigenetic reader domains have been identified, such as the histone methyl mark readers chromodomain, PhD fingers, and Tudor domains, and many others[136].

Epigenetic reader, writer and eraser proteins can reside in larger protein complexes and/or interact with transcriptional factors to elicit site/sequence specific modifications of the epigenome[136]. Additionally, a writer or an eraser can have epigenetic reader activities to further target their activity[136]. In general, epigenetic readers, writers, and erasers play an essential part in the diversity of epigenetic regulation through modifying the chemical structure and interpreting such signals.

1.2.3 Other Examples of Epigenetic Regulation

Similar to histones, DNA can also be chemically modified. The nucleotide cytosine can be methylated into 5-methylcytosine (5mC) by DNA methyltransferase (DNMT), and this reaction can be reversed by the ten-eleven translocation (TET) methylcytosine dioxygenases or through a passive process during cell cycle[211]. Additionally, DNA methylation can be read by reader proteins such as the methyl-binding proteins (MBP)[100]. In particular, the dinucleotide CpG can be methylated and

this sequence is underrepresented because of the mutagenic nature of methylcytosine[57,90]. Interestingly, CpG islands are areas in the genome where the CpG dinucleotide are maintained at a relatively higher frequency compared to the rest of the genome[57,90]. These unique sites in our genome are found around the transcriptional start site (TSS) and also at distal regions to regulate transcription[57,90].

Chromatin remodeling complexes are protein complexes that modulate chromatin structure through nucleosome sliding, histone variant exchange and others[297]. Remodeling chromatin structure is an epigenetic mechanism to control DNA accessibility during processes such as DNA replication, repair, and transcription[45]. Some of the most well-studied chromatin remodelers, such as the SWI/SNF complex, the ISWI ATPase, the CHD family and the INO80 family, are all ATP-dependent[45,297]. Similar to other epigenetic protein complexes, chromatin remodeling complexes also exist in large protein complexes, with members such as epigenetic readers, transcriptional factors, catalytic ATPases, and other adapter proteins[45,297]. In summary, DNA methylation/demethylation and chromatin remodeling complexes represent two epigenetic regulatory mechanisms not mutually exclusive with epigenetic readers, writers, and erasers. All of these processes work in concert to regulate developmental processes such as hematopoiesis, and are often perturbed in hematopoietic malignancies[130].

1.2.4 Trithorax and Polycomb: An Example of Epigenetic Regulation in Development

Epigenetic dysregulation through mutations or translocations is well-documented in AMLs (**Figure 1-3**) and perturb proper regulation of hematopoietic and developmental

transcriptional factors to drive malignant hematopoiesis. A well-studied example is the epigenetic writer MLL, its regulation on the HOX genes, and how this process is hijacked in a subset of AML. The human MLL gene (also known as KMT2a) is a H3K4 methyltransferase homologous to the *Drosophila* Trithorax gene[147,267]. Trithorax belongs under a larger group of *Drosophila* proteins called the Trithorax group proteins (TrxG), and together with the *Drosophila* Polycomb group of proteins (PcG) are important epigenetic regulators of the homeotic gene (HOX genes) complexes (such as the bithorax complex (BX-C) and Antennapedia complex (ANT-C)) in *Drosophila*[147,182,267]. These homeotic gene complexes are important in determining segment identity of *Drosophila*[182]. Mutations in Polycomb caused homeotic transformations similar to that of mutations in homeotic gene complexes. This led to the groundbreaking hypothesis that Polycomb might serve as a global regulator for these homeotic gene complexes[166]. Subsequent screens of epigenetic regulators of these Drosophila homeotic gene complexes identified the two opposing groups of PcG or TrxG[147,267]. These two groups of proteins are not necessary for initiating, but rather are important in maintaining Hox gene expression throughout development[147,267]. Specifically, the PcGs have repressive roles and the TrxG have activating roles on homeotic genes in *Drosophila*[147,267].

Members of the TrxG and PcG belong in large epigenetic complexes, with reader, writer, eraser, and chromatin remodeling activities as well as DNA methyltransferases and demethylases[147,267]. Homologs of TrxG and PcG in human have been identified. For instance, the Drosophila polycomb is homologous to several CBX proteins in the human canonical PRC1 complex, and trithorax is homologous to human MLL and MLL2[147,267]. Similarly, both MLL and MLL2 are regulators of Hox gene in

human[120]. The MLL protein together with its involvement in AML will be discussed further in later sections.

1.2.5 Summary

Epigenetics confers an important spatial-temporal gene regulation mechanism, with the regulation of Trithorax and Polycomb on Hox genes is a well-known example. In human, the Trithorax homolog MLL is found in a subset of aggressive AMLs through translocations involving chromosome 11q23. In the next section, I will provide an overview on our understanding of the key players in MLL fusion leukemias and how these stakeholders are hijacked to dysregulate the epigenome and transcription in MLL-r AMLs.

1.3 MLL Rearranged Leukemia: An Overview

The MLL protein was first identified when studying translocations involving the chromosome 11q23 locus and later identified as a homolog of the Drosophila protein Trithorax[68,115,294,358]. These translocations produce oncogenic MLL fusion proteins and drive a subset of approximately 10% of acute myeloid and lymphoid leukemias. Leukemias with these translocations are collectively referred to as MLL rearranged (MLL-r) leukemias and happen in patients of all age groups, including infant (defined as <12 months of age), pediatric and adult. In certain age groups, MLL-r leukemia accounts for most of the cases. For instance, MLL-r leukemia accounts for up to 80% of infant ALL and 31%-45% of infant AMLs cases. In childhood leukemias, MLL-r leukemias account for 6% of ALL and 14% of AMLs respectfully[8,263]. In adults, MLL-r leukemias account for about 9% of ALL and 5%-11% of AMLs[24].

More than 130 fusion partners of MLL have been identified to date[199], connecting the N-terminal MLL protein with the C-terminal part of the fusion partner. In clinic, MLL-r leukemias manifest in intermediate to poor prognoses, although the prognosis could depend on many different factors such as lineage subtypes, patient age group, and fusion partners. For instance, a study on the prognosis of childhood MLL-r AMLs concluded a wide range of 5 year event free survival (EFS), with several fusion partners identified as individual prognostic predictors[13]. In mice, MLL-r leukemia models with different fusion partners generate different disease latency, further supporting this claim[36]. While most MLL-r leukemias happen *de novo*, therapy-induced MLL-r leukemias are also found in cases following DNA topoisomerase II inhibitor treatment, which is generally associated with poor clinical outcome[109].

Given the diversity of patients, fusion partners, and disease prognosis, there are common attributes which most MLL-r leukemias share. For instance, early studies on the targets of MLL-r leukemias showed that these leukemias depend on MLL targets HOXA7, HOXA9 and their co-factor MEIS1[11,345]. Overexpression of Hoxa9 immortalizes hematopoietic progenitor populations, but Meis1 co-expression is necessary to drive the aggressive leukemic phenotype[105]. Hoxa9 or Meis1 knockout abrogates leukemogenesis and induce *ex vivo* differentiation in MLL-r models[11,321]. Biochemical characterization, together with studies conducted in transcriptional and epigenetic regulation, have generated a broad mechanistic model that aids in our understanding of MLL-r leukemogenesis. These studies revealed transcriptional and epigenetic activation at many MLL targets through the increased localization of the MLL fusion proteins, elongating RNA pol II, the methyltransferase DOT1L, and many others. In this chapter

of my thesis, I will discuss our current understanding of the MLL proteins and MLL-r leukemias, and some of its most common fusion partners. I will also provide a brief overview of the pertinent players involved in our current understanding of MLL-r leukemias, which span across different fields within epigenetics and transcriptional research.

1.3.1 The MLL Protein

MLL is encoded by a 90kb gene[251] and post-translationally processed into a 320kDa and a 180kDa proteins[126]. The two MLL proteins (**Figure 1-6**) together have different DNA-, histone-, and protein- binding domains and H3K4 methyltransferase activity, and serve as the backbone of a greater complex. The MLL-N fragment interacts with both LEDGF and Menin, and these interactions are essential for targeting the MLL complex[206,338]. Menin is a DNA binding scaffold that interacts with a wide variety of proteins[193] and LEDGF is an epigenetic reader for H3K36me3 via the PWWP domain[305]. In this trimeric complex, menin serves as a mediator between MLL and LEDGF and fusing the PWWP reader domain directly with MLL can bypass the need for menin binding[134,338]. Given the importance of Menin in targeting MLL and MLL fusion proteins, small molecules targeting Menin have been of great interest, with several of them currently in clinical trials [153,287].

Figure 1-6: The MLL Protein Domains and Functions

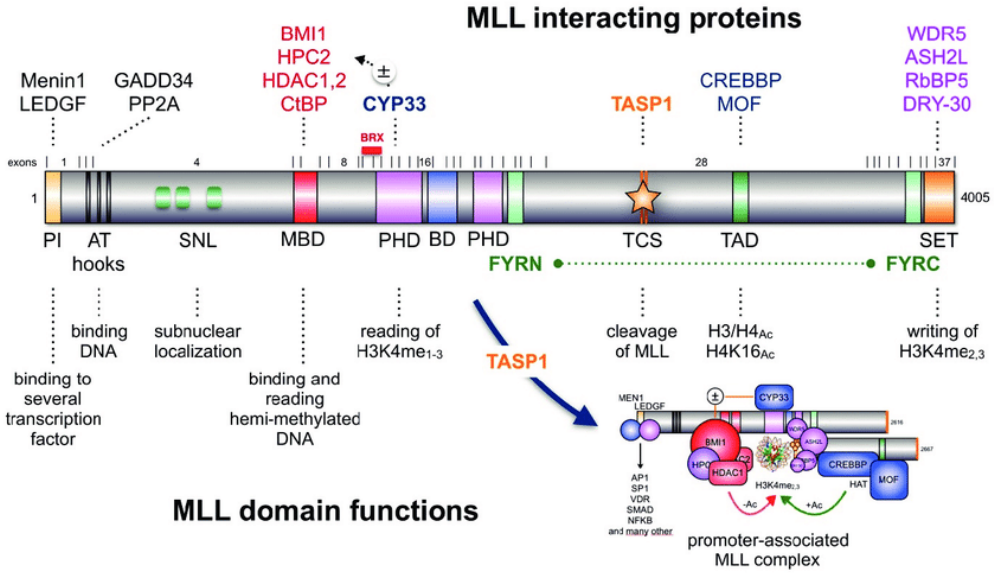


Figure depicts the wild type MLL protein with annotated domains, functions, and binding partners. Figure is taken from Marschalek, Annals of Laboratory Medicines, 2016.[189]

On the N-terminus of the MLL protein, the AT hooks bind to the minor groove of AT-rich DNA[347]. Two repression domains (RD) are located downstream of the AT hooks and were shown to repress transcription in a reporter gene system[347]. The CxxC (RD1) region binds specifically to unmethylated CpG DNA[6,19]. The RD1 and RD2 domain of MLL coimmunoprecipitates with co-repressor complexes such as HDAC1, CtBP, BMI1, and HPC2, several of which are members of the polycomb repressor complex[325]. Interestingly, the CXXC-RD2 region also interact with the Polymerase Associated Factor 1 Complex (PAC1c)[207,217]. Further downstream are a cluster of 3 PHD fingers, a bromodomain, followed by another PHD finger. In general, these PHD fingers are thought to play a co-repressor role. For instance, PHD2 has E3 ubiquitin ligase activity and plays a role in H3, H4 and MLL ubiquitination and subsequent degradation during cell cycle[312]. PHD3, the most characterized PHD finger of MLL, displays epigenetic reader function and binds to trimethylated H3K4 marks[35,317]. It also interacts with

Cyp33[317], and this interaction has been shown to be needed for some of RD interactions with Polycomb group proteins[317,325] as well as repression of Hox genes[88,317]. Most MLL-r leukemia patients (>90%) have breakpoints that are located between MLL exon 9 and exon 11, which is located between the CXXC-RD and the 1st PHD finger of MLL[199]. As a result, MLL fusion proteins retain the ability to bind to DNA, LEDGF/Menin, PAF1c, and certain co-repressor complexes. Interestingly, previous studies showed that PHD finger inclusion destroys MLL-r leukemogenesis using an MLL-AF9 model[216].

Downstream of the PHD fingers are the FYRN region, a transactivation domain (TAD), FYRC region, followed by the catalytic SET domain. The MLL protein is cleaved into two fragments by the protease Taspase 1 post-translationally, and the two protein fragments interact with each other through the FYRN and the FYRC regions[126,339]. The MLL TAD co-immunoprecipitates with the co-activator protein CBP (CREB binding protein) and the histone acetyltransferase MOF complex[77,86]. At the C-terminus of the protein is the catalytic SET domain, which catalyzes the methylation of H3K4[205]. The MLL-C fragment forms a complex with at least 4 other proteins: WDR5, RBBP5, ASH2L and DPY30[250]. WDR5 is necessary for targeting MLL to H3K4me2 to catalyze H3K4 trimethylation and Hox gene activation[324]. RBBP5 is important for the MLL-C complex integrity as it interacts with MLL-C, ASH2L, and WDR5, although both RBBP5 and WDR5 are required for the integrity of MLL-C complex[76].

In MLL-r leukemias, the MLL-N portion is fused to a fusion partner and producing an oncogenic fusion protein. The role of MLL-C from the same allele in leukemogenesis remains to be further investigated. Previous studies have explored the balanced

translocation hypothesis using the MLL-AF4 and AF4-MLL fusions. However, these studies generated conflicting evidence as to how the balanced reciprocal translocation contributes to MLL-r leukemogenesis[27,244]. The balanced translocation hypothesis is further complicated by the discovery of complex MLL rearrangements, which involves more than 2 chromosomal translocation events[201]. Another interesting question is whether and how the wild type MLL allele contribute to MLL-r leukemogenesis. Using the MLL-AF9 model, two publications have come to opposing conclusions on whether wild type MLL is required in MLL-AF9 leukemia[39,293]. Blocking MLL1 methyltransferase activity using the inhibitor MM-401 (which disrupts the MLL1-WDR5 interaction) blocks proliferation and induces cell cycle arrest, differentiation and apoptosis in several MLL-r models[31]. In a separate study, it was demonstrated that wild type MLL and MLL-r regulate distinct targets and their recruitments to target genes are also differentially regulated[329].

MLL is not required to initiate but is required to maintain proper expression of a subset of Hox genes. MLL null mice showed similar expression of Hox genes until E9 but failed to maintain expression and were not viable past E10.5 and have reduced number of HSCs[196,341,342]. Additionally, studies done in adult hematopoiesis determined that MLL is important in adult HSC function and maintenance, as MLL conditional deletion results in bone marrow failure[143]. MLL +/- mutants showed severe growth defects such as homeotic transformation of the anterior and posterior axial skeleton and hematopoietic defects such as anemia and decreased platelet and B- cell counts[342]. In a study aimed at identifying MLL targets, Wang and colleagues identified a few hundred genes requiring MLL to maintain their H3K4 methylation levels and expression. While

only a subset of Hox genes are directly regulated by MLL, its interacting partner Menin is necessary for proper H3K4me3 across the Hox loci[314]. In MLL-r leukemias, the targets of some MLL fusion proteins have been established[18,104,116]. Interestingly, targets of MLL-AF9, MLL-ENL and MLL-AF4 do not completely overlap, suggesting that in the context of MLL fusions the fusion partners might play a role in directing the MLL-r proteins.

1.3.2 Common MLL Fusion Partners

Figure 1-7: MLL-r Fusion Partners in MLL-r Acute Leukemias

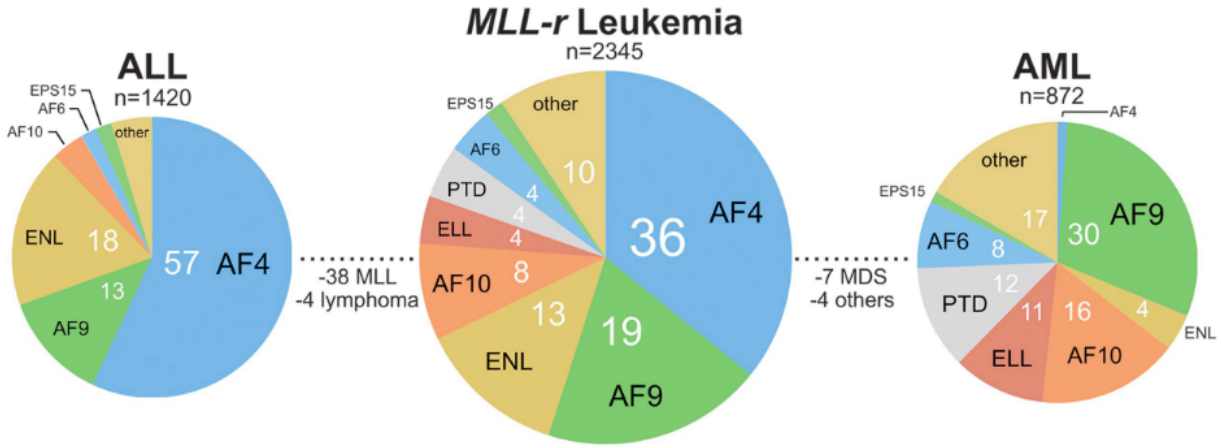


Figure shows the most common MLL fusion partners in MLL-r leukemias in N=2345 patients, broken down into lineage subtype. Numbers in figure represent percentages of patients with the particular fusion partners. Figure is taken from Meyer et al., Leukemia, 2018.[199]

The most common MLL fusion partners can be found in **Figure 1-7.** The first clue of a converging mechanism on how most MLL-r fusion drives leukemogenesis was elucidated when several different groups performed co-immunoprecipitation experiments isolating protein complexes associated with these fusion partners and found similar complexes. Multiple names were used to describe these overlapping protein complex: ENL-associated proteins (EAPs)[214], AF4 family/ENL family/P-TEFb coactivator complex (AEP)[340], and the super elongation complex (SEC)[175]. I will discuss

these common MLL fusion partners immediately below. Additionally, all these studies identified the presence of P-TEFb and DOT1L in their respective protein complexes. The importance of P-TEFb and DOT1L in positively potentiating transcription has been extensively documented in the context of MLL-r leukemia. An abridged version of MLL-r leukemogenesis mechanism can be found in **Figure 1-8.** Further in the subsequent sections, I will discuss the current status quo of our mechanistic knowledge on how these key players are hijacked in MLL-r leukemia to dysregulate pro-leukemic genes.

Figure 1-8: Depiction of MLL-r Leukemogenesis Mechanism

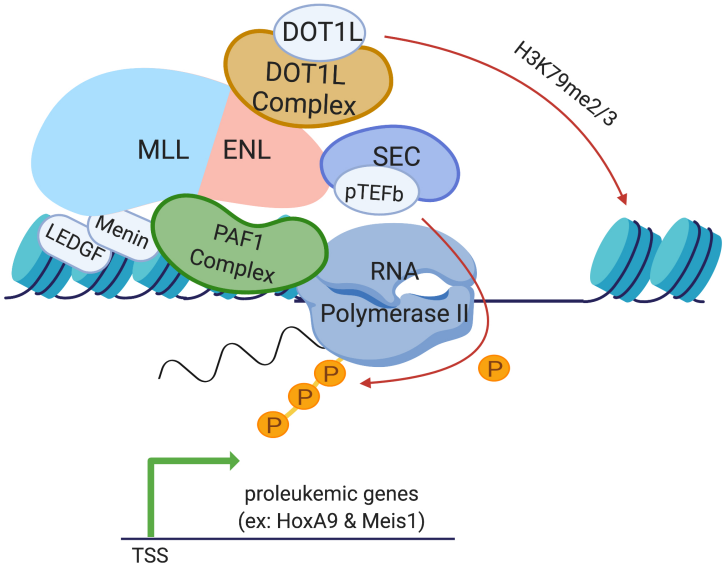


Figure models our current understanding on how the MLL-r oncoproteins hijack several complexes important in RNA polymerase II regulation to promote leukemogenesis. The protein ENL is used as an example for MLL fusion partner in this diagram.

AFF1 (AF4):

AFF1 (AF4) is the most common MLL fusion partner, encompassing about ~36% of MLL-r cases[199]. It belongs to the AFF family, which includes three other members: AFF2, AFF3 (LAF4), and AFF4 (AF5q31)[40]. Other than AFF2, all AFF family members are MLL fusion partners, with MLL-LAF4 and MLL-AF5q31 occurring at a lower frequency[199]. Structurally, AFF family members share the N-terminal homology domain

(NHD), the ALF domain, and the C-terminal homology domain (CHD)[229]. In the SEC, AFF1/AFF4 serve as a scaffolding protein, with the NHD interacting with CyclinT1 of the P-TEFb, CHD as a homodimer/heterodimer binding site, and the middle regions identified as binding sites for ENL/AF9 and ELL[40]. In mice, AF4 null mice showed a defect in lymphoid lineage, affecting both T and B cell development[137]. Interestingly, while AF5q31 null mice showed mostly embryonic lethality, the small fraction that survived (13%) were sterile and showed defect in spermiogenesis[300]

ENL (MLLT3) and AF9 (MLLT1)

Both ENL and AF9 belong to the YEATS family and are the third (~13%) and second (~19%) most common MLL fusion partners[199]. Both proteins share sequence homology in two domains, namely the N-terminal YEATS domain and the C terminal ANC-1 homology domain (AHD)[42]. ENL and AF9 AHD has been shown to form complexes and interact the SEC, DOT1L, and the Polycomb Repressive Complex 1 (PRC1) members BMI1 and CBX8[183,185,299]. It was proposed that interaction with PRC1 neutralizes PRC1's repressive activity[183]. The N-terminal YEATS domain is a chromatin reader that connects the SEC and DOT1L to chromatin[85,172,311,346], and this epigenetic reader function will be discussed in detailed in later sections. ENL knockout is embryonically lethal[75], while AF9 knockout mice die within 2 weeks of birth[46]. In the hematopoietic system, AF9 has been shown as an important factor for HSC maintenance, stemness and transplantation capacity[28].

AF10 (MLLT10)

AF10 is the 4th (~8%) most common MLL fusion partner[199]. AF10 knockout is embryonically lethal in mice[226]. In addition to its role in MLL-AF10 fusions, AF10 is a fusion partner of the Clathrin assembly lymphoid myeloid leukemia (CALM) protein through chromosomal translocations involving chromosomes 10 and 11[61]. Similar to MLL-r leukemias, CALM-AF10 also dysregulates expression of Hoxa genes[33]. Structurally, AF10 is an epigenetic reader through its PHD1-zinc-knuckle-PHD2 (PZP) domain, which contains 2 PHD fingers and recognize unmodified H3K27 marks[38]. The OM-LZ domain of AF10 interacts directly with DOT1L[227]. A previous study on WT AF10 concluded the protein as an essential factor in several AML subtypes, presumably because of its interaction with DOT1L[60].

ELL1

ELL1 (elongation factor for RNA polymerase II) is the 5th (~4%) most common MLL fusion partner[199]. It was first identified and characterized as a factor that increase the Vmax of RNA pol II elongation[278]. ELL knockout is embryonic lethal in mice[209]. Out of the three ELL family members, only ELL1 is an MLL fusion partner[199]. Crystal structure of ELL2 revealed that its occluding homology domain directly interacts with AFF4[245], with both proteins as components of the SEC. In addition to the SEC, ELL is a member of the Little Elongation Complex (LEC), which is a positive potentiating factor for RNA pol II-dependent transcription of small nuclear RNA (snRNA)[280].

In conclusion, the common fusion partners of MLL are important in embryonic development as shown in the knockout studies. These partners participate in several

overlapping complexes including the SEC and DotComm (which includes DOT1L). The SEC plays an important role in RNA pol II proximal-pause release, and how the SEC regulate such process will be discussed later. DotCom contains the methyltransferse DOT1L, which is extensively characterized for its important role in MLL-r AMLs demonstrated through genetic and pharmaceutical studies. In my next section, I will discuss the protein DOT1L and its importance in MLL-r AMLs.

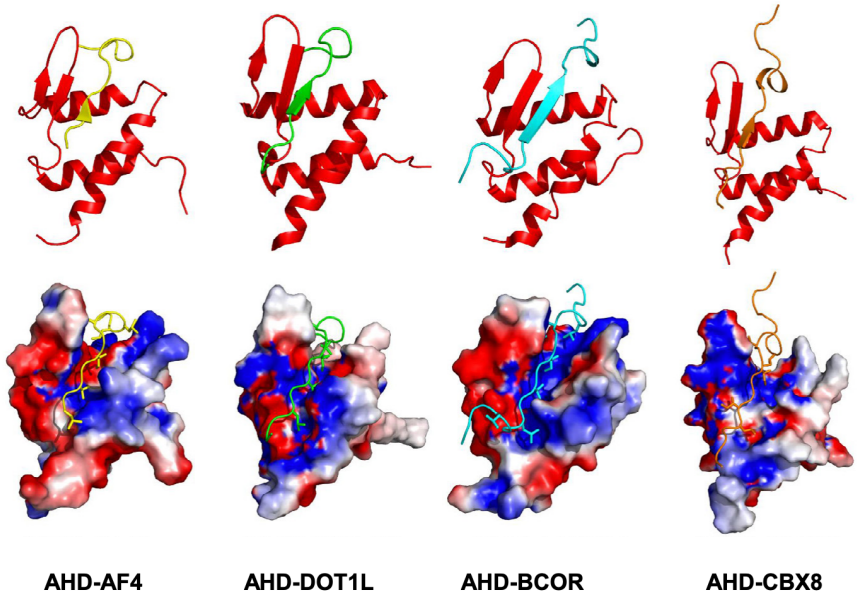
1.3.3 The DOT1L Histone Methyltransferase

DOT1L is the first and only H3K79 methyltransferase identified in human[91]. DOT1L differs from other methyltransferases in that it lacks the catalytic SET domain which confers the methyltransferase activity, and instead uses the N-terminal catalytic domain for H3K79 methyltransferase activity[208]. Early studies in MLL-r leukemias established that DOT1L interacts with or exist in the same protein complex with many MLL fusion partners[210,214,227,340]. More importantly, DOT1L's importance was shown when several independent groups showed that it is necessary for MLL-r leukemogenesis through both genetic and chemical studies[18,34,55,59,156,219]. Because of the observation that MLL-r leukemia cells are highly dependent on DOT1L and its activity, DOT1L is an attractive target for small molecule inhibitor development targeting the enzymatic activity or protein-protein interactions[54,274]. The DOT1L inhibitor EPZ-5676 was shown to be effective against MLL-r leukemia xenograft in mouse and synergizes with other epigenetic targeting drugs[54,151]. However, it only had a modest effect in clinical trials[288].

The term Dot1 complex (DotCom) was coined by Shilatifard's group when they identified proteins that coimmunoprecipitated with DOT1L, which included several

members of the SEC[210]. The observation that both AFF1/AFF4 and DOT1L interact with ENL/AF9 at the AHD, and that DOT1L-ENL and AFF4-ENL coimmunoprecipitations seem to be mutually exclusive[340] raises the question of whether SEC and DotCom are two separate complexes that function synergistically to activate target gene transcription. This idea is further modelled in **Figure 1-9**, whereby the AF9 AHD interacts with AFF1/AF4 and DOT1L in very similar conformations.

Figure 1-9: MLLT3/AF9 AHD Interactions



The ribbon and surface representations of AF9/MLLT3 AHD interactions with AFF1/AF4, DOT1L, BCOR and CBX8. Figure is taken from Kabra and Bushweller, Journal of Molecular Biology, 2022.[144]

Structurally, DOT1L interacts directly with the ANC-1 Homology Domain (AHD) of ENL/AF9[274,340] and the OM-LZ domain of AF10[351]. Disrupting the key residues in the AHD of ENL/AF9 compromises colony formation capacity and pro-leukemic gene expression in MLL-ENL and MLL-AF9 cells[340]. In mice, DOT1L knocked out is embryonically lethal[142]. Analysis of the yolk sac indicated increased apoptosis and cell cycle arrest in DOT1L null mice compared to wild type[92]. Additionally in adult

hematopoiesis study, conditional DOT1L knockout resulted in reduced HSPCs and lineage-specific progenitors[141]. Mechanistically, the levels of DOT1L and H3K79 di- and tri-methylation on the chromatin generally correlate with the degree of active transcription[220]. However, the mechanism by which H3K79 di- and tri-methylation drive active transcription and how these marks are removed remain unknown. In summary, DOT1L is an essential epigenetic writer in development and in MLL-r leukemia, although the mechanism of its contribution to gene regulation is unclear and remains currently at the correlative stage.

1.3.4 RNA Polymerase II Promoter-Proximal Pausing and Release

RNA pol II-mediated transcription is a highly regulated process, and transitions among the different phases are highly regulated. RNA pol II promoter-proximal pausing, first described in Drosophila heat shock protein (hsp) gene transcription[257], involves the RNA pol 2 machinery synthesizing a fragment of nascent RNA (20-60 bps) and pausing 25-50bp downstream of the transcriptional start site[48]. The RNA pol II then remains stably attached to the DNA and can either resume into elongation phase or terminate transcription upon receiving further signals. Promoter-proximal pausing is a wide-spread mechanism of gene regulation[48]. Using GRO-seq to map nascent RNA to promoter proximal RNA pol 2, Core and colleagues determined that promoter proximal pausing is a transcriptional regulatory mechanism in at least 30% of human genes[49]. Two factors, the negative elongation factor (NELF) and the DRB sensitivity-inducing factor (DSIF, a dimer of two proteins Spt4 and Spt5), are required for promoter proximal pausing[307,333]. Phosphorylation on three proteins, including RNA pol II C-terminal S2, Spt5 and NELF, triggers the release and transition of RNA pol II into a productive elongation phase[243].

The phosphorylation events are mediated at least by the positive transcription elongation factor (P-TEFb) complex, which is composed of the cyclin T1 and CDK9 subunits[239]. One well-studied mechanism of P-TEFb regulation is its release from the 7SK snRNP complex. A majority of nuclear P-TEFb is sequestered by the small RNA-protein complex called 7SK snRNP together with RNA binding proteins HEXIM1 or HEXIM2[221,335,336]. The release of P-TEFb from 7SK snRNP has been demonstrated through many mechanisms, such as post-translational modifications on either P-TEFb or other components of the 7SK snRNP complex[37,47]. Specifically, the HIV tat protein can interact with P-TEFb via its activation domain and release P-TEFb from 7SK snRNP, hijacking it to HIV LTR sequence for viral transcription[187,269,357]. Two independent studies on the Tat- P-TEFb complex revealed several other protein components, including ENL, AF9, AFF1/AF4, AFF4/AF5q31, ELL, ELL2 and the PAF1c[124,283]. In addition to the HIV Tat protein, the Bromodomain-containing protein 4 (BRD4) have also been shown to be able to release P-TEFb from the 7SK snRNP complex[139,334]. BRD4 is an epigenetic reader protein through its two N-terminal bromodomains and is widely regarded as a transcriptional activator. The bromodomains of BRD4 can interact with acetylated H3 and H4 histones[63]. Genome-wide studies revealed high level of colocalization between BRD4 and RNA pol II[179], suggesting broad distribution of BRD4 across the genome. In addition, BRD4 has been shown to localize at a subset of enhancers and interact with lineage specific transcriptional factors to regulate target expression[73]. Additionally, BRD4 also interacts with several epigenetic writers and chromatin remodelers to decompact chromatin and facilitate active transcription[73]. Interestingly, studies identifying interacting partners of Tat- P-TEFb did

not identify BRD4 as part of the complex[124]. This result is consistent with the idea that BRD4- P-TEFb and Tat- P-TEFb exist in different complexes and that BRD4 and Tat compete for P-TEFb[20,301]. Using mouse ES cells stimulated with retinoic acid, Lin and colleagues showed that SEC formation and interaction with P-TEFb can happen independent of Tat to drive target transcription[174]. In the same study, BRD4 localized to a subset of SEC targets but had little to no impact on the expression of these targets when knocked down, suggesting that the functions of BRD4- P-TEFb and SEC-P-TEFb might overlap little to none.

The most common MLL fusion partners are members of the SEC and DotCom, and in turn hijacks the SEC and DotCom to MLL fusion targets to drive hyperactive transcription of these genes. The history behind the discovery of SEC, RNA pol II-dependent transcription, BRD4 and P-TEFb is a fascinating one and provides important mechanistic insight in our understanding of MLL-r leukemias. Another important protein complex is the PAF1c, which has also been shown to be essential in MLL-r leukemias. PAF1c is a complex implicated in multiple different stages of transcriptional regulation. In my next section, I will discuss briefly about the PAF1c, and our currently knowledge on how it fosters a conducive epigenetic landscape around the transcriptional start site for RNA pol II pause-release.

1.3.5 The PAF1c in MLL-r Leukemias

The Polymerase Associated Factor 1 Complex (PAF1c) is a protein complex extensively studied in the context of RNA pol II transcription and influences gene regulation through a myriad of mechanisms such as epigenetic modification, chromatin structure, DNA repair, mRNA transport and genomic stability[101,302]. Early work done in

S. cerevisiae established the core components of the PAF1c to include Paf1, Ctr9, Cdc73, Leo1 and Rtf1[213,277,308]. In higher organisms such as human, another subunit WDR61 is also a part of the PAF1c[356]. Deletion of PAF1c components results in a wide range of yeast mutants albeit not essential for yeast viability[101]. Importantly, these yeast mutants showed phenotypes consistent with transcriptional and chromatin structural defects[101]. Early studies have established a role for the PAF1c with RNA pol II during transcriptional elongation[50,285]. In general, the PAF1c localization is correlated with sites of active transcription, although PAF1c has also been shown to act as a transcriptional repressor[344]. In *S. cerevisiae* at least, PAF1c recruitment to RNA pol II is proposed to occur after RNA pol II phosphorylation at the CTD, both independent and dependent of Spt5 (part of the DSIF)[246,318]. In higher organisms, PAF1c recruitment could be mediated by transcriptional activators to promoter and enhancer sequences. For instance, post-transcriptionally modified CDC73 has been shown to interact with transcriptional factors in the Wnt, Hedgehog and Notch pathways[148,289]. These findings suggest that the PAF1c recruitment might be cell-type specific and regulated in a spatial-temporal manner. In concert with this idea is the observation that the PAF1c only regulates RNA pol II transcription in a subset of RNA pol II targets, as PAF1 knockdown did not cause global transcriptional shutdown[344]. Different PAF1c subunits have been assigned with different functions based on biochemical characterization. For instance, the subunits PAF1 and CTR9 are highlighted for their necessity in maintaining the PAF1c integrity[43]. At least two PAF1c two contact points with RNA pol II have been identified on the RTF1 and CDC73 subunits[195,246,318] . The LEO1 subunit has been

implicated in PAF1c recruitment[58] and together with PAF1 bind to H3 to facilitate PAF1c-chromatin interaction[43].

One mechanism by which the PAF1c regulates transcription is through influencing the epigenetic landscape of its targets. The ubiquitination of H2B at K123 (K120 in human) is an epigenetic modification implicated in RNA pol II elongation[326]. This epigenetic mark is catalyzed by the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin-protein ligase Bre1 (RNF20 and RNF40 in human)[135,254]. In yeast studies, Bre1 and Rad6 interact with the PAF1c[150,303] and this interaction is essential for H2B ubiquitination[322]. The PAF1c has also been shown to promote ubiquitination of H2BK34, which is deposited by the E3 ubiquitin ligase MSL2[323]. Mechanistically, H2B ubiquitination has been shown to destabilize chromatin compaction, leading to a chromatin conformation more conducive for transcription[93]. Both H2BK120ub and H2BK34ub have been shown to be prerequisite for downstream histone H3K4 and H3K79 methylation[157,322], two epigenetic marks associated with active transcription. In addition to H2B ubiquitination's role in promoting downstream epigenetic changes, it also promotes change in chromatin structure, with one well-studied example of cooperation with the FACT histone chaperone[237]. The FACT histone chaperone has been shown to displace the H2A/H2B histones from the histone octamer[17], promote further H2B ubiquitination[96], and potentiate transcriptional elongation[96,237]. In summary, the PAF1c is recruited to phosphorylated RNA pol II likely through multiple mechanisms (such as through transcriptional factors) and plays an essential role in regulating the epigenetic landscape transiting RNA pol II from the proximal-paused state to the elongation state.

In humans, PAF1c dysregulation has been associated with several different cancers. PAF1 is found to be overexpressed in poorly differentiated pancreatic cancer[146]. In a subset of Wilm's tumor, CTR9 is mutated and proposed to be a tumor suppressor gene[119]. CDC73 autosomal dominant mutations or deletion has been found in hyperparathyroidism jaw tumor (HPT-JT) syndrome patients[296]. In MLL-r AMLs, the PAF1c interacts with MLL at the CxxC-RD2 region and this interaction is essential in MLL-r leukemia[215,217]. The recruitment of the MLL-r fusion to its target, such as Hoxa9, is at least partially mediated by the PAF1c[217]. Using a *Cdc73* knockout, our lab demonstrated that while the PAF1c is important in both the hematopoietic stem cells and MLL-r AMLs, PAF1c seems to demonstrate functions unique to MLL-r leukemia[262]. Together, these lines of evidence demonstrate the importance of the PAF1c in MLL-r leukemias.

1.3.6 Alternative MLL-r Leukemogenesis Mechanism

In the above sections, I have discussed the key players in MLL-r leukemogenesis and how they contribute to hijacking RNA pol II in activating pro-leukemic gene transcription. A summary of this canonical model can be found in **Figure 1-8.** Another observation and leukemogenesis mechanism in a subset of MLL-r leukemias is the ability of the fusion partners to induce MLL dimerization. Although the precise molecular mechanism remains unclear on how MLL-r dimers drive leukemogenesis, MLL-r dimers have been observed when MLL-N is fused to several different partners: AF6 (Afadin), ESP15 (AF1P), GAS7, and SEPT6[173,231,281]. In these leukemias, similar upregulation of pro-leukemic target genes has been reported[231,281]. Most of these fusion partners are cytoplasmic proteins and are forced into the nucleus when fused to MLL[186,231]. The

domains in these proteins important for the dimerization have been reported, such as the coiled-coil domain of AF1P, GAS7, and SEPT7 and the Ras association (RA) domain of AF6[173,231,281]. Interestingly, MLL-N dimerization induced by an artificial receptor and ligand system is enough to transform HSPCs, blocking myeloid differentiation and inducing expression of targets such as Hoxa7/9 and Meis1 while the common MLL fusion MLL-AF9 is not able to induce MLL dimers[192]. In the interesting case of MLL-AF6, MLL-AF6 proteins have been shown to dimerize, hijack wild type AF6 into the nucleus and dysregulate the Ras signaling pathway[173,186]. At the same time, MLL-AF6 leukemias have also been shown to require DOT1L activity[59]. This line of evidence suggests that MLL fusion-mediated dimerization and the dysregulation of SEC/DOT1L activity might not be mutually exclusive in driving MLL-r leukemogenesis. Another common driver of MLL-r leukemia is the MLL partial tandem duplication (PTD). MLL-PTD usually involves an in-frame duplication of E2/E3 to E6-E11[154]. It has been shown that MLL-PTD driven leukemia aberrantly upregulate Hox gene[74]. Because of the partially duplicated MLL-N proteins in MLL-PTD, it is hypothesized that MLL-PTD shares similar leukemogenesis mechanism with MLL-r dimerization[337].

1.3.7 Summary

The MLL protein is an epigenetic writer for methylated H3K4 marks and can interact with transcriptional factors, histone, and DNA. In about 10% of acute leukemias, MLL is fused to a fusion partner and thereby acquiring the functions of the fusion partner. Biochemical studies have concluded that most of these fusion partners belong to several protein complexes relevant in regulating RNA polymerase II mediated transcription. In this section, I provided an overview on several common MLL fusion

partners as well as key contributing complexes to MLL-r leukemogenesis. These key complexes are hijacked to MLL targets via protein-protein interactions with the MLL fusion protein, and continued investigation to identify and characterize these protein-protein interactions will supply clues on how MLL-r leukemias can be targeted. Two of the most common MLL fusion partners, ENL and AF9, are epigenetic readers of acetylated histone lysine marks. Given the ample evidence of epigenetic dysregulation in AMLs, one curious question is whether this epigenetic reader function of ENL and AF9 could contribute to MLL-r leukemia. In the next section, I will briefly discuss readers and writers of histone acetylations, and shift focus to the epigenetic readers of histone acetylation.

1.4 Histone Acetylation: An Overview on Readers

Histone acetylation is one of the most well-studied histone post-translational modifications which can be found across all four histones[1]. Histone acetylation largely happens at lysine residues, although other amino acids, such as serine, threonine, and tyrosine can also be acetylated[133]. Epigenetic dysregulation involving histone acetylation has been well documented[64,352]. In MLL-r AMLs, two of the most common fusion partners are epigenetic readers of acetylated histones. In this section, I will provide an overview of histone acetylation, with an emphasis on the epigenetic readers of acetylated histone marks.

1.4.1 The Deposition and Removal of Histone Acetylation Marks

In H3 and H4 alone, over 22 lysine residues can be acetylated by a group of epigenetic writers collectively referred to as histone acetyltransferase (HATs)[133]. The

HATs catalyze histone acetylation by transferring the acetyl group from acetyl-CoA to the ε-amino group on lysine residues of histones[15,188]. In general, histone lysine acetylation is thought to be associated with active sites of transcription, as acetylation neutralizes the lysine residue's positive charge, reducing the interactions between histones and DNAs[15].

Based on their function, HATs can be grouped into type-A and type-B. Type-B HATs reside predominately in the cytoplasm and acetylate free histones that haven't been incorporated into the chromatin[15], and this acetylation might be important for histone incorporation into the nucleosom[282]. Type-A HATs are found predominately in the nucleus and often associated with larger protein complexes. The 9 mammalian members can be further grouped into 3 groups based on the structure of their catalytic and substrate binding sites[306]. While knockout and knockdown studies have provided some evidence of assigning residue-specific HAT activity, the results are confounded by assay system and conditions as well as model organisms[306].

Acetylation of histone lysine are reversible and catalyzed by a group of enzymes called histone deacetylases (HDACs), thereby restoring the positive charge on the lysines and formation of compact chromatin structure. HDACs can be divided into roughly 4 main classes based on the co-factors (Zn2+ (the classical HDACs) or NAD+) and sequence homology[204,271]. Classes I, II, and IV HDACs use Zn2+ as their co-factor, while class III HDACs use NAD+ as their co-factor[204,271]. In human, there are a total of 18 HDACs identified[271]. Because HDACs lack DNA binding activity in general, they are usually found in complexes with transcriptional factors or large transcriptional complexes. HDAC I and II, two of the most well studied class I HDACs, participate in a

wide array of transcriptional co-repressor complexes (such as the Sin3, CoREST, and the NuRD complexes)[10,204,271]. Additionally, these protein complexes often include members with DNA binding and chromatin remodeling activities. Lastly, assigning residue specific HDAC functions has been difficult. Class I HDACs have been shown to deacetylate all 4 histone subunits in the histone octamer at multiple lysines, and some of these lysine residues can be deacetylated by multiple Class I HDACs[169,204].

1.4.2 The Bromodomain Epigenetic Readers

The bromodomain is a protein domain consists of many α-helices connected by loop structure, forming a hydrophobic cavity that binds to acetylated lysines[65]. In human to date, there are a total of 61 bromodomains identified across 46 proteins, which are roughly grouped into 8 families through sequence and structural homology[138]. Most of these bromodomain proteins are subunits of transcriptional regulator and chromatin remodeling complexes[138]. Additionally, many epigenetic writers with methyltransferase or acetyltransferase activities also have bromodomains[138]. Bromodomain-containing proteins can be categorized into bromodomain and extra-terminal (BET) vs non-BET families, depending on the presence or absence of extra-terminal domains[138]. There are 4 human species in the BET family: BRD1 through BRD4. These 4 proteins share 2 N-terminal bromodomains (BD1 and BD2) and a C-terminal extra-terminal (ET) domain. Specifically, the bromodomains of BET family can bind to diacetylated KXXK motif in histones and transcriptional factors[159,236], linking BET family members with lineage-specific transcriptional factors and acetylated chromatin[160]. The ET domain of BET family members has been shown to interact with a myriad of histone modifiers and chromatin remodeling complexes such as NSD3, JMJD3, CHD4 and GLTSCR1[248].

Additionally, studies done on BRD3's ET domain hypothesized that the ET domain recognizes chromatin remodeling complexes through a conserved KIKL motif on one of the complex subunits[309]. In addition to BRD4's role in RNA pol II promoter pausing and releasing (discussed above), the BET family members also function to modulate chromatin 3D structure. BET family members can establish transcriptional boundaries through CTCF/cohesin interactions[129], enhance histone acetylation and chromatin remodeler recruitment through HAT interaction[1], and evict histones[62]. In summary, the BET family is a group of well-studied bromodomain-containing proteins, which can interact with transcriptional factors, chromatin remodelers and histone modifiers to direct transcriptional changes through several sequence-specific mechanisms.

1.4.3 The YEATS Domain on Epigenetic Readers

The YEATS domain is a highly conserved epigenetic reader domain that recognize histone acylations (such as acetylation, propionylation, butyrylation, and crotonylation) with a stronger preference for crotonylation over acetylation[355]. In this section, I will focus on the YEATS domain's role as an epigenetic reader for acetylated histone lysines, and the YEATS domain's role as a histone crotonylation reader will be discussed briefly later.

The name YEATS is derived from 5 different proteins sharing this epigenetic reader capacity: Yaf9, ENL, AF9, Taf14, Sas5[268,355]. Over 100 YEATS domain proteins have been identified in over 50 eukaryotic species[268]. In *S. cerevisiae,* three YEATS proteins Yaf9, Taf14, and Sas5 have been identified. These *S. cerevisiae* YEATS proteins participates in at least 8 histone-modifying, chromatin remodeling, and transcription regulatory complexes involved in many cellular processes such as DNA

damage response, telomere silencing, and cell cycle progression among others[53,268,328,350]. In human, 4 species of the YEATS domain family have been identified: ENL (YEATS1), YEATS2, AF9 (YEATS3), and GAS41 (YEATS4). Works from the Shi lab have demonstrated that all four members of the human YEATS proteins have acetylated reader functions[127,172,203,311]. In particular, the human YEATS domains show the strongest interaction with acetylated H3 lysines and weak to no interactions with acetylated H4 or H2B lysines[127,172,203,311]. However, the specificity differs. For instance, YEATS2 binds to H3K18ac and H3K27ac but not H3K9ac nor H3K14ac[203]. GAS41 YEATS domain recognizes H3K14ac and H3K27ac the strongest, with much weaker interactions with H3K9ac and H3K18ac[127]. Both ENL and AF9 YEATS domains show affinity for H3K9ac, H318ac and H327ac and have a much weaker interaction with H3K14ac[172,311].

The structure of the human YEATS domains binding to H3K27ac have been solved[128,172,203,311]. The YEATS domain adopts a β-sandwich with an aromatic cage that binds to the acetylated lysine residues and the key residues of the aromatic cage responsible for the YEATS interaction have been identified[127,171,172,203,311]. Interestingly, Hsu and colleagues pointed out that there are two distinct binding modes across the four different YEATS domains[127]. In ENL, the YEATS domain adopts an N-to-C orientation when binding to H3K27ac while in GAS41 and YEATS2 the binding adopts a C-to-N orientation[127], suggesting a difference in the four YEATS domain and potentially providing an explanation for differential affinity to different acetylated lysines.

The YEATS domain family members have been implicated in different cancers. GAS41 is amplified in at least a subset of non-small cell lung cancer and

glioblastoma[95,127], and is a part of the SRCAP and TIP60 chromatin remodeling complexes[235]. GAS41 has been shown to be an epigenetic reader of acetylated H3K14 and H3K27 and recruit these two chromatin remodeling complexes for the deposition of histone variant H2A.Z[128], and this function is important in maintaining ESC identity and hijacked in non-small cell lung cancer model[127,128]. YEATS2 is amplified in a subset of lung squamous cell carcinoma, ovarian serous cystadenocarcinoma, and head and neck squamous cell carcinoma[203]. It is a part of the Ada-two-A-containing (ATAC) complex with histone acetyltransferase activity and regulates H3K9ac levels at target promoters[203].

ENL and AF9's roles as SEC and DotCom components, as well as MLL fusion partners, have been discussed in previous sections. The YEATS domain of ENL and AF9, other than their role as an epigenetic reader, also bind to the PAF1c[123,125,283]. This function of their YEATS domain has been proposed as a targeting mechanism connecting the SEC with the PAF1c[123]. In Wilm's tumor, ENL somatic mutations have been identified[125]. These patient-specific mutations are characterized with short insertion and deletion within the YEATS domain[125,310], and increases ENL's occupancy at its genomic targets[310,284], and increase recruitment of the SEC through a process called phase separation **(Figure 1-10)** without altering its epigenetic reader function nor interaction with the PAF1c[125,284]. Interestingly, ENL has previously been implicated as a component of the EBAFb complex, which belongs to the chromatin remodeler SWI/SNF family[222], however the exact ENL protein domain that contribute to this interaction remains unknown.

Figure 1-10: Pathogenesis Model of Wilm's Tumor Patients with ENL YEATS Mutations

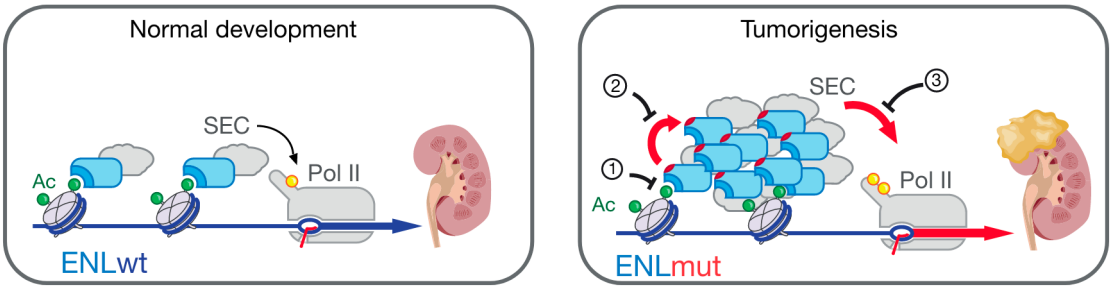


Figure depicts current model on how ENL YEATS mutants in Wilm's tumor patients drive tumorigenesis through a pathogenic level of ENL self-aggregation and SEC recruitment. Figure is taken from Wan et al., Nature, 2020.[310]

1.4.4 Histone Crotonylations: Epigenetic Marks Read by the YEATS Domain

The human YEATS domain proteins have also been shown to bind to histone crotonylations[140,171,354]. Histone crotonylation was first identified in male germ cell as a marker of active promoter using mass spectrometry-based proteomics approach and generally coincides with sites of acetylated histones[291]. The crotonylation modification is very similar to an acetylation, except the length of the hydrocarbon[224]. Like acetylation, crotonylation happens at the ε-amino group on lysine residues and involves a substrate called Crotonyl-CoA. The increased hydrocarbon length in a crotonylated histone mark, compared to an acetylated histone mark, means that the hydrophobicity of modified lysine increases[224,261]. Histone crotonylation or decrotonylation is relatively less efficient compared to histone acetylation or deacetylation because steric hinderance[145,261]. To date, no enzymes have been identified to exclusively modify histone crotonylation. HATs such as p300/CBP and class I HDACs have been identified to catalyze the crotonylation of histone marks[181,260].

Interestingly, the YEATS domain and the double PHD fingers are the only domains identified with higher affinity to histone crotonylation than acetylation[152,327,355].

A subset of bromodomain containing proteins can also bind to histone crotonylation, with affinity close to or less than histone acetylation binding[97]. More studies are needed to elucidate the epigenetic significance of different histone acylation marks and their differential affinity to the various epigenetic readers to decipher the biological and pathological significance.

1.4.5 Other Readers of Acetylated Histones

Several less studied protein domains also have acetylated histone lysines reader functions. The double/tandem PHD fingers in the MORF/MOZ HAT complex and the protein DPF3 have been shown to interact with acetylated H3K14 and H3K9[5,247,348]. Interestingly, the MOZ tandem PHD finger binding to H3K14ac creates a positive loop of increased H3K14ac level at targets such as *HOXA9*[247]. Additionally, a bromodomain-like domain has been identified in DNA-dependent protein kinase catalytic subunits (DNA-PKcs), which binds to acetylated H2AX K5 and promote the formation of γH2AX and facilitate NHEJ repair in response to double strand break[313]. Additional research is needed to further elucidate the roles of these acetylated histone lysine readers in normal development and diseases.

1.4.6 Summary

Histone acetylation is generally considered an epigenetic modification demarcating actively transcribed regions of the genome. These marks are written by histone acetyltransferases, removed by deacetylases, and read by the YEATS and bromodomain families of proteins and others. Several members of the bromodomain and YEATS domain family members have been implicated in human diseases such as

AMLs. Understanding the underlying mechanisms dysregulating these acetylated histone readers will contribute to the development of personalized therapeutics.

1.5 Summary and Goals

Hematopoiesis is a tightly regulated process essential for a functional blood system, and its perturbations can be pathogenic and lead to hematological malignancies. Many mechanisms of dysregulation in cytokine signaling, hematopoietic transcriptional factors, and epigenetic pathways have been observed through screens of somatic mutations and chromosomal translocations in AMLs. These genetic abnormalities often result in sustained proliferative signals and differentiation block and confer survival advantages for AML cells. Characterizing these genetic perturbations therefore is essential in understanding and developing personalized therapeutics for AML patients.

Multiple epigenetic modifiers are recurrently mutated in AMLs. MLL, a H3K4 methyltransferase, is involved in chromosomal translocations in some AMLs. In this subset of aggressive diseases, the MLL fusion proteins obtain C-terminal fusion partners and gain new molecular functions. Two of its most common fusion partners, ENL and AF9, are YEATS epigenetic readers of acetylated histone lysines. All 4 human YEATS epigenetic reader proteins have been implicated in human cancers. For instance, wild type ENL and its epigenetic reader function are important for a wide panel of AML cells. However, the presence, importance, and function of the YEATS domain in MLL-ENL and MLL-AF9 fusion proteins are yet to be elucidated. These questions will be addressed by my thesis work and delineated in the next chapter of my thesis.

Chapter 2 : The YEATS Epigenetic Reading Domain Links MLL-ENL to Leukemic Stem Cell Frequency in t(11;19) Leukemia

2.1 Introduction

11q23 translocations involving *MLL (KMT2a)* are found in pediatric, adult and therapy-related leukemias and about 10% of leukemias overall. Patients harboring *MLL* translocations have a poor prognosis, but this varies depending on fusion partner, leukemia subtype and age[201]. The *MLL* gene codes for a large H3K4 histone methyltransferase that positively regulates *HOX* gene expression[205]. Translocation events result in fusion proteins consisting of N-terminal *MLL* sequence with C-terminal sequence of one of >100 partners[199]. Despite a vast number of translocation partners, most cases (>80%) involve *MLL* fusion to one of six common partners: *AF4, AF9, ENL, AF10, ELL* and *AF6*. Apart from AF6 these proteins cooperate within several similar transcriptional activation complexes including the Super Elongation Complex (SEC)[175,214,340]. The SEC interacts and recruits positive transcriptional elongation factor b (P-TEFb), which is implicated in activation of MLL target genes[21,175,214]. P-TEFb phosphorylates the C-terminus of RNA polymerase II to release it into a productive transcriptional elongation phase[190]. Components of the SEC, including ENL, also interact with the DOT1L complex responsible for H3K79 methylation and necessary for MLL-fusion mediated gene activation[18,210,227,228]. MLL fusion proteins localize the SEC and DOT1L to pro-leukemic target genes like *Hoxa9* and its co-factor *Meis1* to drive transcriptional elongation and MLL-fusion mediated transformation[11,321,345]. Thus,

targeting these complexes has become an attractive strategy for treatment of MLL-rearranged leukemia. Indeed, both genetic and small molecule inhibitor studies targeting DOT1L have demonstrated promise in acute leukemia mouse models, however DOT1L catalytic inhibitors displayed modest activity in clinical trials[18,34,55,82,112,156,227,288].

Eleven-Nineteen-Leukemia (*ENL*, also known as *MLLT1*) is the third most common *MLL* fusion partner[199] and shares high homology with another common *MLL* fusion partner AF9 (~74% gene sequence homology)[42]. Recently, *ENL* was identified in a CRISPR-Cas9 screen to be essential for acute myeloid leukemia cell growth, where loss of ENL results in decreased RNA polymerase II occupancy and decreased transcriptional initiation and elongation at ENL-enriched targets[84,311]. In contrast, AF9 was found dispensable in several acute myeloid leukemia cell lines but essential for hematopoietic stem cell self-renewal and expansion[28,84,311]. Structurally, both ENL and AF9 contain an N-terminal YEATS domain and an intrinsically disordered C-terminal Anc1-Homology Domain (AHD). The C-terminus of ENL and/or AF9 undergoes coupled binding and folding upon interaction with members of the SEC and DOT1L complexes[123,158,164,274,286,346]. Indeed, the AHD is essential for MLL-ENL and MLL-AF9 mediated leukemogenesis[279,340] and we have reported the first peptidomimetic inhibitors of the AF9/ENL AHD, demonstrating the druggability of this protein-protein interaction[82]. The ENL and AF9 YEATS domains interact with histone H3 acetylated at K9, K18 or K27 and mutations disrupting the YEATS epigenetic reader function affects recruitment to target loci[1–3]. Recurrent mutations in the ENL YEATS domain occur in children with Wilms tumor, which induce increased binding and spreading of ENL at target

genes[240,310]. The ENL YEATS domain also directly interacts with PAF1 of the Polymerase Associated Factor 1 complex (PAF1c)[123]. The PAF1c functions by recruiting epigenetic and transcription factors to influence transcriptional elongation[295]. We and others have shown that the PAF1c directly interacts with MLL and is essential for MLL-fusion mediated leukemogenesis[1–3]. These studies suggest multiple functions of the ENL/AF9 YEATS domain may be critically important in regulating ENL/AF9 function in leukemic cells and several YEATS domain inhibitors have been reported[170,177,212]. However, the importance of the YEATS domain in the context of MLL-ENL fusion proteins and leukemogenesis and its prospect as a potential therapeutic target has not been addressed.

In this study, we investigate the clinical relevance and leukemic importance of the ENL YEATS domain in MLL-ENL leukemias and our results reveal a critical role for the YEATS domain in t(11;19) patients with MLL-ENL translocations. We demonstrate the preferential inclusion of the YEATS domain in most MLL-ENL fusions (>84% of MLL-ENL patients) and exclusion of the AF9 YEATS domain in MLL-AF9 patients (>98% of MLL-AF9 patients). We report the importance of the YEATS domain on MLL-ENL localization and leukemogenesis *in vivo* and its impact on leukemic stem cell frequency that may be exploited for therapeutic intervention.

2.2 Methods

2.2.1 Patient Data

Chromosomal breakpoint data was derived by targeted long distance inverse PCR (LDI-PCR) experiments as previously described[202]. All t(11;19) patients have been described previously[199]. Briefly, 1 μg of genomic DNA is digested with restriction

enzymes and ligated to form DNA circles followed by LDI-PCR analysis. PCR amplimers are isolated from gel and subjected to DNA sequence analysis to identify patient specific fusion sequences and breakpoints.

2.2.2 Transformation of Lin- Progenitor Cells for Proliferation, Colony Formation Assay, and Leukemogenesis Assay

Bone marrow was collected from 12 week old C57Bl/6 mice (Taconic Farms) and lineage depleted using the Easysep mouse hematopoietic progenitor cell isolation kit (Stemcell Technologies) 5 days following 5-FU injection (Sigma Aldrich). Lin- cells were subjected to spinoculation (25^{o}C, 90 minutes, 3200xg) with filtered viral supernatant and polybrene (final concentration: 5μg/mL; Sigma Aldrich) on consecutive days. Cells were recovered in IMEM + 15% stem cell FBS + 1X P/S + recombinant (r)mIL3 (10ng/mL; R&D) + rmSCF (100ng/mL; R&D) for at least two days before neomycin (50mg/mL; Sigma Aldrich) selection. For cell line generation, virally transduced cells were weaned from rmSCF over two weeks and maintained in growth media (IMEM + 15% stem cell FBS + 1X P/S + rmIL3). For proliferation assay, 2500-5000 cells were seeded, and proliferation quantified by trypan blue exclusion. For colony formation assay, virally transduced cells were plated in MethoCult- media (Stemcell Technologies cat#03234) with rmIL-3, rmSCF, rmIL-6 (10ng/mL; PeproTech), rmGM-CSF (10ng/mL; R&D Systems) and neomycin. Cells were grown in duplicate at 37^{o}C, 5% CO_2 for 5-7 days and colonies were quantified. After the 3rd round, representative colony images were obtained by iodonitrotetrazolium chloride (INT) solution staining (1% in Ethanol; Sigma Aldrich) for 1 hour at 37 ^{o}C. For *in vivo* leukemogenesis assay, retrovirally transduced Lin- cells from 5-FU treated C57/Bl6 mice were injected into lethally irradiated (950 rad)

syngeneic C57Bl/6 recipient mice. An equal number of cells were injected for each condition per experiment. Either 80K or 150K cells were injected depending of the experiment. Untransduced cells served as support marrow. Mice were kept on 13% D-sucrose baytril (enrofloxacin) water (130g D-sucrose + 3.4mL enrofloxacin/1L of ddH_20) for two weeks and monitored for course of the experiment. Moribund mice were sacrificed, followed by isolation of the bone marrow, spleen, and liver.

2.2.3 Plasmid Cloning and Mutagenesis

The MSCVneoMLL-ENL construct was kindly provided by Dr. Michael Cleary. The ENL sequence and variants were cloned into XhoI and PmlI sites of MSCVneoMLL-pl. MLL-ENL ΔYEATS (ENL aa 373-559) was published[340] and cloned into MSCVneoMLL-pl using the TOPO TA cloning kit (ThermoFisher Scientific). F59A, Y78A, and Δ21-26[125,311] were generated using QuikChangeXL kit with manufacturer's protocol (Agilent) and the TOPO TA cloning kit (ThermoFisher Scientific). FLAG-tagged ENL constructs (wild type, F59A, Y78A, Δ21-26, and the triple mutant) were cloned into pcDNA3.1 (+) vector using Platinum Taq DNA polymerase high fidelity kit (ThermoFisher Scientific), TOPO TA cloning kit, and QuikChangeXL kit. MigR1-HA-CDC73 was previously published[255], MigR1-HA-PAF1 was made using a similar strategy.

2.2.4 Retrovirus Production and Transfection

The retroviral packaging cell line PlatE cells (maintained in DMEM + 10% FBS) were plated to ~60% confluency and transfected with MSCV retroviral vectors using the

FuGENE6 protocol (Promega). Viral supernatants were harvested at 24- or 48-hours post-transfection, spun down and filtered.

2.2.5 Immunoprecipitation (IP), Western Blot and Antibodies, In vitro Binding Assay

Immunoprecipitations were carried out using MSCVneoMLL-pl-ENL and stated mutants in the pcDNA3.1 (+) expression plasmid. HEK 293T cells were transfected with the corresponding mammalian expression vectors using the Fugene6 protocol. Cells were lysed in BC300 lysis buffer 48 hours post-transfection. For FLAG-IP experiment, FLAG-tagged proteins were enriched by incubating lysate overnight at 4^{o}C with anti-FLAG M2 affinity gel (Millipore Sigma). For *in vitro* binding assays, His-tagged YEATS and mutants with H3K27ac peptides, Scrambled, histone H3 (21-44), and [Lys(Ac) 27] histone H3 (21-43) (Anaspec) were used. Enrichment was performed using Dynabeads^{TM} M-280 streptavidin (Invitrogen) following manufacturer protocol. For Western blotting, blocking was performed in 5% milk overnight in 4^{o}C. Primary antibodies used: anti-MLL-N (Millipore Sigma, cat#05764), overnight in 4^{o}c; Anti-FLAG (Sigma, cat#F7425), room temperature for an hour; Anti-HA (Abcam, cat#ab9110), room temperature for an hour; Anti-His (Cell Signaling, cat#2365T), room temperature for an hour; Anti-PAF1 (Bethyl, cat#A300-173A), room temperature for an hour. Secondary antibodies used: goat-anti-mouse IgG (EMD Millipore cat#AP308P), room temperature for an hour; donkey-anti-rabbit IgG (Sigma cat#SAB3700928), room temperature for an hour.

2.2.6 RNA-Seq Analysis

Total RNA was harvested from MLL-ENL and MLL-ENL ΔYEATS cells using the RNeasy kit (Qiagen). mRNA Library was generated using the poly(A) RNA selection kit (Lexogen) and Swift RNA library kit (Swift Biosciences) by the University of Michigan Advanced Genomics Core and sequenced on Illumina NovaSeq S4 for paired-end sequencing. Adapter sequence trimming was done using Cutadapt v2.3[191], and the processed reads were mapped to reference genome GRCm38/mm10 using STAR v2.7.8a[69]. Data quality was monitored using FastQC v0.11.8 and Fastq Screen[320]. Count estimates were obtained through RSEM v1.3.3[167] and analyzed on DESeq2[178] for differential expression analysis. The argument lfcThreshold was used to identify genes that are at least 1.5-fold differentially expressed (significance: padj. < 0.05). List of MLL-ENL targets was obtained[104] and cross-referenced with 1.5-fold differentially expressed genes to identify disrupted MLL-ENL targets in MLL-ENL ΔYEATS cells. Gene Ontology (GO) analysis was completed by using the clusterProfiler package[343] to look at molecular functions (MF) GO terms enriched by 1.5-fold differentially expressed genes. GO analysis results were plotted using the barplot function from the clusterProfiler package. Gene Set Enrichment Analysis (GSEA) was performed using the fast gene set enrichment analysis package (fgsea)[155]. Ranked list of the genes was generated using Wald statistics from DESeq2 analysis after testing for differential expression. The gene sets used in our analysis were curated in the C2: chemical and genetic perturbations (CGP) collection from the Molecular Signatures Database (MSigDB), and the results were plotted using the plotEnrichment function from the fgsea package. RNA-seq gene tracks were generated by converting BAM files into bigwig files using the bamCoverage tool on the Galaxy open platform (https://galaxyproject.org/)[249] and visualized on the

Integrative Genomics Viewer (the Broad Institute). All analyses were completed in R version 4.0.4. RNA-Seq data has been deposited into GEO: accession GSE211523.

2.2.7 Gene Expression by qPCR

Cells were harvested and processed for RNA using the RNeasy mini and micro kits (Qiagen). Synthesis of cDNA was done using the First Strand cDNA Synthesis System kit (Sigma Aldrich) according to protocol. Quantitative PCR (qPCR) was done using the Fast SYBR Green Master Mix protocol (ThermoFisher Scientific). For cell line gene expression, analysis was done using the $\Delta\Delta$Ct method using β-actin for housekeeper expression and MLL-ENL as the control. Each data point in the cell line expression analysis represents a biological cell line generated independently. For gene expression following SGC-iMLLT treatment, analysis was first done using the $\Delta\Delta$Ct method using β-actin for housekeeper expression and DMSO sample as the control. The $\Delta\Delta$Ct values were then transformed by multiplying to a cell line-specific factor (the average ΔCt of all the DMSO-treated sample of the cell line) to compare expression levels across different cell lines. Primer sequences are listed below in **Table 2-1**.

Table 2-1: Primer Sets and Sequence for qPCR

Name	Forward Primer	Reverse Primer
mHoxA9	GAATGAGAGCGGCGGAGAC	GAGCGAGCATGTAGCCAGTTG
mMeis1	ATCAGAGCGCCAGGACCTAT	CTTCCCCCTGGCTTTCGATT
mMyc	TCCTGTACCTCGTCCGATTC	TTGCTCTTCTTCAGAGTCGCT
mBcl-2	CTGAGTACCTGAACCGGCAT	AGGGTCTTCAGAGACAGCCA
mBcl-xl	GCGTGGAAAGCGTAGACAAG	CTGCTGCATTGTTCCCGTAG

mMcl-1	AAGAGGCTGGGATGGGTTTG	CTGATGCCGCCTTCTAGGTC
mCd80	TATTGCTGCCTTGCCGTTAC	GACCAGGCCCAGGATGATA
mNab2	AGGACAGCGCCAGTCTGT	GTGTCTGCTGCAAGATGTGG
mId2	GTCCTTGCAGGCATCTGAAT	CTCCTGGTGAAATGGCTGA
mItgam	CCCATGACCTTCCAAGAGAA	ACACCGGCTTGTGCTGTAGT
hMLL	ACCCCATCAGCAAGAGAGG	TTCGTGGAGGAGGCTCAC
mβ-actin	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC
mEya1	TAGTAGCGAATCCCCCAGTG	CCGAGAGCTGAACCTGAGAA

2.2.8 Flow Cytometry

A total of 5x10^5-1x10^6 cells were washed with and resuspended in flow buffer (1X PBS + 2% FBS + 2mM EDTA). For differentiation assays, the antibodies and conditions are listed as below: APC anti-mouse cKIT (1:100; BioLegend, cat# 135108), APC anti-mouse CD11B (1:100; BD Biosciences, cat# 553312), APC anti-mouse CD14 (1:100; BioLegend, cat#123312), APC-isotype (1:100; BD Bioscienes, cat#47403180), and DAPI (final concentration 1ug/mL; Sigma, cat#D9542-10mg). For apoptosis assay, the antibodies and conditions are listed as below: APC- AnnexinV (1:100; Invitrogen; cat#17800772) and DAPI (final concentration 1ug/mL; Sigma). For cell cycle assay, the cells were fixed in the BD Cytofix/Cytoperm kit (BD Biosciences) according to protocol and stained with DAPI. For homing assay, the antibodies and conditions are listed as below: APC anti-mouse CD45.2 (1:50; BioLegend, cat#109814), APC/Cy7 anti-mouse CD45.1 (1:50; BioLegend, cat#110715) and DAPI (final concentration 1ug/mL; Sigma).

2.2.9 Dual Luciferase Assay

HEK 293T cells were co-transfected with MSCVneoMLL-pl-ENL or derivative mutant constructs, a previously described *Hoxa9-Luc* expression vector[217], and renilla luciferase expression vector according to the Fugene6 protocol. Cells were changed into low serum media (Opti-MEM+ 0.05% FBS) 24-hours post-transfection. The dual luciferase expression was detected using the Dual-Luciferase Reporter Assay system with the GloMax 20/20 luminometer (Promega). Analysis was performed by normalizing the firefly luciferase expression to renilla luciferase expression and then against the MSCVneoMLL-pl-ENL transfected sample.

2.2.10 Leukemic Stem Cell Frequency Assay and Homing Assay

Leukemic cells collected from the bone marrow of primary leukemogenesis assays were thawed and counted before injecting into sublethally irradiated (650 rad) syngeneic recipients (1000, 200, 50, 20 or 5 cells were injected). The assay was conducted twice. We first compared an MLL-ENL primary leukemia to an MLL-ENLF59A primary leukemia. A second experiment was conducted comparing a different MLL-ENL to an MLL-ENLY78A primary leukemia. Mice were kept on 13% D-sucrose baytril (enrofloxacin) water (130g D-sucrose and 3.4mL enrofloxacin per 1L of ddH_20) for two weeks and monitored over the course of the experiment. Moribund mice were scarified, and spleen and liver were collected. Leukemic stem cell frequency results were analyzed via the extreme limiting dilution analysis[132]. For homing assay, 2 million CD45.2+ leukemic cells harvested from primary leukemogenesis assays were injected into sublethally irradiated (650 rad) B6.SJL congenic recipient mice (Taconic). Recipient mice were sacrificed 24 hours post-injection. Bone marrow was collected, and the red

blood cells lysed using ACK lysing buffer (ThermoFisher Scientific) according to protocol. Samples were then washed in 1X PBS before flow cytometry analysis.

2.2.11 ChIP qPCR

Chromatin immunoprecipitation (ChIP) was performed using standard protocol. Briefly, 30 million cells were crosslinked using 1% paraformaldehyde for 15 minutes at room temperature and the crosslinking reaction was quenched using freshly prepared glycine solution. Cell pellets from crosslinking reactions were snap frozen for further downstream processing. Pellets were thawed on ice and lysed using standard SDS lysis buffer for 10 minutes in ice. Lysed samples were subjected to sonication for shearing of chromatin. Sonication of chromatin was optimized to prepare chromatin size of 200 bp to 500 bp with Q800 sonicator from QSonica. Sonicated chromatin samples were diluted by standard dilution buffer. SDS lysis buffer and dilution buffer were supplemented with proteinase and phosphatase inhibitors. Chromatin samples were either used for immunoprecipitation assay or frozen. 1% chromatin sample was used for preparing input DNA. Chromatin sample from approximately $2x10^6$ cells was subjected to each ChIP reaction. Chromatin samples were incubated with 5 ug of anti-FLAG (Sigma cat#F7425), anti-PAF1 (Bethyl cat#A300-172A), anti-Histone H3 (Abcam cat#ab1791), anti-histone H3K4me3 (Abcam cat#ab8580), anti-histone H3K9Ac (Epicypher cat#13-0033), anti-histone H3K79me2 (Abcam cat#ab3594) and anti-IgG-rabbit (Millipore cat#12-370) overnight shaking at 4^0C. ChIP reactions were incubated with 30 ug of Protein G-Dynabeads (Invitrogen) and incubated for 2 hours shaking at 4^0C. Beads were washed at 4^0C with standard low salt buffer, standard high salt buffer, Lithium chloride buffer, and TE buffer once. DNA samples were eluted using freshly

prepared elution buffer at 65^0C for 30 minutes with intermittent vortexing. Input DNA and ChIP eluted DNA were incubated with RNaseA (Roche) and NaCl at 65^0C overnight for reverse crosslinking. Following reverse-crosslinking, DNA samples were incubated with proteinase K (thermoscientific) for 2 hours at 65^0C for 1 hour and DNA was purified using standard DNA isolation technique followed by analysis using real-time quantitative PCR. The sequence of ChIP-qPCR primers Primers for Eya1 are listed below. Additionally, Meis1 and gene desert (GD) were previously published[215,270]used were listed below in **Table 2-2.**

Table 2-2: Primer Sets and Sequence for ChIP-qPCR

Name	Forward Primer	Reverse Primer
Eya1 P1	TGGCTAAACCCTTGACTTGG	TTCTTTGGCTAGGACCCAGA
Eya1 P3	CAGTCCCATTCCCTGTCACT	AAACATGCGAACACATGGAA
Eya1 P7	AGCAGCCATTTCTGGTGACT	TCATTCCCTGCTCTCTCTGC
Meis1 P1	TCAAAGTGACAAAATGCAAGCA	CCCCCCGCTGTCAGAAG
Meis1 P2	GAAGAAGACAGAACGGACGATCA	GCCACTCCAGCTGTCAATCA
GD	TAAACAGTCCCTCAAACCACC	GAGGATTATGTGCTGCCCTAA

2.2.12 Cloning of Maltose Binding Protein (MBP)-ENL YEATS into pET28-His-SUMO Vector

Two step polymerase chain reaction (PCR) was performed to prepare MBP and ENL YEATS domain (1-148) fusion gene for ENL YEATS WT and F59A, Y78A, Δ21-26, and F59A/Y78A/Δ21-26 mutants with a Tobacco Etch Virus (TEV) cleavage site linking

the MBP and ENL YEATS domain. The fusion products carrying a BamHI site at the 5' ends and an XhoI site at the 3' ends were then digested with BamHI/XhoI (NEB), purified and ligated into a pET28-His-SUMO vector pre-treated with BamHI/XhoI. The ligation products were transformed into DH5α cells for plasmid amplification and extraction. The DNA sequences for all constructs were confirmed via Sanger sequencing. The plasmids were transformed into Rosetta™ (DE3) cells for protein expression.

2.2.13 Expression and Purification of ENL-YEATS Domain and ENL-YEATS-His Protein

Six colonies for each ENL-YEATS or ENL-YEATS-His protein were picked from plate to inoculate 100 ml of Luria Broth (LB) media, which was allowed to grow at 30°C with shaking at 225 rpm overnight. Two flasks of 0.5 L of Terrific Broth (TB) media were inoculated with 5 ml of overnight culture, which was allowed to grow at 37°C with shaking at 225 rpm until OD600 reached 0.9-1.0. The flasks were cooled to room temperature and protein expression was induced with 100 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein expression was allowed to continue for 16 hr at 18°C with shaking at a decreased speed of 140 rpm. Cells were harvested by centrifugation at 6000g for 20 minutes at 4°C. Pellets from 1L cell culture was re-suspended into 50 ml of lysis buffer containing 50 mM Tris 8.0, 200 mM NaCl, 10% glycerol, 1:100 protease inhibitor cocktail (Bimake, B14002), 100 ug/ml lysozyme, 8 mM imidazole, 1 mM TCEP. The suspended cells were lysed by four pulses of 45 second sonication at 50% amplitude on ice. The cell lysate was centrifuged at 38,000g for one hour at 4°C. The supernatant was then incubated at 4°C for 2 hours with 3 ml of Ni^{2+}-

nitrilotriacetic acid (Ni-NTA, HisPurTM Ni-NTA, Thermo Scientific 88222) resin pre-equilibrated with lysis buffer. The resin was sequentially washed with 150 ml of wash 1 buffer containing 50 mM Tris 8.0, 200 mM NaCl, 10% glycerol, 8 mM imidazole, 100 ml of wash 2 buffer containing 50 mM Tris 8.0, 1 M NaCl, 10% glycerol, 8 mM imidazole, followed by an additional 50 ml of wash 1 buffer. Protein was eluted with elution buffer containing 50 mM Tris 8.0, 200 mM NaCl, 10% glycerol, and 250 mM imidazole. The eluted protein was treated with TEV at a TEV: total protein weight ratio 1:100 and dialyzed at 4°C overnight against 25 mM HEPES 7.0, 150 mM NaCl, 5% glycerol, 1 mM DTT. The protein was further purified via cation exchange column Hi-trap S and gel-filtration Superdex 75. The final protein was concentrated and stored at -80°C in the buffer containing 25 mM HEPES 7.0, 150 mM NaCl, 5% glycerol, and 1 mM TCEP.

2.2.14 Fluorescence Polarization Binding Assay

Fluorescence polarization (FP) based binding assay was developed and optimized to determine the binding affinity of the reported ENL YEATS domain inhibitor[212] to different wild type ENL YEATS domain and following mutants: F59A, Y78A, Δ21-26 and triple mutant. We designed and synthesized fluorescein conjugated-SGC-iMLLT which used as a fluorescent probe for the FP binding assay developed based on our previously reported FP binding assay[223] in 5 nM concentration. The experiments were performed in black round-bottom 96-well plates (Corning #3792) and analyzed by BioTek Synergy H1 Hybrid plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Dissociation constant (K_d) values were determined by protein saturation experiments where fixed concentrations of the Flu-SGC-iMLLT fluorescent probe (5 nM) was mixed with two-fold serially diluted different

YEATS recombinant proteins, wild type and mutants, starting from 8 μM in assay buffer (20 mM phosphate pH 7.5, 250 mM NaCl, 0.005% Tween-20) and the plates were incubated for 3 hours at room temperature. An equilibrium binding isotherm was plotted with the FP values in millipolarization units (mP) as a function of the ENL YEATS domain protein concentrations and the K_d values were calculated by nonlinear regression fitting of the curves by *GraphPad Prism* 8.0.

2.2.15 Bio-Layer Interferometry (BLI) Based Binding Assay

For differential interactions of the H3K27Ac peptide with ENL YEATS domain recombinant proteins (wild type, F59A, Y78A, Δ21-26 and triple mutant), the BLI experiments were performed at room temperature on OctetRED96 (PALL/ForteBio) in black 96-well plates (Greiner bio-one, # 655209). The biotinylated H3K27Ac (5.0 μM) (AnaSpec, #AS-64846-1) was immobilized on Streptavidin (SA) Biosensors (Sartorius, # 18-5019) for 45 minutes. Association and dissociation cycles were fixed for 3 and 6 minutes respectively. The assay buffer (20 mM phosphate pH 7.4, 150 mM NaCl, 0.1 % BSA, 0.01% Tween-20) was used for custom, baseline, and dissociation steps, while wells containing serially diluted proteins were used for association. Collected raw kinetic data were processed with the Data Analysis software provided by the manufacturer by reference subtraction of the buffer sensograms. Plotting the response nm values with the respective protein concentrations was used to calculate steady state K_d values.

2.2.16 Statistical Analysis

Statistical tests, number of replicates, error bar information and test statistics are reported in each figure legend. It is summarized here briefly. Chi-square tests were

performed to determine statistical significance of YEATS domain inclusion among different subgroups of MLL-ENL patients. The log-rank (Mantel-Cox) test was used to assess significant differences in leukemic disease latency driven by MLL-ENL mutant fusion proteins. Paired or unpaired student's t-test (two tailed) was used to assess the differences in colony formation assay, spleen weight, gene expression, luciferase transcriptional activation, and apoptosis assays. One way ANOVA followed by Dunnett's multiple comparisons test for post hoc analysis was used to compare the difference in colony formation post SGC-iMLLT treatment of different cell lines and the associated gene expression evaluation. Two way ANOVA followed by post hoc analysis was used to assess the proliferation of cell lines.

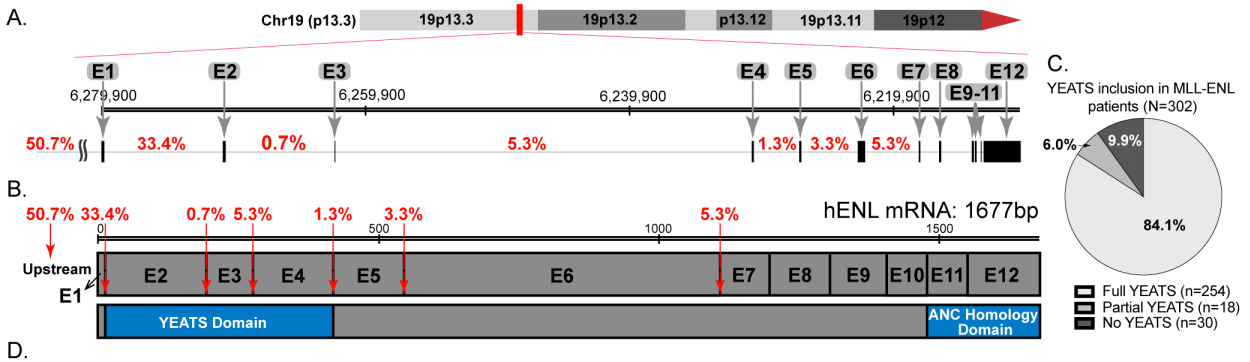
2.3 Results

2.3.1 The Majority of t(11;19) Patients Retain the YEATS Domain in Resultant MLL-ENL Fusion Proteins

t(11;19) and t(9;11) translocations are common MLL translocations that fuse *MLL (KMT2a)* with *ENL (MLLT1)* or *AF9 (MLLT3)*, respectively. ENL and AF9 contain N-terminal YEATS domains, which share 88% alignment[212] that may impact MLL-fusion protein function through protein interactions[84,123,172,311]. To understand the functional outcomes of these protein-protein interactions, we asked whether t(11;19) patients retain the YEATS domain in resultant MLL-ENL fusion proteins. *ENL* is composed of 12 exons and located on chromosome 19p13.3 **(Figure 2-1A)**. The YEATS domain is coded from exons 2-4 (amino acids 5-140) **(Figure 2-1B)**[355]. Thus, all t(11;19) translocations with ENL breakpoints upstream of exon 2 produce MLL-ENL fusion proteins containing the YEATS domain. We mined breakpoint data from a previously

described clinical cohort of 302 t(11;19) patients[199] and found that 50.7% (n=153) of all MLL-ENL patients harbor genomic breaks 5' upstream of the *MLLT1* gene but 3' downstream of *ACER1* **(Figure 2-1A)**. Another 33.4% (n=101) patients harbor genomic breaks within the first *MLLT1* intron **(Figure 2-1A)**. In combination, 84.1% (n=254) of t(11;19) leukemia patients retain the YEATS domain in resultant MLL-ENL fusion proteins. The remaining 15.9% (n=48) t(11;19) patients have genomic breakpoints downstream of exon 2 **(Figure 2-1A)**, leading to partial inclusion (6.0%) or exclusion of the YEATS domain (9.9%) in MLL-ENL fusion proteins **(Figure 2-1A, B, C)**. These findings align with previous studies investigating ENL breakpoints in t(11;19) patients reporting YEATS domain retention in 19 of 23[258] and 10 of 15[102] t(11;19) patients. Patient age group is not significantly correlated with YEATS domain retention status in MLL-ENL leukemia (Chi-sq p=0.33, df=2) **(Figure 2-1D)**. Fewer patient samples prohibited us from comparing YEATS retention in mixed lineage leukemia, however significantly fewer AML than ALL patients retained the YEATS domain (Chi-sq p<.0001, df=1) **(Figure 2-1E)**.

Figure 2-1: ENL Breakpoint Locations in t(11;19) Patients



D.

	5′ Upstream	Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8	Intron 9	Intron 10	Intron 11	Total
Infant	96	44	1	9	3	3	4	0	0	0	0	0	160
Pediatric	33	30	1	4	1	4	5	0	0	0	0	0	78
Adult	24	27	0	3	0	3	1	0	0	0	0	0	58

E.

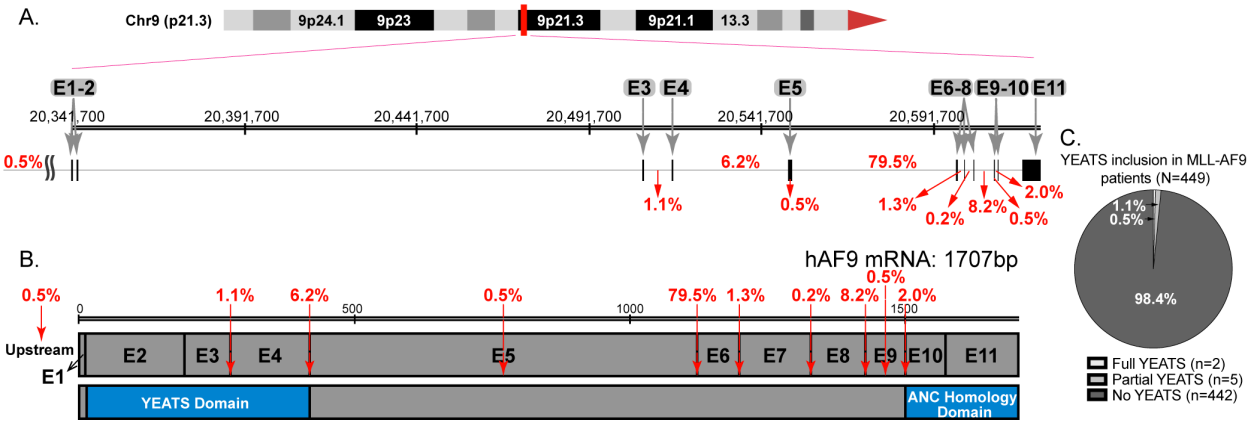
	5′ Upstream	Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8	Intron 9	Intron 10	Intron 11	Total
ALL	150	87	2	12	3	3	6	0	0	0	0	0	263
AML	1	13	0	4	1	7	10	0	0	0	0	0	36
MLL	2	1	0	0	0	0	0	0	0	0	0	0	3

(A) Genomic location of the ENL gene and distribution of ENL breakpoints in t(11;19) (MLL-ENL) patients described previously (7). N=302. (B) The location of ENL breakpoints described in A is shown in relation to ENL mRNA and protein. The YEATS domain and AHD are indicated on the ENL protein schematic. (C) Pie chart summary of patient data shown in A showing percentage of t(11;19) patients harboring MLL-ENL fusion proteins containing: the full YEATS domain (n=254/302; 84.1%), partial YEATS domain inclusion (n=22/302; 7.3%), and no YEATS domain inclusion (n=26/302; 8.6%). (D and E) Summary table of ENL breakpoint data shown in A broken down by (D) infant, pediatric and adult patients or (E) leukemia subtype (AML=acute myeloid leukemia, ALL=acute lymphoid leukemia, MLL=mixed lineage leukemia).

Given the ENL and AF9 (*MLLT3*) YEATS domain homology, we investigated *AF9* breakpoints in t(9;11) MLL-AF9 leukemia patients **(Figure 2-2)**. In contrast to *ENL*, *AF9* genomic breaks occur almost exclusively downstream of exon 4 (98.4%, n=442) and exclude the AF9 YEATS domain from the resultant MLL-AF9 fusion proteins **(Figure 2-2A, B)**. Only two MLL-AF9 patients (0.5%) had breakpoints upstream of *MLLT3* exon 1, resulting in YEATS domain inclusion in the MLL-AF9 fusion product **(Figure 2-2B, C)**. Together, these data suggest that the YEATS domain is specifically retained in the

majority of the MLL-ENL fusion proteins from t(11;19) patients but absent in the majority of the MLL-AF9 fusion proteins in t(9;11) patients.

Figure 2-2: AF9 Breakpoint Locations in t(9;11) Patients



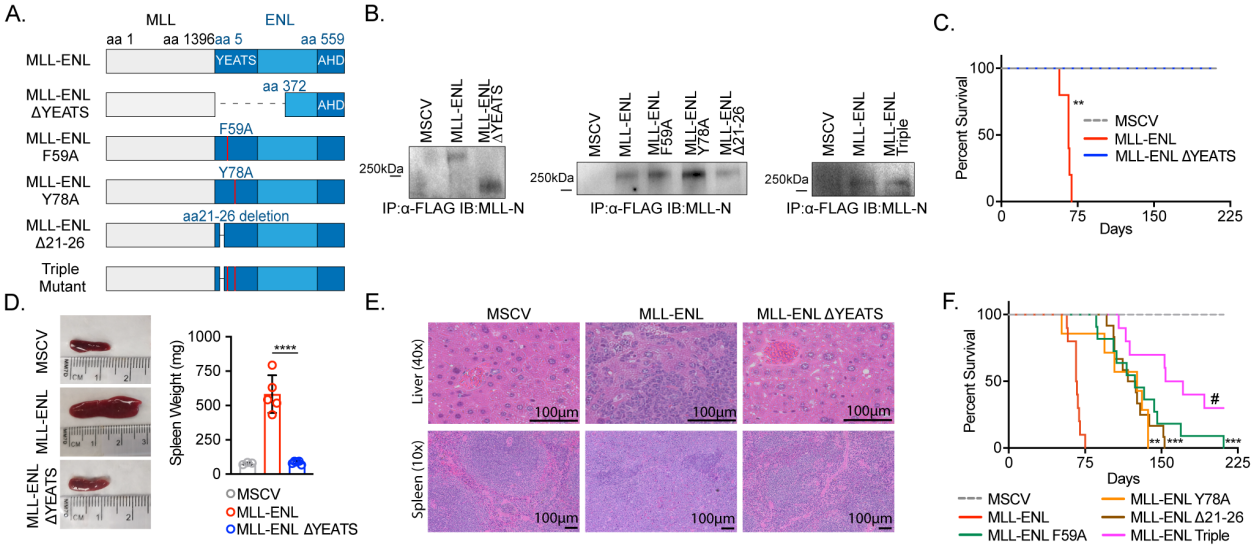
(A) Genomic location of the AF9 gene and distribution of AF9 breakpoints in t(9;11) patients. N=449. (B) The location of AF9 breakpoints described in A is shown in relation to AF9 mRNA and protein. The YEATS domain and AHD are indicated on the AF9 protein schematic. (C) Pie chart summary of patient data shown in A showing percentage of t(9;11) patients harboring MLL-AF9 fusion proteins containing: the full YEATS domain (n=2/449; 0.5%), partial YEATS domain inclusion (n=5/449; 1.1%), and no YEATS domain inclusion (n=442/449; 98.4%).

2.3.2 The YEATS Domain and Downstream Sequence is Important for MLL-ENL Mediated Leukemogenesis

We next investigated the importance of the ENL YEATS domain in MLL-ENL mediated transformation and leukemogenesis using retroviral MLL-ENL vectors with (MLL-ENL) or without the YEATS domain (MLL-ENLΔYEATS) **(Figure 2-3A)**. The MLL-ENLΔYEATS construct includes the C-terminal AHD minimally required for MLL-ENL colony formation *ex vivo*[340]. Both MLL-ENL fusion constructs displayed similar mRNA and protein levels **(Figure 2-3B, 2-4A)** and were sufficient for serial colony replating as evidenced by third round colony formation **(Figure 2-4B)**, consistent with previous reports[279,340]. However, MLL-ENL cells showed significantly higher colony forming potential and proliferation rates compared to MLL-ENLΔYEATS cells **(Figure 2-4B, C)**.

To interrogate the function of the YEATS domain in MLL-ENL leukemogenesis *in vivo*, we injected MSCV, MLL-ENL, or MLL-ENLΔYEATS transduced cells into lethally irradiated syngeneic C57Bl/6 recipient mice. Mice receiving MLL-ENL transduced cells succumbed to leukemia with a median survival of 66 days, displaying splenomegaly and leukemic infiltration in the spleen and liver **(Figure 2-3C, D, E)**. Strikingly, mice receiving MLL-ENLΔYEATS transduced cells failed to develop leukemia *in vivo* **(Figure 2-3C, D, E)**. These data suggest amino acids 5-371 of ENL, which contain the YEATS domain, are critical for MLL-ENL mediated leukemogenesis.

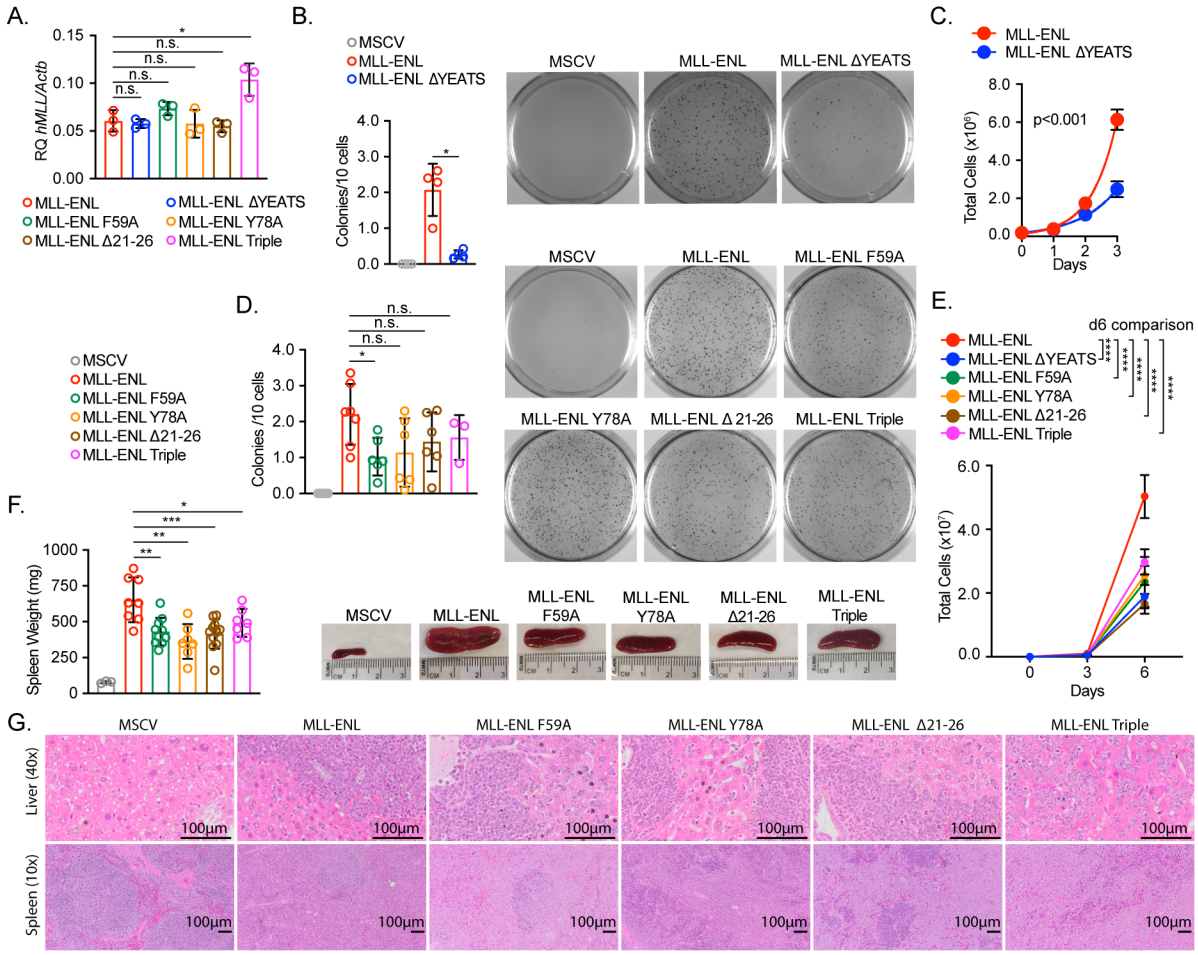
Figure 2-3: The ENL YEATS Domain and Downstream Sequence is Required for MLL-ENL Mediated Leukemogenesis



*(A) Schematic of MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutants constructs used in this study. (B) IP-Western blots of MLL-ENL, MLL-ENLΔYEATS, MLL-ENL YEATS mutants demonstrate expression of the fusion proteins. (C) In vivo leukemogenesis assay performed by retroviral transduction of lin- mouse bone marrow cells from 5-FU treated C57/Bl6 mice with MSCV (n=3), MLL-ENL (n=5), or MLL-ENLΔYEATS (n=5). 80K cells were injected into lethally irradiated (950 rads) syngeneic recipients where untransduced cells served as support marrow. Statistical significance was calculated using the log-rank (Mantel-Cox) test (**: p=0.0018). (D) Spleen comparison of sacrificed animals from the MSCV (n=3), MLL-ENL (n=5) and MLL-ENLΔYEATS (n=5). (Left) Representative spleen images. (Right) Spleen weight comparison. Error bars represent SD. Statistical significance was calculated using unpaired student's t-test (****: p<0.0001). (E) Representative images of H&E stained liver and spleen of sacrificed animals from the MSCV, MLL-ENL and MLL-ENLΔYEATS groups. (F) Combination of two independent in vivo leukemogenesis assays performed as above with bone marrow cells retrovirally transduced with MSCV (n=6), MLL-ENL (n=10), MLL-ENLF59A (n=12), MLL-ENLY78A (n=7), MLL-ENLΔ21-26 (n=12) or MLL-*

*ENL triple mutant (n=10). 80K cells were injected/mouse for th first experiment and 150K cells/mouse for the second experiment. Statistical test: log-rank (Mantel-Cox) (***: p<0.001; **: p<0.01). # = MLL-ENL triple vs MLL-ENLF59A: p=0.027; MLL-ENL triple vs MLL-ENLY78A: p=0.008 MLL-ENL triple vs MLL-ENLΔ21-26: p=0.002.*

Figure 2-4: The YEATS Domain Impacts MLL-ENL Colony Formation, Proliferation, and Spleen Size



(A) RT-qPCR detection of the MLL-ENL, MLL-ENLΔYEATS, or MLL-ENL YEATS mutants' fusion transcripts in leukemic cell lines. Statistical test: unpaired student's t-test. (: p<0.05; n.s.: not significant) (B) (Left) Third round colony formation assay results from MSCV, MLL-ENL and MLL-ENLΔYEATS transduced lin- mouse bone marrow cells. Error bars represent standard deviation. Statistical test: unpaired student's T-test (*: p<0.05) (Right) Representative images of INT-stained colonies. (C) Proliferation assay of MLL-ENL and MLL-ENLΔYEATS cell lines. Figure contains 3 biological replicates. Error bars represent standard deviation. Statistical test: Two-way ANOVA. (Source of variance: time and cell line: p<0.001; post hoc analysis of difference at day 3: p<0.001). (D) (Left) Third round colony formation assay results from MSCV, MLL-ENL and MLL-ENL YEATS mutants transduced lin- mouse bone marrow cells. Error bars represent standard deviation. Statistical test: unpaired student's t-test (*: p<0.05; n.s.: not significant) (Right) Representative images of INT-stained colonies. (E) Proliferation assay of MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutant cell lines. Figure contains 3 biological replicates. Error bars represent standard deviation. Statistical test: Two-way ANOVA. (Source of variance: time and cell line: p<0.0001; post hoc analysis of difference at day 6: shown in graph. ****:*

p<0.0001). (F) (Left) Spleen weight comparison of sacrificed animals from leukemogenesis assay utilizing bone marrow transduced with MSCV (n=3), MLL-ENL (n=8), MLL-ENLF59A (n=10), MLL-ENLY78A (n=7), MLL-ENLΔ21-26 (n=12) and MLL-ENL triple mutant (n=7). Error bars represent standard deviation. Statistical test: unpaired student's t-test (: p<0.05; **: p<0.01 ***: p<0.001). (Right) Representative images of spleen from each group. (G) Representative images of H&E stained liver and spleen of sacrificed animals from the MSCV, MLL-ENL and MLL-ENL YEATS mutants groups.*

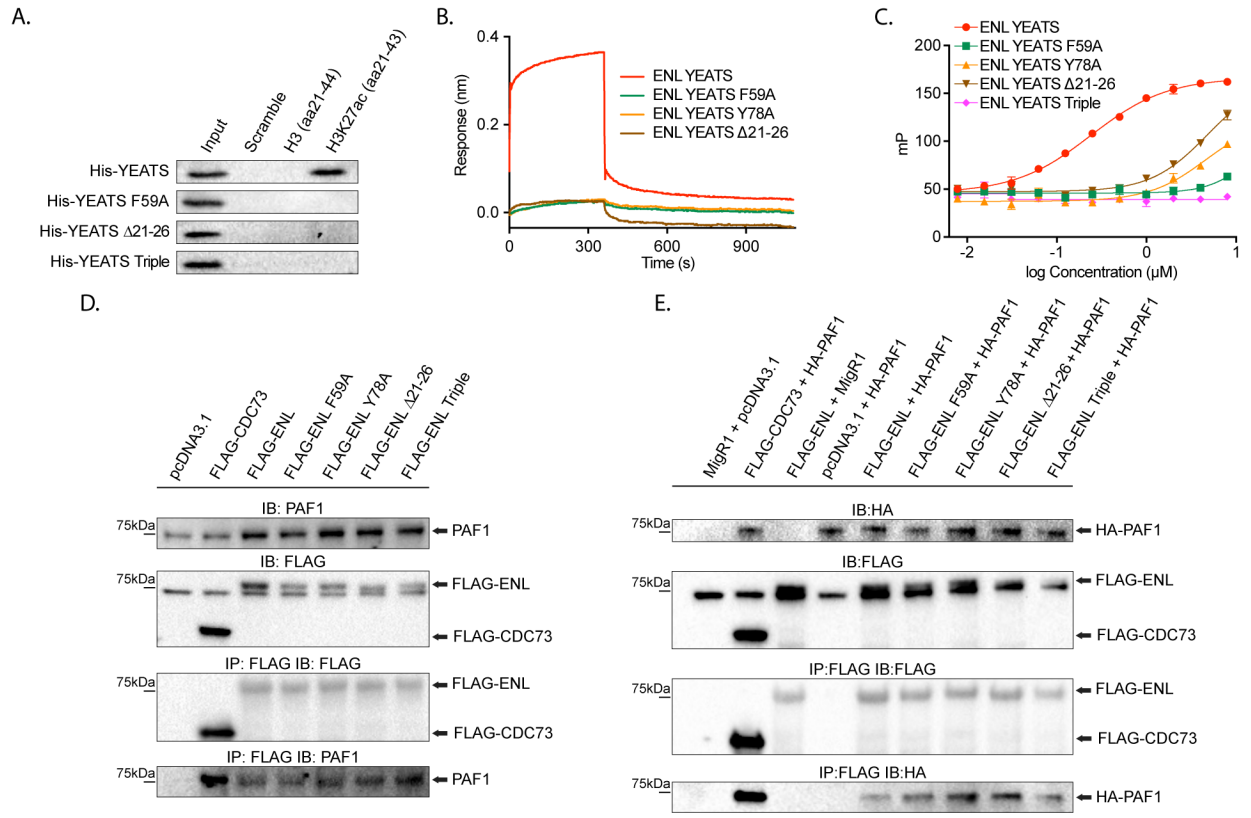
We next addressed specific YEATS domain functions in MLL-ENL leukemogenesis. Specifically, ENL F59A and Y78A mutants were introduced to disrupt recognition of acetylated H3 lysine marks (H3K9, K18 and K27) **(Figure 2-3A)**[311]. We also generated a deletion construct (ENL Δ21-26) that mitigates binding to PAF1 of the PAF1c[125] and a triple mutant that disrupts both (F59A, Y78A and Δ21-26) **(Figure 2-3A)**. These mutations do not impact transcript and protein expression **(Figure 2-3B, 2-4A)**. To assess their transformative capabilities, we performed colony and proliferation assays with MLL-ENL or MLL-ENL-mutant-YEATS transduced cells. All YEATS domain mutants displayed slower proliferation rates and a modest decrease in colony forming potential compared to MLL-ENL **(Figure 2-4D, E)**. To definitively assess the impact of these mutations, we performed *in vivo* leukemogenesis assays utilizing MLL-ENL fusion proteins containing YEATS mutations. All mutations significantly extended leukemic disease latency *in vivo* compared to MLL-ENL **(Figure 2-3F)**. Interestingly, combining these mutations (MLL-ENL Triple, **Figure 2-3A**) resulted in a significant extension of disease latency compared to MLL-ENL single YEATS mutations **(Figure 2-3F)**. All groups eventually succumbed to leukemic disease burden accompanied with splenomegaly **(Figure 2-4F)** and leukemic infiltration in the spleen and the liver **(Figure 2-4G)**, although decreased spleen weights were observed in MLL-ENL YEATS point mutant diseased mice **(Figure 2-4F)**. These results suggest that YEATS domain protein interactions are important for MLL-ENL leukemogenesis.

2.3.3 Mutation of the YEATS Domain Alters Binding to Acetylated Histone H3

We characterized how YEATS domain mutations impact binding to H3Kac and PAF1 using immunoprecipitation and quantitative biolayer interferometry (BLI) assays. Similar to previous reports[311], the YEATS domain binds preferably to immobilized biotin labeled acetylated H3K27ac peptide **(Figure 2-5A, B, 2-6A, B)** with a binding affinity K_d of 66 μM to immobilized biotinylated H3K27ac peptide as previously reported. F59A and Y78A mutations abrogate binding to H3K27ac **(Figure 2-5A, B)**. Interestingly, the Δ21-26 also disrupted interaction with acetylated H3K27 peptide **(Figure 2-5A, B)**. This result contrasts with a previous report indicating this deletion does not alter YEATS interaction with histone H3; albeit using a different biochemical assay[125]. Not surprisingly, the triple mutation (F59A, Y78A and Δ21-26) failed to bind acetylated H3K27 peptide **(Figure 2-5A, B)**. To further analyze the impact of YEATS mutations on H3Kac binding, we utilized the ENL/AF9 YEATS small molecule inhibitor, SGC-iMLLT[212], which binds within the H3Kac binding groove of the ENL/AF9 YEATS domain in the nanomolar range allowing quantitative analysis of the interactions with wild type and mutated YEATS domains. Based on the complex structure between SGC-iMLLT and ENL (PDB ID: 6HT1) we designed a fluorescein labeled inhibitor, Flu-SGC-iMLLT **(Figure 2-6C)** and developed a fluorescence-polarization binding assay to determine its binding affinity to wild type and mutated ENL YEATS domain. Consistent with previous studies, Flu-SGC-iMLLT binds to the ENL YEATS domain with a K_d of 0.26 μM **(Figure 2-5C)**[212] demonstrating the fluorescein label does not affect interaction with the YEATS domain. Importantly, we observed significantly decreased binding affinity to all YEATS mutants in the following order: Δ21-26 (K_d = 4.6 μM) > Y78A (K_d = 8.82 μM) > F59A (K_d

> 8 μM) > triple mutation (K_d > 8 μM). The obtained binding affinities correlated with the binding preferences to the H3K27ac peptide, and as expected the YEATS triple mutant had the most significant impact on Flu-SGC-iMLLT binding, suggesting an additive effect **(Figure 2-5C, 2-6D)**.

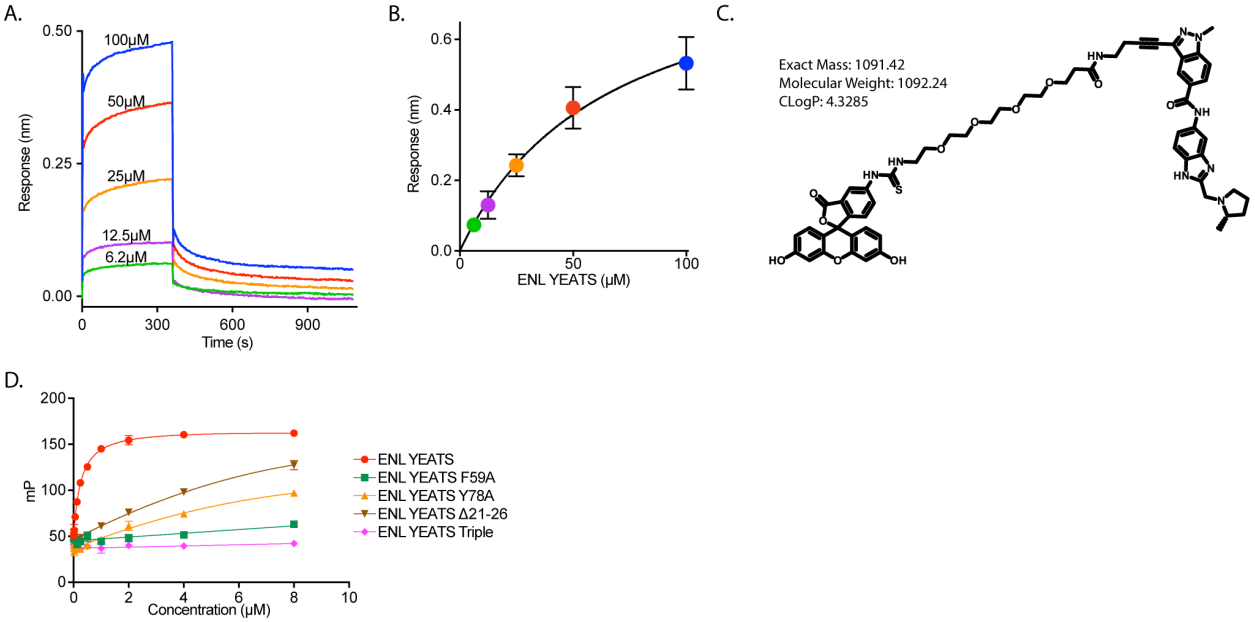
Figure 2-5: YEATS Domain Mutations Impact H3Kac Binding



(A) In vitro binding assay of His-tagged ENL YEATS domain or mutants (F59A, Δ21-26, triple) with histone H3 peptide (aa21-44), H3K27ac peptide (aa21-43) or a scrambled peptide control. (B) Representative bio-layer interferometry (BLI) experiment of the wild type ENL YEATS domain or mutants (F59A, Y78A, and Δ21-26) with 50μM H3K27ac peptide. K_d for ENL YEATS is estimated to be 66.56 ± 7.07 μM (n=2 independent results). Binding data are obtained as the average of two or more independent experiments. (C) Fluorescence polarization experiment using fluorescently labelled SGC-iMLLT compound with the wild type ENL YEATS domain or YEATS mutants (F59A, Y78A, Δ21-26, triple). Means with SD values were plotted. Estimated K_d values: ENL YEATS: 0.26 ± 0.02 μM; ENL YEATS F59A: >8 μM; ENL YEATS Y78A: 8.82 ± 0.48 μM; ENL YEATS Δ21-26: 4.6 μM; ENL YEATS Triple: >8 μM. K_d values for ENL YEATS Y78A and Δ21-26 were calculated by constrained fitting using the Klotz plot (semi-log plot). (D) Representative co-immunoprecipitation experiment performed by immunoprecipitating FLAG-tagged ENL or ENL mutants from transiently transfected HEK293T cells and blotting for endogenous PAF1. (E) Representative co-immunoprecipitation experiment performed by immunoprecipitating FLAG-tagged ENL or ENL mutants from HEK293T transiently transfected with FLAG-ENL or ENL mutants and HA-PAF1. Precipitate was immunoblotted with either anti-HA or anti-FLAG.

We then addressed how YEATS domain mutations disrupt interaction with PAF1 of the PAF1c using co-immunoprecipitation experiments. Our positive control, FLAG-CDC73, a known PAF1 binding partner, efficiently co-immunoprecipitated with endogenous or exogenous PAF1 **(Figure 2-5D, E)**. As expected, FLAG-ENL co-immunoprecipitated endogenous PAF1 **(Figure 2-5D)** or exogenous HA-PAF1 **(Figure 2-5E)**. Interestingly, all mutations of the YEATS domain (F59A, Y78A, Δ21-26, or Triple mutation) do not disrupt interaction with PAF1 **(Figure 2-5D, E)**. These results provide evidence that the H3K27ac and PAF1 binding sites are not overlapping suggesting they are not mutually exclusive. Together, these data demonstrate that the single YEATS mutations independently or additively disrupt the YEATS domain epigenetic reader function but do not alter interaction with PAF1 of the PAF1c.

Figure 2-6: Biochemical Analysis of the ENL YEATS Domain



(A) Representative control BLI experiment of wild type ENL YEATS domain concentration dependent binding to H3K27ac peptide. (B) Representative binding curve derived from the BLI experiment shown in (A). K_D = 66.56 ± 7.07, n=2. (C) Chemical structure of Flu-SGC-iMLLT. (D) Saturation binding curves of Flu-SGC-iMLLT to wild type and mutants ENL YEATS domain. Flu-SGC-iMLLT (5 nM) with increasing concentrations of YEATS domain recombinant proteins (from 0 to 8 μM). Estimated K_D values: ENL

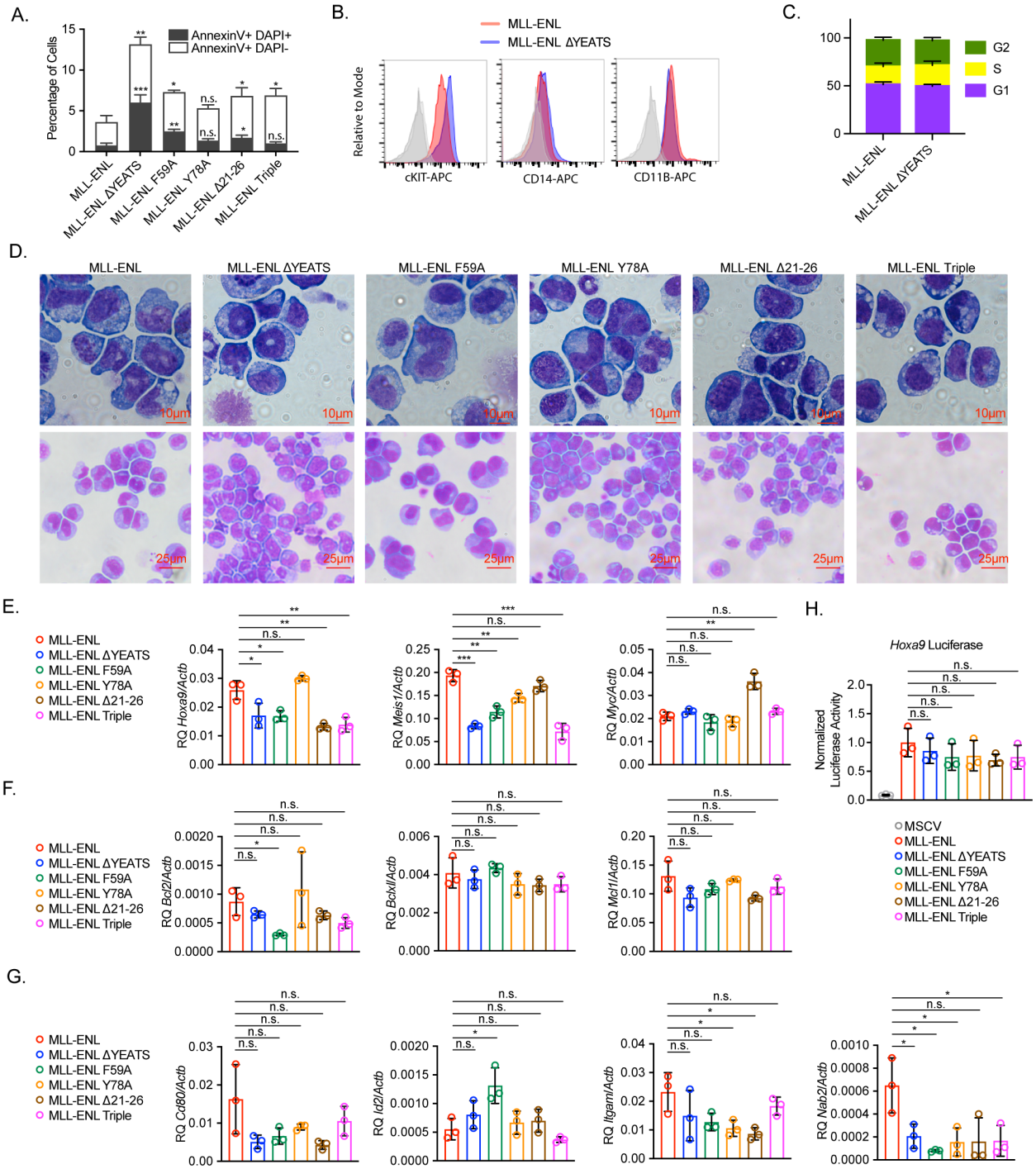
YEATS: 0.25 ± 0.004 μM; ENL YEATS F59A: >8 μM; ENL YEATS Y78A: >8 μM; ENL YEATS Δ21-26: >8 μM; ENL YEATS Triple: >8 μM.

2.3.4 Loss of YEATS Domain Function Affects Transcription of Selected MLL-ENL Targets

We characterized how YEATS domain mutations impair MLL-ENL leukemogenesis by investigating apoptosis, cell cycle and differentiation. We observed a modest but statistically significant increase in apoptotic populations when comparing murine MLL-ENLΔYEATS transformed cells to MLL-ENL transformed cells **(Figure 2-7A)**. A less pronounced increase in apoptotic cells is observed in MLL-ENL YEATS point mutants **(Figure 2-7A)**. Interestingly, we did not observe a significant change in expression of cell surface markers associated with myeloid differentiation (CD14 and CD11b), cell cycle or cellular morphology in MLL-ENLΔYEATS cells compared to MLL-ENL cells **(Figure 2-7B, C, D)**. Thus, loss of the YEATS domain and downstream sequence in MLL-ENL cells only modestly alters apoptosis *in vitro*. Next, we addressed how loss or mutation of the YEATS domain affects MLL-ENL-mediated transcriptional activation. We investigated the expression of confirmed MLL-ENL target genes (*Hoxa9, Meis1* and *Myc*) by qPCR. A modest expression difference was detected for *Hoxa9* and *Meis1* but not *Myc* **(Figure 2-7E)**. Given the modest change in apoptosis, we examined the expression of *Bcl2, Bclxl* and *Mcl1*, which showed mostly insignificant changes with loss or mutation of the YEATS domain **(Figure 2-7F)**. We did not detect significant increases in genes associated with myeloid differentiation (*Cd80, Id2, Itgam* and *Nab2*)[162] consistent with our results showing loss or mutation of the YEATS domain does not affect differentiation **(Figure 2-7B, D, G)**. Finally, we tested the transcriptional

activity of MLL-ENL fusion proteins with and without YEATS mutations on the *Hoxa9* promoter using a luciferase reporter. MLL-ENLΔYEATS, F59A, Y78A, Δ21-26 or triple mutant fusion proteins displayed no reduction in transcriptional activation of the *Hoxa9* promoter **(Figure 2-7H)**, suggesting transcription per se is not altered by mutation (or loss) of the YEATS domain.

Figure 2-7:Cellular Characterization Following Mutations of the YEATS Domain



*(A) Assessment of apoptosis using AnnexinV and DAPI double stains in MLL-ENL, MLL-ENLΔYEATS and MLL-ENL YEATS mutants (n=3 biological replicates). Error bars represent standard deviation. Statistical test: student's paired t-test. *: p<0.05; **: p<0.01; ***: p<0.001; n.s.: not significant. (B) Flow cytometry detection of cKIT, CD14, and CD11B on MLL-ENL and MLL-ENLΔYEATS cell lines. Gray area in each image shows isotype control. (C) Flow cytometric cell cycle analysis showing G1, S and G2 populations in MLL-ENL and MLL-ENLΔYEATS cell lines. (D) Representative 100x and 40x hemastain images of MLL-ENL, MLL-ENL mutants cell lines. (E) RT-qPCR gene expression analysis of Hoxa9,*

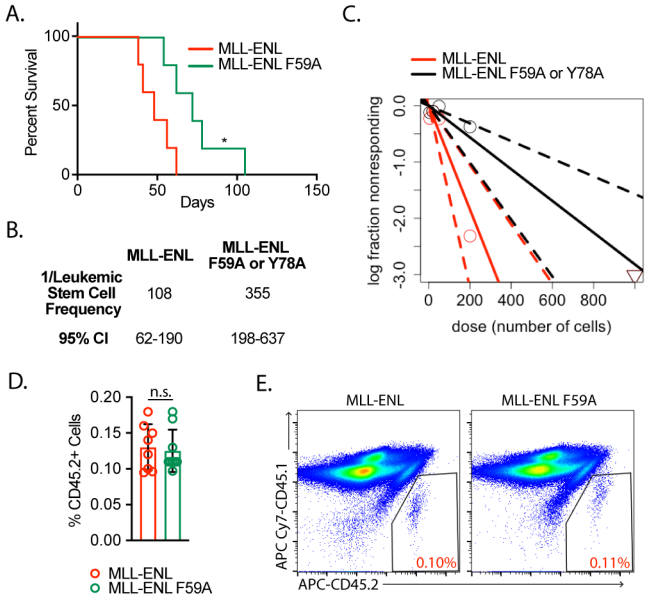
Meis1, and Myc in MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutants cell lines. Error bars represent standard deviation. Statistical test: unpaired student's t-test. (: p<0.05; **: p<0.01; ***: p<0.001; n.s. not significant) (F) RT-qPCR gene expression analysis of Bcl2, Bclxl, and Mcl1 in MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutants cell lines. Error bars represent standard deviation. Statistical test: unpaired student's T-test. *: p<0.05; n.s. not significant. (G) RT-qPCR gene expression analysis of Cd80, Id2, Itgam, and Nab2 in MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutants cell lines. Error bars represent standard deviation. Statistical test: unpaired student's t-test. (*: p<0.05; n.s. not significant) (H) Normalized luciferase activity (MLL-ENL) of 293T lysate 48 hours post-transfection. 293T cells were co-transfected with Firefly-Renilla luciferase, HoxA9-luciferase-luciferase, and the indicated MLL-ENL fusion constructs. Error bars represent standard deviation. Statistical test: unpaired student's t-test. (n.s. = not significant)*

2.3.5 Mutation of the YEATS Domain Alters MLL-ENL Leukemic Stem Cell Frequency

We hypothesized that the YEATS domain epigenetic reader function impacts MLL-ENL leukemic stem cell frequency. First, we investigated whether the disease latency extension following mutation of the YEATS domain **(Figure 2-3F)** was transplantable. Indeed, we observed a significant disease extension in secondary recipients following transplantation of primary MLL-ENL YEATS mutant leukemic cells (F59A or Y78A) compared to MLL-ENL leukemic cells **(Figure 2-8A, 2-9A)**. We then performed extreme limiting dilution analysis by transplanting primary MLL-ENL or MLL-ENL YEATS mutant (F59A or Y78A) leukemic cells into irradiated syngeneic recipients. We detected a significant decrease in leukemic stem cell frequency in MLL-ENL YEATS mutant leukemias (1/355 cells, 95% CI:1/198-1/637 cells) compared to MLL-ENL leukemia (1/108 cells, 95% CI: 1/62-1/190 cells) **(Figure 2-8B, C)**. To rule out differences in leukemic cell homing to the bone marrow we injected CD45.2 donor leukemic cells (MLL-ENL or MLL-ENL F59A) into sublethally irradiated CD45.1 syngeneic recipients and observed no difference in the percentage of donor MLL-ENL or MLL-ENL F59A leukemic cells in the bone marrow **(Figure 2-8D, E, 2-9B)**. Thus,

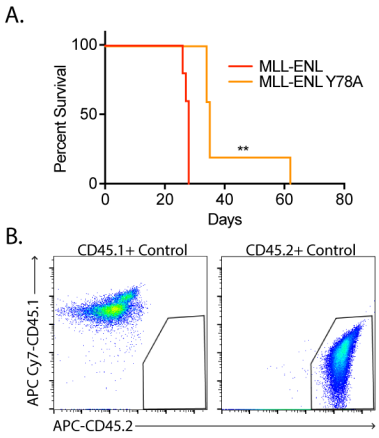
disrupting the YEATS domain epigenetic reader function on MLL-ENL fusion proteins impacts leukemic stem cell frequency.

Figure 2-8: Disruption of the YEATS Domain Epigenetic Reader Function Impacts MLL-ENL Leukemic Stem Cell Frequency



(A) Secondary leukemogenesis assay comparing primary MLL-ENL (n=5) or MLL-ENLF59A (n=5) leukemias. 1000 primary leukemic cells were injected into sublethally (650 rads) irradiated syngeneic recipients without support bone marrow. Statistical test: log-rank (Mantel-Cox). (: p<0.05) (B) Summary of the estimated leukemic stem cell frequencies with 95% confidence interval derived from extreme limiting dilution analysis of MLL-ENL leukemia compared to MLL-ENLF59A or Y78A leukemia. Analysis represents a combination of two independent experiments. In each experiment, a total of 1000, 200, 50, 20 or 5 primary mouse leukemic cells were injected into sublethally (650 rads) irradiated syngeneic recipients (n=5) without support marrow. (C) Log-fraction plot showing the leukemic stem cell frequencies in MLL-ENL and MLL-ENLF59A or Y78A leukemias according to the Extreme Limiting Dilution Analysis (ELDA). (D) Homing assay comparing the leukemic homing capacity in recipients (CD45.1+) injected with MLL-ENL (n=8) or MLL-ENLF59A (n=9) leukemias (CD45.2+). Figure shows mean with SD. Statistical test: unpaired student's t-test. n.s.: not significant. (E) Representative flow plot showing the gating for CD45.2+ leukemic cells in recipient mice injected with either MLL-ENL or MLL-ENLF59A leukemias.*

Figure 2-9: Characterization the YEATS Domain on LSC Frequency



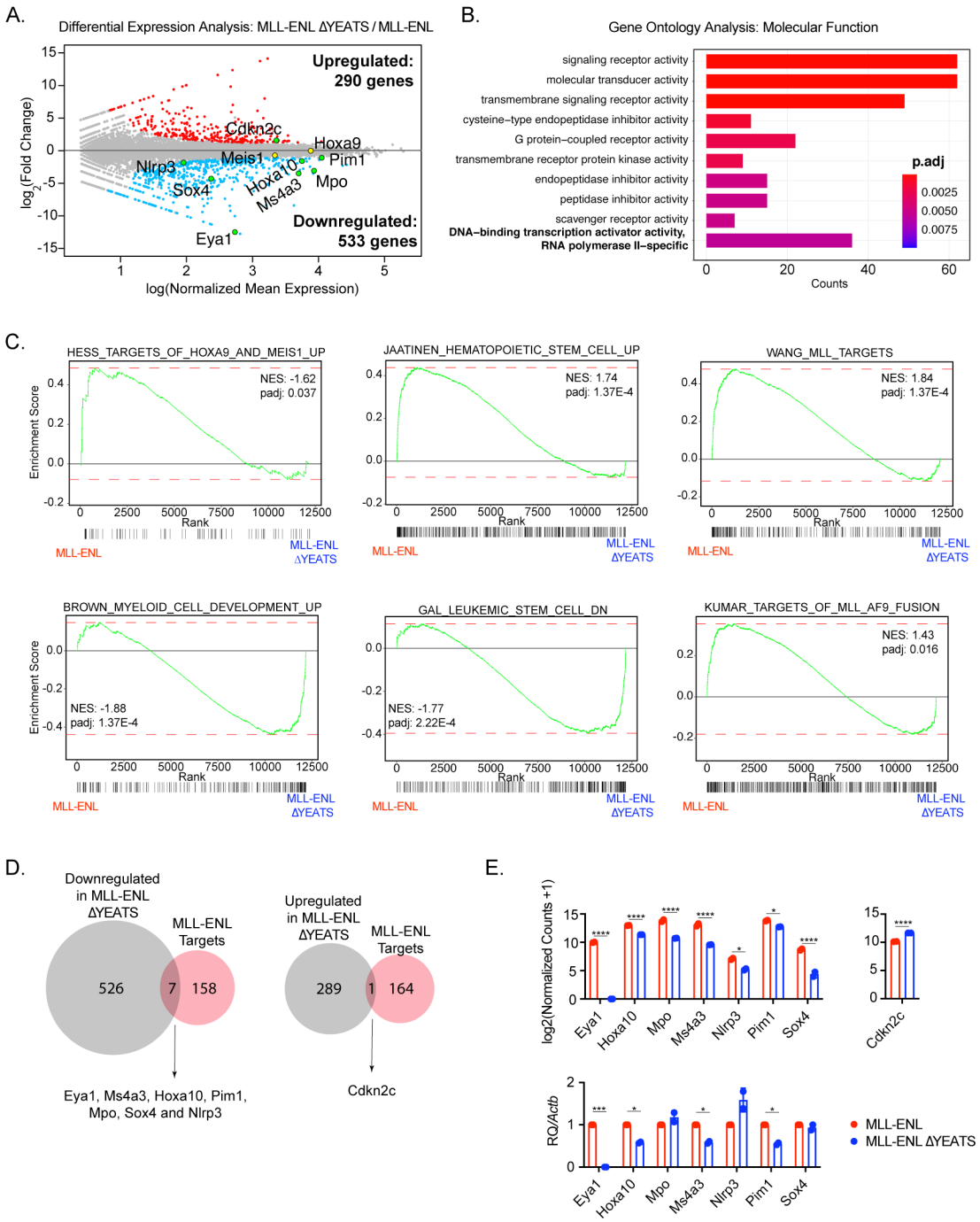
*(A) Secondary leukemogenesis assay comparing primary MLL-ENL (n=5) and MLL-ENLY78A (n=5) leukemias (1000 cells). Statistical test: log-rank (Mantel-Cox) (**: p<0.01). (B) Flow cytometry plots showing the gating strategy for CD45.1+ and CD45.2+ controls.*

2.3.6 Loss of the YEATS Domain Impacts Expression of Select MLL-ENL Target Genes

To understand how the YEATS domain impacts MLL-ENL target gene expression we performed RNA-Seq analysis on RNA prepared from murine MLL-ENL and MLL-ENLΔYEATS cell lines. Differential expression analysis using a 1.5 fold cut off revealed 533 downregulated and 290 upregulated genes in MLL-ENLΔYEATS compared to MLL-ENL cells **(Figure 2-10A)**. Gene ontology analysis revealed deregulation of several GO terms under the molecular function aspect including "DNA-binding transcription activator activity" **(Figure 2-10B)**. Next, we used Gene Set Enrichment Analysis (GSEA) to investigate gene programs misregulated in MLL-ENLΔYEATS cells **(Table 2-1)**. Importantly, we found decreased expression of a HOXA9 and MEIS1 gene program, previously described in MLL-ENL cells, in MLL-ENLΔYEATS cells **(Figure 2-10C)**. A myeloid development phenotype was also more

associated with MLL-ENLΔYEATS cells **(Figure 2-10C)**. Gene programs associated with hematopoietic and leukemic stem cells were enriched in MLL-ENL cells compared to MLL-ENLΔYEATS cells **(Figure 2-10C)**. Finally, direct transcriptional targets of MLL and MLL-AF9 were more enriched in MLL-ENL cells compared to MLL-ENLΔYEATS cells **(Figure 2-10C)**. Thus, we examined how many direct MLL-ENL target genes[104] were downregulated or upregulated in MLL-ENLΔYEATS cells. We identified downregulation of seven direct MLL-ENL targets: *Eya1, Ms4a3, HoxA10, Pim1, Mpo, Sox4* and *Nlp3* and upregulation of *Cdkn2c* in MLL-ENLΔYEATS cells **(Figure 2-10D, E)**. Downregulation of *Eya1, Hoxa10, Ms4a3* and *Pim1* were confirmed by qPCR from freshly prepared mRNA from MLL-ENL and MLL-ENLΔYEATS cells **(Figure 2-10E)**. These data point to differential regulation of MLL-ENL transcriptional targets depending on the presence or absence of the ENL YEATS domain.

Figure 2-10: Transcriptomic Changes Associated with MLL-ENLΔYEATS Cells



(A) MA-plot showing significant differentially expressed (defined as 1.5-fold upregulated or downregulated) genes in MLL-ENLΔYEATS cells compared to MLL-ENL cells. Grey dots mark genes that are non-significant while red and light blue dots mark genes that are significantly up- or downregulated respectfully. Hoxa9 and Meis1 are highlighted in yellow. Green dots signify genes that are differentially expressed and targets of MLL-ENL (52). (B) Gene ontology analysis of the molecular function aspect using 1.5-fold differentially expressed genes. Figure captures top 10 GO terms with the lowest padj value (represented with the color gradient bar). Count indicates number of differentially expressed genes mapped to the GO term. (C) Gene Set Enrichment Analysis of the C2: CGP (chemical and genomic perturbations) curation from MSigDB using differentially expressed genes. Selected pathways were

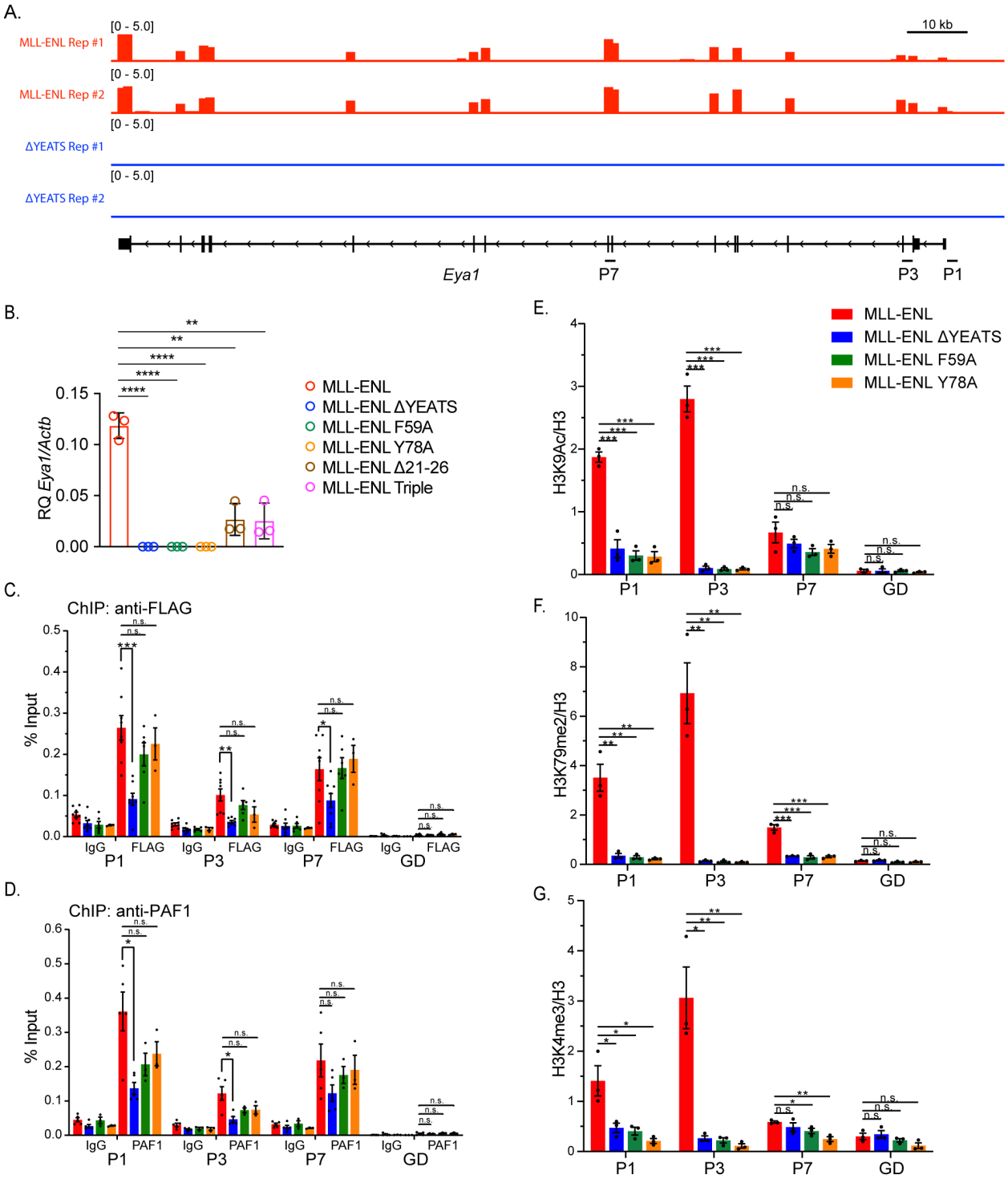
*highlighted in the figure shown with normalized enrichment score (NES) and adjusted p-values. (D) Venn diagram of 1.5-fold differentially expressed genes (either up- or downregulated) with targets of MLL-ENL targets (52). (E) (Top) Relative expression (presented as log2(Normalized Counts + 1)) values of differentially expressed MLL-ENL targets. Normalized Counts and statistics were obtained from DESeq2 results. Figure shows mean values with SD. (p < 0.05 *; p < 0.0001 ****). (Bottom) RT-qPCR data (shown in ΔΔCt) of two biological replicates probing for downregulated MLL-ENL targets identified by RNAseq. Figure shows mean values with SD. Statistics: student's unpaired T-test on ΔCt values. (*:p < 0.05; ***:p < 0.001)*

2.3.7 Loss of the YEATS Domain Impairs MLL-ENL Localization on Target Genes

We next performed chromatin immunoprecipitation (ChIP) assays to assess MLL fusion protein binding and changes to the epigenetic landscape on *Eya1* and *Meis1* loci. Impaired transcriptional activation of *Eya1* and *Meis1* in murine MLL-ENLΔYEATS cells observed by RNA-seq was also detected in MLL-ENL F59A, -Y78A, -Δ21-26 and -Triple mutants compared to MLL-ENL cells **(Figure 2-11A, B, 2-12A, B)**. We examined the promoter regions (P1 and P3) and intragenic region (P7) of *Eya1* **(Figure 2-11A)** and the regulatory region (P1 and P2) of *Meis1* **(Figure 2-12A)**. ChIP for the N-terminal FLAG-tag revealed reduced binding of MLL-ENLΔYEATS fusion protein compared to MLL-ENL on the *Eya1* and *Meis1* loci **(Figure 2-11C, 2-12C)**. Interestingly, MLL-ENL F59A or -Y78A fusion proteins bound with similar affinity as MLL-ENL despite significantly lower expression levels **(Figure 2-11C, 2-12C)**. The binding pattern of the PAF1c subunit PAF1 mirrored the MLL-fusion proteins suggesting the PAF1c and MLL-ENL fusion proteins cooperatively assemble on these loci **(Figure 2-11D, 2-12D)**. Next, we interrogated histone modifications associated with transcriptional activation by MLL-ENL fusion proteins, including H3K9ac, H3K79me2 and H3K4me3, which were detected in MLL-ENL cells at the promoter region for *Eya1* and *Meis1.* Consistent with transcriptional changes observed from these loci, H3K9ac, H3K79me2 and H3K4me3

was reduced proportionally to *Eya1* and *Meis1* transcriptional output in MLL-ENLΔYEATS, -F59A and -Y78A cells **(Figure 2-11E, F, G, 2-12E, F, G)**. These data suggest that deletion of ENL that includes the YEATS domain impairs binding and transcriptional activation, whereas point mutations to the YEATS domain impacting H3Kac binding do not alter fusion protein localization but inhibit downstream epigenetic function.

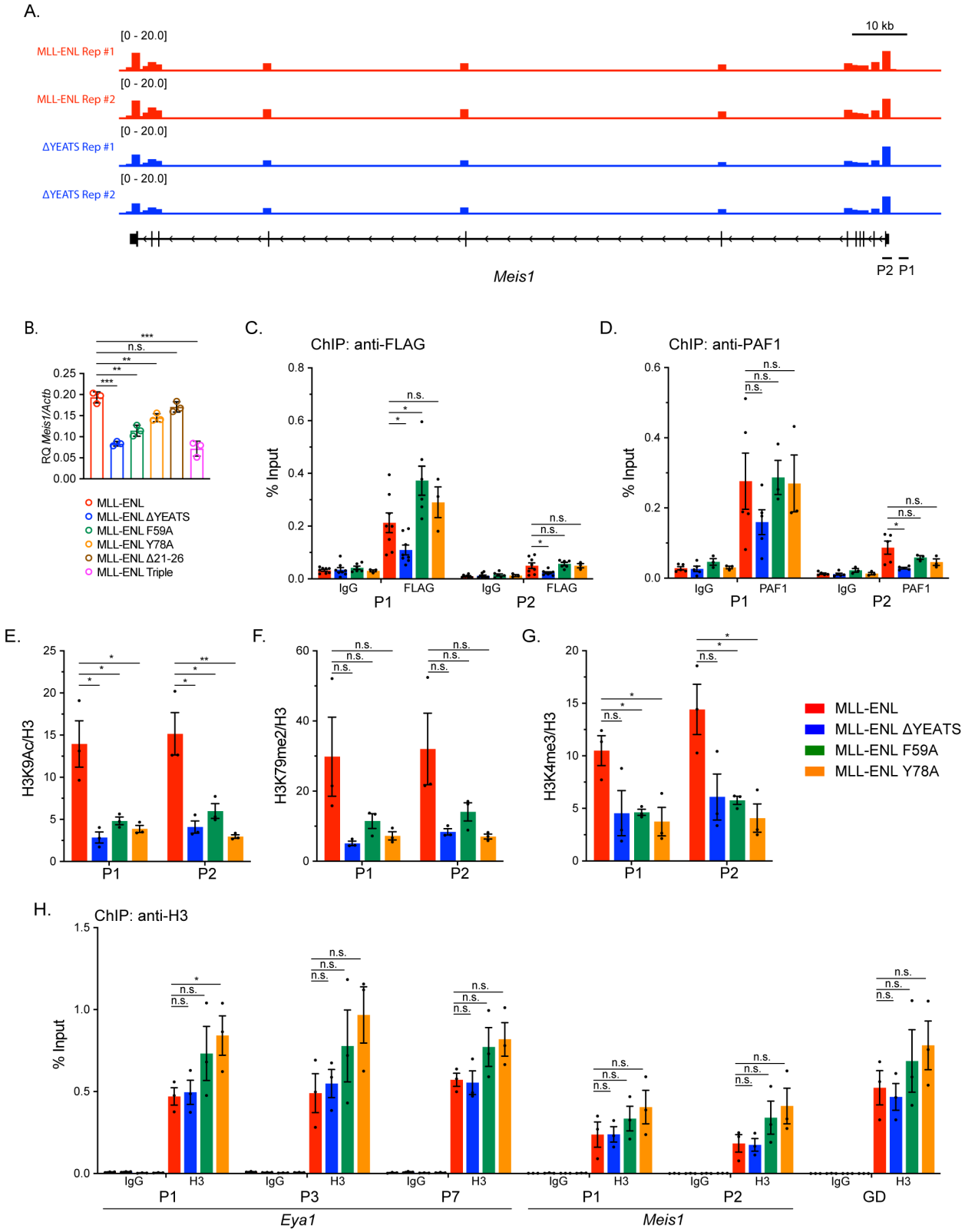
Figure 2-11: YEATS Domain of ENL is Required for Epigenetic Regulation of *Eya1*



*(A) RNA-seq genomic track showing expression of Eya1 in MLL-ENL or MLL-ENLΔYEATS cell lines. (B) RT-qPCR analysis showing expression of Eya1 in MLL-ENL or MLL-ENL mutant cell lines. RT-qPCR analysis is represented as mean with SD. Statistical test: unpaired student's t-test. (****: p<0.0001; **: p<0.01) (C-G) ChIP-qPCR analysis of anti-FLAG (C), anti-PAF1 (D), anti-H3K9Ac (E), anti-H3K79me2 (F) and anti-H3K4me3 (G) on Eya1 locus in MLL-ENL, MLL-ENLΔYEATS, MLL-ENL F59A or MLL-ENL Y78A mutant cell lines. Anti-FLAG and anti-PAF1 ChIP are represented as percent input and histone*

modification ChIP assays are represented as normalized value to histone H3 ChIP. qPCR amplicons are designated as P1, P3, and P7 and their locations are represented in (A) as black bars. GD represents an amplicon at a non-specific gene desert region. ChIP-qPCR analysis is represented as mean with SEM (C-G). All experiments represent at least 3 independent biological replicates. Unpaired t-test was performed for (E-G) and Welch's t-test was performed for (C-D). (:p < 0.05; **:p < 0.01; ***:p < 0.001; n.s: not significant)*

Figure 2-12: *Meis1* is Regulated by MLL-ENL via YEATS Domain



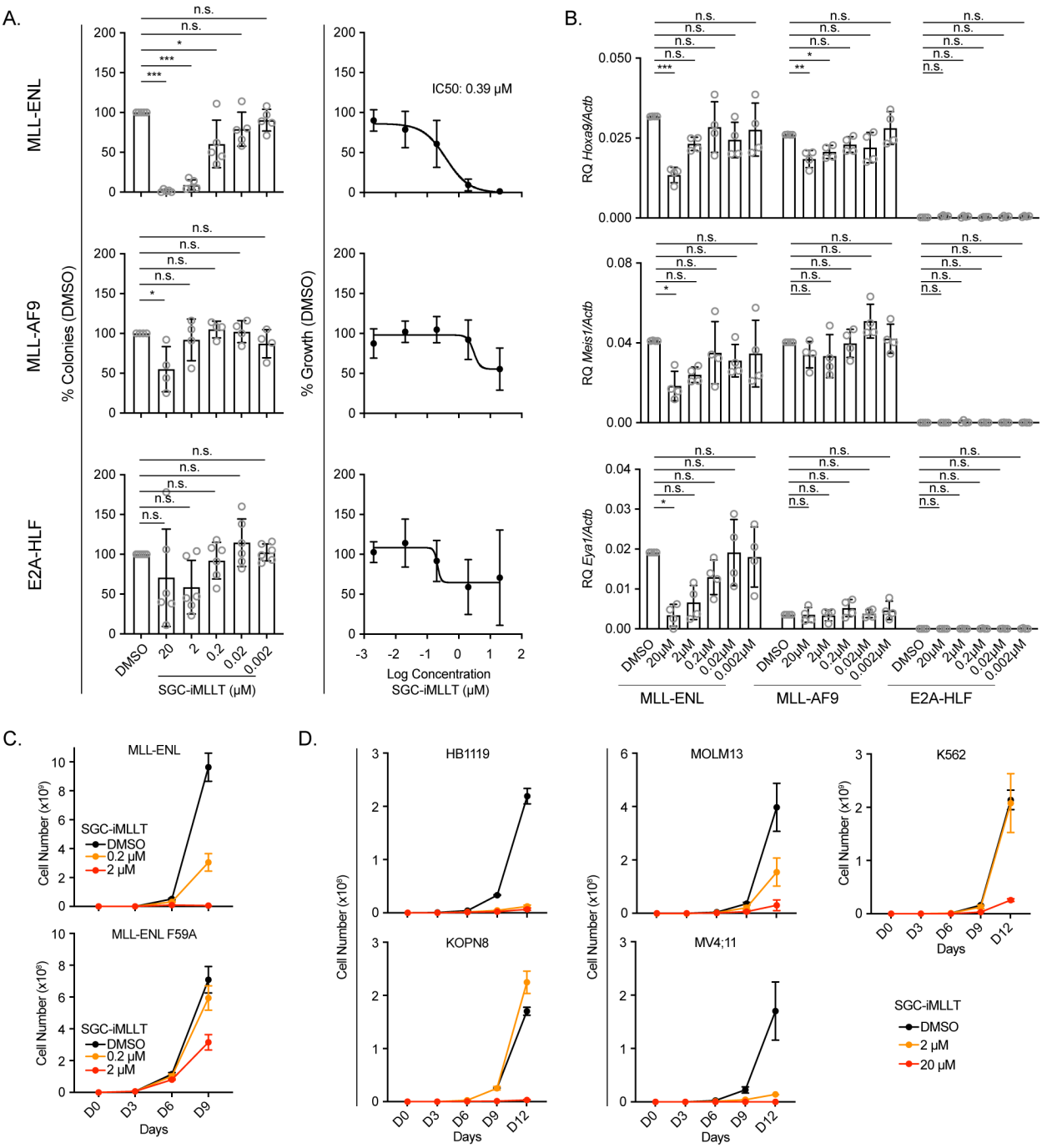
*(A) RNA-seq genomic track showing expression of Meis1 in MLL-ENL or MLL-ENL∆YEATS cell lines. (B) RT-qPCR analysis showing expression of Meis1 in MLL-ENL, MLL-ENL∆YEATS or MLL-ENL YEATS mutants cell lines. RT-qPCR analysis is represented as mean with SD. Statistical test: student's unpaired T-test. (**: p < 0.01; ***: p < 0.001; n.s.: not significant) (C-G) ChIP-qPCR analysis of anti-FLAG (C), anti-PAF1 (D), anti-H3K9Ac (E), anti-H3K79me2 (F) and anti-H3K4me3 (G) on the Meis1 locus in MLL-ENL MLL-ENL∆YEATS or MLL-ENL YEATS mutants cell lines. qPCR amplicons are designated as P1 and P2 and their locations are represented in (A) as black bars. (H) ChIP-qPCR analysis of histone H3 on Eya1, Meis1 or GD genomic loci. GD represents an amplicon at a non-specific gene desert region. anti-FLAG, anti-PAF1, and anti-H3 ChIP are represented as percent input and histone modification ChIP are represented as normalized value to histone H3 ChIP. ChIP-qPCR analysis is represented as mean with SEM. All experiments represent at least 3 independent biological replicates (C-H). Unpaired t-test was performed for (E-H) and Welch's t-test was performed for (C-D). (*:p < 0.05; **:p < 0.01; ***:p < 0.001; n.s.: not significant)*

2.3.8 Targeting YEATS Domain Binding to H3Kac Impairs MLL-ENL Proliferation and Target Gene Expression

Given our *in vivo* data **(Fig. 2-3)**, we hypothesized that MLL-ENL cells may be sensitive to small molecule inhibitors disrupting the YEATS-H3Kac interaction. We utilized SGC-iMLLT to test colony formation capacity of murine MLL-ENL described above (containing the YEATS domain), MLL-AF9 (without YEATS domain) and E2A-HLF AML cell lines in the presence of escalating doses of SGC-iMLLT (0.002μM to 20μM) **(Figure 2-13A)**. MLL-ENL cells showed the highest sensitivity to SGC-iMLLT (IC50=0.39μM) without a noticeable change in cell morphology after second round plating **(Figure 2-13A, 2-14A, B)**. In contrast, MLL-AF9 and E2A-HLF transformed cells showed modest sensitivity to SGC-iMLLT at the highest concentration (20μM) **(Figure 2-13A, 2-14A, B)**. Gene expression analysis revealed a dose dependent reduction of *HoxA9, Meis1* and *Eya1* expression in MLL-ENL cells treated with SGC-iMLLT without changes in myeloid differentiation genes **(Figure 2-13B, 2-14C)**. SGC-iMLLT exposure did not impact *Bcl2* or *Bclxl* expression in E2A-HLF cells **(Figure 2-14C)**. We next compared the sensitivity of murine cell lines generated by transduction with either MLL-

ENL or MLL-ENL F59A to SGC-iMLLT in liquid proliferation assays. The F59A mutation rendered MLL-ENL cells less sensitive to SGC-iMLLT treatment **(Figure 2-13C)**, consistent with its abolished binding affinity to this mutated YEATS domain **(Figure 2-5C)**. Finally, we examined the sensitivity of human leukemic cell lines to SGC-iMLLT. We compared HB1119 cells driven by MLL-ENL fusion proteins containing the YEATS domain[294], KOPN8 cells harboring MLL-ENL fusion proteins lacking the YEATS domain[319], MOLM13 cells (MLL-AF9), MV4;11 cells (MLL-AF4) and K562 cells (BCR-ABL). All human cells were sensitive to 20 μM SGC-iMLLT, which may be cytotoxic **(Figure 2-13D)**. However, greater sensitivity was detected for HB1119 and MV4;11 cells at 2 μM consistent with a role for wild type ENL in leukemic cell survival[84]. Increased sensitivity of HB1119 cells compared to KOPN8 cells is also consistent with the reliance of HB1119 cells on the YEATS domain present in the driving MLL-ENL fusion protein **(Figure 2-13D)**. Thus, leukemic cells are sensitive to targeted chemical inhibition of the YEATS domain on wild type ENL. Further, leukemic cells driven by MLL-ENL fusion proteins harboring the YEATS domain display greater sensitivity suggesting this may be an ideal target for t(11;19) patients.

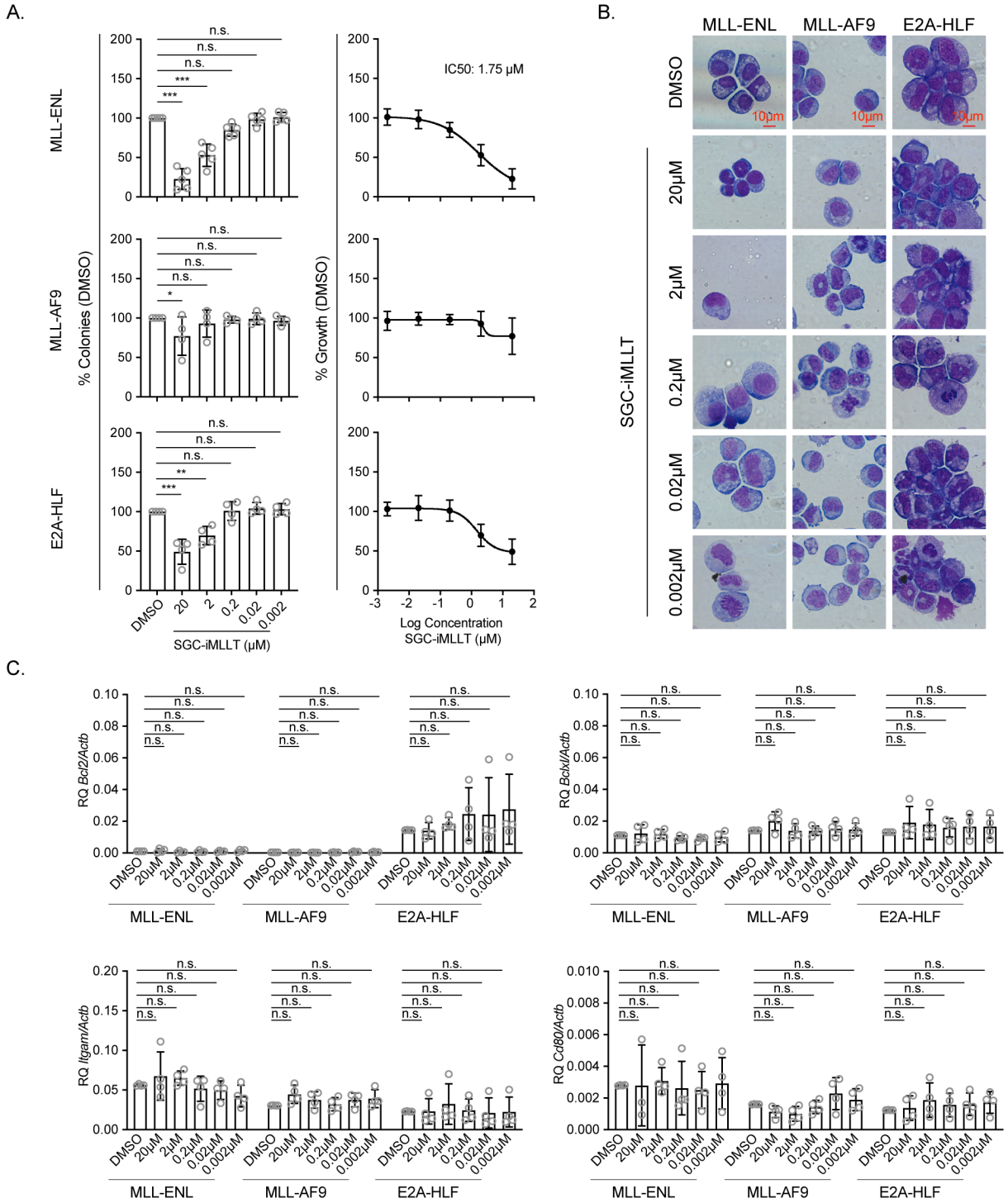
Figure 2-13: MLL-ENL Cells Display Increased Sensitivity to SGC-iMLLT



(A) Left: results from second round colony formation of simultaneously generated murine MLL-ENL, MLL-AF9 and E2A-HLF cells treated with SGC-iMLLT. Results are displayed normalized to DMSO. Statistical test: one-way ANOVA test comparing treatments within each cell line (colony counts were used for significance calculation). Figure represents mean values with SD. (: p<0.05; ***: p<0.001; n.s. not significant) Right: SGC-iMLLT dose-response curve of MLL-ENL, MLL-AF9 and E2A-HLF cell lines normalized to DMSO. Means with SD values were plotted. (B) RT-qPCR analysis of Hoxa9, Meis1, and Eya1 expression in MLL-ENL, MLL-AF9 and E2A-HLF colonies harvested 6 days post SGC-iMLLT treatment. Figure represents mean values with SD. Data presented as ΔΔCt value (normalized to DMSO-treated samples) multiplied by a cell line-specific factor to compare expression across different cell lines.*

Cell line specific factors equal the average transformed ΔCt value from four biological replicates treated with DMSO. Statistical test: one way ANOVA test of ΔCt values comparing treatments within each cell line. (: p<0.05; **: p<0.01; ***: p<0.001; n.s. not significant). (C) Cell culture proliferation assay of MLL-ENL or MLL-ENLF59A cell lines treated with SGC-iMLLT. 20,000 cells were seeded on D0 and counted on D3, D6, and D9. Means with SD values were plotted. (D) Cell culture proliferation assay of HB1119, KOPN8, MOLM13, MV4;11, and K562 cell lines treated with SGC-iMLLT. 20,000 cells were seeded on D0 and counted on D3, D6, D9 and D12. Means with SD values were plotted.*

Figure 2-14: Cellular and Transcriptional Effects of SGC-iMLLT Treatment



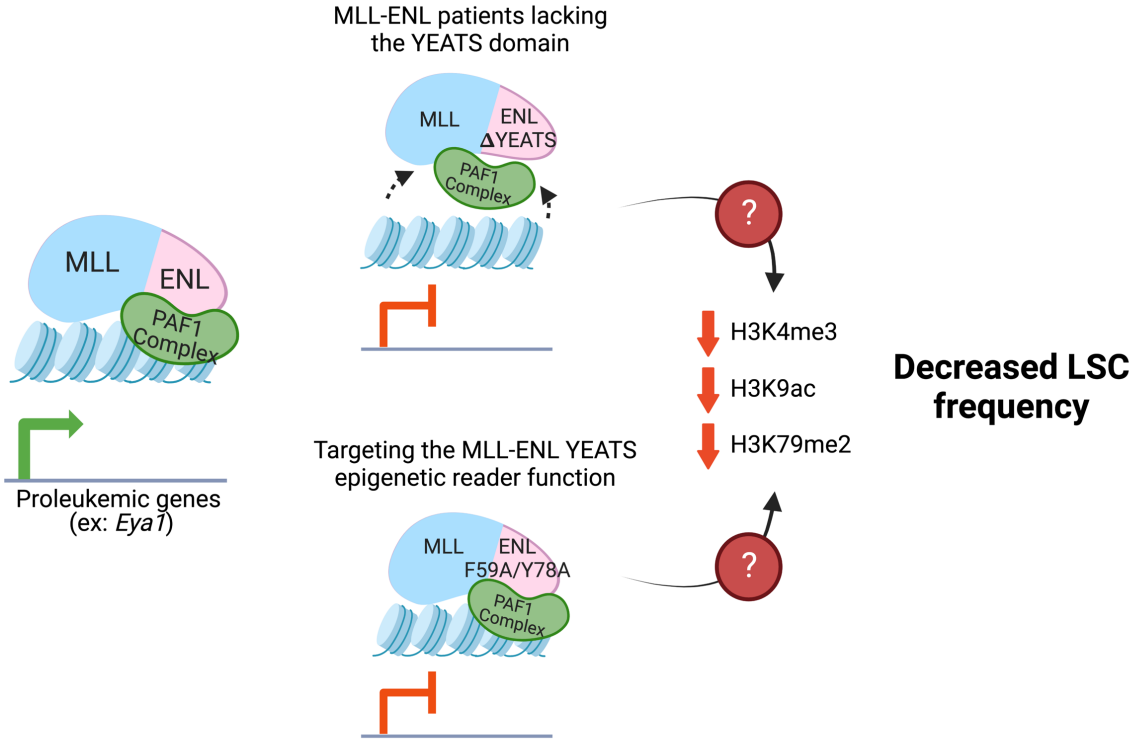
*(A) (Left) First round colony formation assay of MLL-ENL, MLL-AF9 and E2A-HLF cell lines treated with SGC-iMLLT. Results were normalized to DMSO. Error bars represent standard deviation. Statistical test: repeated measure one-way ANOVA test followed by Dunnett's multiple comparisons test for post hoc analysis comparing treatments within each cell line (colony counts were used for significance calculation). (RM one way ANOVA result: DF=29; MLL-ENL: F=57.37, p<0.0001; MLL-AF9: F=2.25, p=0.10; E2A-HLF: F=17.19, p<0.0001; post hoc analysis results presented in panel: *: p<0.05; **: p<0.01 ***: p<0.001, n.s.*

not significant). (Right) SGC-iMLLT dose-response curve of MLL-ENL, MLL-AF9 and E2A-HLF cell lines normalized to DMSO. Error bars represent standard deviation. (B) Representative 100x hemastain images of MLL-ENL, MLL-AF9, and E2A-HLF cells treated with SGC-iMLLT for 11 days. (C) qPCR expression analysis of anti-apoptotic genes (Bcl2 and Bclxl) and differentiation genes (Itgam and Cd80) in MLL-ENL, MLL-AF9 and E2A-HLF colonies harvested 6 days post SGC-iMLLT treatment. Data presented as ΔΔCt value (normalized to DMSO-treated samples) multiplied by a cell line-specific factor to compare expression across different cell lines. Cell line specific factors were calculated as the average transformed ΔCt from four biological replicates of each cell line's DMSO-treated sample. Error bars represent standard deviation. Statistical test: repeated measure one way ANOVA followed by Dunnett's multiple comparisons test for post hoc analysis of ΔCt values comparing treatments within each cell line. (RM one way ANOVA result: DF=23, no significant difference detected. post hoc analysis results presented in panel: n.s.: not significant).

2.4 Conclusions and Discussion

ENL and AF9 are YEATS domain proteins and *MLL* translocation partners in ~32% of MLL-rearranged leukemia[199]. However, investigation of the YEATS domain inclusion in t(11;19) MLL-ENL and t(9;11) MLL-AF9 patients was lacking. We present data from t(11;19) and t(9;11) leukemia patients that shows the YEATS domain is retained in >84% of MLL-ENL fusion proteins but lost in almost all MLL-AF9 fusion proteins **(Figure 2-1, 2-2)**. *In vivo* leukemogenesis assays demonstrated an important role for the YEATS domain and downstream sequence in MLL-ENL leukemias **(Figure 2-3)**. Our biochemical analysis suggests a model whereby disrupting the YEATS domain epigenetic reader function impacts MLL-ENL target activation and leukemic stem cell frequency **(Figure 2-15)**. These data identify a potential "Achilles heel" that may render MLL-ENL leukemias more susceptible to therapeutics targeting the YEATS domain.

Figure 2-15: Working Model of MLL-ENL YEATS Epigenetic Reader Function in MLL-ENL Leukemogenesis



The schematic of current working model on how the YEATS domain contribute to MLL-ENL leukemogenesis. The absence or abrogation of the YEATS epigenetic reader function of the fusion severely perturbs the epigenetic landscape and expression of a subset of MLL-ENL targets, leading to change in leukemic stem cell frequency.

Interestingly, our cellular characterization revealed a LSC defect following mutation of the YEATS domain in MLL-ENL fusion proteins. Transcriptomic analysis revealed differential expression of *Meis1* and *Eya1* comparing MLL-ENL and MLL-ENLΔYEATS or point mutant cells **(Figure 2-10, 2-11, 2-12)**. *Meis1* is implicated in leukemic stem cell self-renewal, differentiation arrest, and cycling and *Eya1* can immortalize hematopoietic progenitors[315,321]. We observed lower LSC frequency following mutation of the YEATS domain affecting H3Kac binding in MLL-ENL fusion proteins **(Figure 2-8)**. It is possible that the YEATS domain directly impacts LSC frequency by affecting MLL-ENL mediated transcription of *Meis1, Eya1* and/or others.

The MLL-ENLΔYEATS construct used in this study closely models t(11;19) leukemia patients harboring ENL breakpoints between exon 6 and 7 **(Figure 2-1)**. These patients constitute ~5.3% of MLL-ENL patients (n=16/302) and fuse ENL to MLL starting at amino acid 371. In total, 15.9% of t(11;19) patients (n=48/302) express MLL-ENL fusion proteins that lack the full YEATS domain, consistent with observations from smaller cohorts[102,258]. What is the mechanism of transformation for an MLL-ENL fusion protein lacking the YEATS domain? Screening of genomic mutations in MLL-rearranged acute leukemia patients revealed secondary mutations in FLT3-ITD, KRAS/NRAS and others[8,14]. Cooperating mutations may play a more prominent role in MLL-ENL leukemias lacking the YEATS domain and downstream sequences. Further, an intrinsically disordered region (IDR; aa171-448) in ENL can initiate a liquid-liquid phase separation of P-TEFb to induce transcriptional induction[117]. Thus, it is noteworthy that our MLL-ENLΔYEATS constructs (aa372-559) remove the YEATS domain and part of the IDR **(Figure 2-3)**. It is possible that part of the leukemic phenotype associated with MLL-ENLΔYEATS **(Figure 2-3)** results from phase separation defects due to partial deletion of the IDR. However, our *in vivo* experiments using single point mutations in the YEATS domain confirm that the YEATS epigenetic reader function is important for leukemogenesis. Further, our ChIP analysis suggests deletion of the YEATS domain affects fusion protein binding, whereas point mutations do not. This may point to impaired recruitment of co-activating proteins, however further experiments are needed.

Targeting the ENL YEATS domain has been established as a potential treatment for AML[84]. Our data predicts targeting the ENL YEATS domain may be effective against MLL-ENL cells **(Figure 2-3, 2-13)**. We used the SGC-iMLLT molecule reported to bind

specifically to the ENL/AF9 YEATS domain forming complementary pi-pi stacking interactions with residues F59 and Y78[212]. We observed varied sensitivity of murine and human AML cell lines to SGC-iMLLT treatment. It is noteworthy that cell lines harboring MLL-ENL fusion proteins containing the YEATS domain were amongst the most sensitive **(Figure 2-13)**. cell lines harboring MLL-ENL fusion proteins that contain the YEATS domain (HB1119 and murine MLL-ENL cells) displayed greater sensitivity compared to other AML cell lines **(Figure 2-13)**. We postulate SGC-iMLLT targets the YEATS domain of both MLL-ENL fusion proteins and endogenous ENL to inhibit cell growth. Indeed, differential transcriptional effects on MLL-ENL targets observed following genetic or pharmacological inhibition of the YEATS domain may result from inhibition of wild type ENL by SGC-iMLLT **(Figure 2-7, 2-13)**. A model of dual contribution of wild type ENL and MLL-ENL fusion proteins may contribute to transcription of pro-leukemic targets. Together, our study reveals the YEATS domain is retained in the vast majority of t(11;19) MLL-ENL patients and plays a critical role during leukemogenesis that may be exploited therapeutically.

2.5 Authorship and Acknowledgements

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leukemia." It is a collaborative work involving laboratories led by Drs. Andrew G. Muntean, Zaneta Nikolovska-Coleska, and Rolf Marschalek.

HH, NS, ZNC and AGM designed the experiments. All authors listed on the publication participated in conducting experiments and collecting or analyzing the data. YY conducted cloning, expression, and purification of recombinant proteins; EA conducted FP and BLI binding assays; US performed synthesis of the fluorescent-labeled inhibitor. RM provided chromosomal breakpoint data and analysis. HH, ZNC and AGM wrote the manuscript. The project was overseen by AGM.

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Chapter 3 : Conclusions and Future Directions

3.1 Summary and Conclusions

MLL-r leukemia is a subset of aggressive leukemia driven by oncogenic MLL fusion proteins. Over 100 unique chromosome 11q23 translocations have been identified in MLL-r patients, producing a large curation of different MLL fusion proteins[199]. Through research from several independent laboratories, we now understand that several common MLL fusion partners form protein complexes important in regulating RNA pol II mediated transcription[175,214,340]. Research efforts continue to build upon this knowledge, with the ultimate goal of successfully leveraging our theoretical knowledge in patient treatment.

I joined this endeavor by looking at the inclusion of the epigenetic reader YEATS domain in the MLL-ENL fusions. ENL is the 3rd most common fusion partner of MLL[199], and its YEATS domain had recently been proposed as an essential component for the growth and proliferation in several AML cell lines[84,311]. We found that the significant majority of MLL-ENL fusion proteins retain the YEATS domain. This was intriguing to us, as the YEATS domain was thought to be excluded from the minimal MLL-ENL sequence required for MLL-ENL leukemias[279,340]. Along the same line, we observed YEATS domain exclusion when MLL is fused to the ENL paralog AF9. All these fascinating observations prompted us to zoom in and interrogate the roles of the YEATS domain in MLL-ENL leukemias.

Using MLL-ENL fusion proteins found in patients, we found that the YEATS domain, together with its downstream sequence, is essential for MLL-ENL leukemogenesis. To our knowledge, the ENL YEATS domain is both a histone acylation reader and an interacting partner with PAF1[123,125,311]. We utilized published YEATS mutations perturbing these interactions and asked how these functions contribute to MLL-ENL leukemogenesis. Our biochemical data, together with our leukemogenesis assay, linked the YEATS epigenetic reader function as an important contributor to MLL-ENL leukemia. Perturbation of the YEATS epigenetic reader function significantly delayed MLL-ENL disease onset in mice.

How does the YEATS domain impact the biology of MLL-ENL leukemic cells? We tested several possible culprits including apoptosis, differentiation, and cell cycle changes, all of which did not produce a satisfactory explanation for the significant disease extension we saw in vivo. Our breakthrough happened when we found a strong association between the YEATS domain and LSC frequency in MLL-ENL leukemia. YEATS epigenetic reader mutations reduced the MLL-ENL LSCs by ~3.5 fold, providing an explanation for the increase in disease latency we saw in mouse models.

Given the YEATS domain's impact on MLL-ENL LSCs, we performed transcriptomic characterization to identify possible MLL-ENL targets responsible for the LSC phenotype. Our RNA-seq and qPCR data identified the transcriptional factor Eya1 as a major player. While the fusion protein and PAF1 showed various degree of displacement, several active epigenetic marks were diminished at *Eya1*, consistent with its lack of expression in YEATS mutant and ΔYEATS cell lines.

Is targeting the YEATS epigenetic reader function a therapeutically sound idea? Our analysis using the YEATS domain inhibitor SGC-iMLLT showed that MLL-ENL cell line HB11;19, which has a YEATS domain in the fusion protein, is among the most sensitive to YEATS inhibitor treatment in vitro. Remarkably, MLL-ENL human and mouse cell lines with functional YEATS epigenetic reader on the fusion protein demonstrated higher sensitivity than their non-functional counterpart.

In summary, our study identified the unique inclusion of the YEATS domain in MLL-ENL fusion proteins and linked its epigenetic reader function with LSC frequency in MLL-ENL leukemias. Further, MLL-ENL leukemias harboring the YEATS domain are more sensitive to YEATS inhibitors. All together, these results demonstrated a unique and potentially "druggable" opportunity for most MLL-ENL patients.

3.2 Future Directions

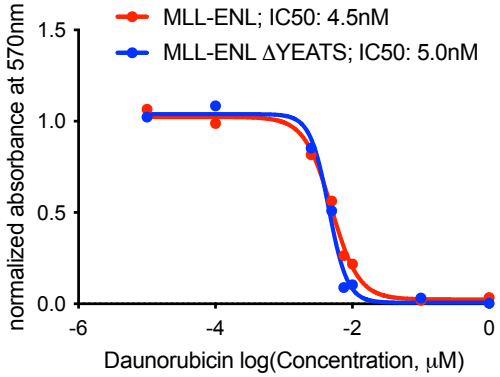
In our study, we characterized the inclusion, importance, and epigenetic reader function of the YEATS domain in MLL-ENL fusion protein and leukemia. There are many questions surrounding MLL-ENL leukemia, wild type ENL and its YEATS domain, and MLL-r AMLs yet to be answered. In this section, I will provide an overview of some ideas that are of interest to us as well as ongoing future directions for this project.

3.2.1 Exploring Secondary Mutations and Their Contributions to MLL-ENL-ΔYEATS Mediated Leukemogenesis

One of the major conclusions from my thesis work is defining the importance of the YEATS domain in the context of MLL-ENL leukemias. We found that MLL-ENLΔYEATS, which lacks the YEATS domain and part of the IDR sequence, fails to

give rise to MLL-ENL leukemias *in vivo*. A subset of MLL-ENL leukemia patients, however, has complete exclusion of the YEATS domain and part of the IDR sequence **(Figure 2-1)**. One future direction is to address this clinical observation concerning disease prognosis, treatment, and molecular characterization. For instance, what is the disease prognosis associated with MLL-ENL patients lacking the YEATS domain vs MLL-ENL patients with fully intact YEATS domain? Are human MLL-ENL leukemias lacking the YEATS domain more or less sensitive to approved chemotherapy drugs? While we lack the relevant clinical data to address these questions, our preliminary data using retrovirally derived mouse cell lines showed that there is no sensitivity difference between MLL-ENL and MLL-ENLΔYEATS cells *in vitro* to daunorubicin (**Figure 3-1**). The question of sensitivity to approved chemotherapy agents between MLL-ENL vs MLL-ENL lacking the YEATS domain should be further addressed using human MLL-ENL cell line models such as HB11;19 and KOPN8. The prognosis and aggressiveness of different MLL-ENL leukemias could be assessed through xenograft experiments involving these two cell lines. Additionally, a longitudinal study tracking the clinical prognosis of MLL-ENL patients with or without the YEATS domain could directly address this hypothesis.

Figure 3-1: Daunorubicin Sensitivity in MLL-ENL and MLL-ENLΔYEATS Cells



Daunorubicin sensitivity (IC50 calculated) in MLL-ENL and MLL-ENLΔYEATS transformed mouse cells as calculated in MTT assay. Briefly, 10,000 cells were seeded and treated with daunorubicin for 24 hours, followed by assessment of cell viability through MTT assay. Absorbances at 570nm were normalized to water-treated control for each cell line.

In my thesis, we showed that human MLL-ENL with an intact YEATS domain (i.e. HB11;19) showed higher sensitivity to SGC-iMLLT (an established YEATS domain inhibitor for ENL and AF9) compared to other AML models including KOPN8, which lacks the YEATS domain. We hypothesized that this increased sensitivity is due to YEATS domain targeting on the HB11;19 MLL-ENL fusion, and that such perturbation would interrupt the fusion protein function. Indeed, we saw varying degrees of decrease in fusion protein and PAF1 localization at a subset of pro-leukemic targets, together with a decrease in key activating epigenetic marks (H3K79me2, H3K9ac, and H3K4me3) in MLL-ENLΔYEATS and the YEATS mutants compared to MLL-ENL at target loci. These data provide a preliminary molecular model on how the YEATS domain's epigenetic reader function drives MLL-ENL fusion protein functions (**Figure 2-15**). At the same time, these results could be further supported and validated using human MLL-ENL cell lines (ex: HB11;19 and KOPN8) and/or through YEATS inhibitor-treated MLL-ENL cell lines.

Our mechanistic data regarding the MLL-ENL fusion and PAF1 recruitment raises some interesting insights. Previously, our lab and others have demonstrated that PAF1 interacts with both MLL at the RD2-CXXC domain and the ENL YEATS domain[123,217]. The interaction between MLL RD2-CXXC and PAF1 is important for binding of the MLL fusion to its genomic targets[215]. We showed that loss of the YEATS domain perturbed MLL-ENL fusion protein and PAF1 localization, which points to a model of perhaps reciprocal stabilization between the PAF1c and MLL fusion proteins at a subset of genomic targets. However, perhaps the most peculiar observation is that even minimal (and non-significant) perturbation in MLL-ENL fusion protein and PAF1 localization, as exemplified in MLL-ENL YEATS point mutants, can produce significant change in the transcription and the epigenetic landscape at a subset of pro-leukemic targets (such as *Eya1*). How does the YEATS epigenetic reader function of MLL-ENL influence the deposition of these marks? Does binding of the YEATS domain to acetylated H3 protect histones from HDACs? Does chromatin accessibility change as a result of perturbing the YEATS domain epigenetic reader function? All of these questions together with others remain yet to be answered. Future experiments assessing change in global histone mark/protein distribution (ex: ChIP-seq) and chromatin accessibility (ex: ATAC-seq) could be done in YEATS genetic mutants and/or pharmaceutically disrupted MLL-ENL cell models in order to address these questions.

Our clinical data show a small subset of MLL-ENL patients lack the YEATS domain. How do patients bearing MLL-ENL fusions similar to our ΔYEATS construct develop leukemias? One hypothesis is through the acquisition of secondary mutations. Some of the most frequently acquired secondary mutations in MLL-r leukemias are the

MAPK pathway members NRAS, KRAS, or BRAF[8,79]. Interestingly, RAS mutations have been associated with an adverse prognosis in adult and pediatric MLL-r AML and infant ALL but not in non-MLL-r AML[79,194]. Exploring this hypothesis, I found that both KOPN8 and HB11;19 have documented secondary mutations, with KRAS G12D in KOPN8 and FLT3 D835H in HB11;19[79,241]. In another study, common secondary mutations such as FLT3 (mutations or ITD) and KRAS are mutated in 36.4% and 27.3% of MLL-ENL AMLs[194], much higher than the percentage of patients lacking a full YEATS domain (15.9%; Figure 2.1). Another way of exploring this hypothesis is through the idea that mutational burden increases over age, so infant and pediatric MLL-ENL leukemias might show a higher percentage of YEATS domain inclusion given the low mutational burden. However, this was not the case in our patient profile as there was no correlation between patient age and YEATS domain inclusion (Figure 2.1E). Perhaps this points to a model where a co-transforming secondary oncogenic mutation is only a selective pressure in MLL-ENL leukemias lacking the YEATS domain. Experimentally, this point could be tested by introducing common secondary mutations in our MLL-ENLΔYEATS transduced cell lines and see how this impacts leukemogenesis *in vivo*. Additionally, a comprehensive mutational screen on MLL-ENL patients would provide more definitive answers.

Another potential explanation for MLL-ENL fusion proteins lacking the YEATS domain is the potential involvement of ENL-MLL reciprocal fusion proteins in these patients. Amongst all MLL fusion partners, only the reverse fusion of MLL-AF4 (AF4-MLL) is more extensively characterized in its leukemogenic potential, although results remain conflicting[27,244]. The reciprocal chromosomal translocation in t(11;19) leukemias

has been documented[201]. The resulting reverse fusion protein products, or lack thereof (spliced fusion in ~50% of MLL-ENL[200]), have not been characterized to my knowledge. How or whether the ENL-MLL reciprocal fusion contribute to MLL-ENL leukemogenesis remains to be elucidated.

3.2.2 Further Exploring YEATS-PAF1 Interaction in MLL-ENL Leukemia

In addition to the YEATS domain's epigenetic reader function, it also interacts with PAF1 of the PAF1c[123,125]. The importance and requirement of the PAF1c in MLL-r AMLs has been previously established by our lab and others[207,217]. We started out asking the question whether the MLL-ENL YEATS domain's ability to interact with PAF1 is important in its leukemogenesis activity. In an attempt to address this question, we used a previously published YEATS mutation ∆21-26 shown to disrupt ENL interaction with PAF1. This mutation phenocopies the YEATS epigenetic reader mutants in the leukemogenesis assay, and in our hands disrupts the YEATS domain's epigenetic reader function but not interaction with PAF1. Therefore, the role and importance of the YEATS-PAF1 interaction in the context of the MLL-ENL fusion remains largely unanswered.

How does studying the YEATS-PAF1 interaction aid in our understanding of MLL-ENL leukemogenesis? We showed that disrupting the YEATS domain's epigenetic reader function in MLL-ENL extended leukemic disease latency, and we observed a further additive disease extension with additional point mutations. In our mouse models, MLL-ENL∆YEATS completely lacked leukemia potential. All these data suggest that additional factors, perhaps the unperturbed YEATS-PAF1 interaction or the ENL IDR (further discussed in the next section), might play a role in MLL-ENL leukemogenesis.

To address this question, we have created MLL-ENL ΔYEATS+IDR to ask whether the YEATS-PAF1 interaction (or perhaps other undiscovered protein-protein interaction by the ENL YEATS domain) contribute to MLL-ENL leukemogenesis. Our lab previously demonstrated the differential roles of PAF1c component CDC73 in MLL-r AMLs vs in hematopoiesis[262]. Additionally, our lab showed that perturbation of MLL-PAF1 interaction using a dominant negative strategy selectively targets MLL-r leukemia but not lin- progenitors[215]. Given the multiple lines of evidence of MLL-r leukemia's dependency on the PAF1c, elucidating the role of the YEATS-PAF1 interaction might provide new insights on targeting the PAF1c in MLL-ENL leukemia.

3.2.3 Exploring the Role of the IDR in MLL-ENL and Other MLL-r Leukemias

The intrinsically disordered region (IDR) of ENL is an unstructured region flanked by the YEATS domain and the AHD. The phenomenon of regulatory protein assembly on chromatin through multivalent and non-stoichiometric interactions has been recently discovered and characterized. Commonly referred as nuclear biocondensate and puncta, these "hubs" of regulatory proteins on the chromatin are thought to associate through a process called liquid-liquid phase separation often mediated by the IDR of transcriptional factors and co-activators. The IDRs of two MLL fusion partners, ENL and AF4, has been shown to drive phase separation and SEC/P-TEFb nuclear biocondensate formation in overexpression models[117,284]. In a subsequent study by Song and colleagues, it was proposed that the increased SEC/P-TEFb nuclear biocondensate formation was driven by ENL oligomerization on chromatin[284]. They showed that the pathogenic oligomerization of ENL on chromatin was caused by changes in the YEATS domain β-sheet conformation caused by YEATS mutations

observed in Wilm's tumor patients[284]. In the same study, it was demonstrated for the first time that ENL IDR's contribution in phase separation is mediated through IDR's regional net charges and serine-rich sequence[284].

While these overexpression studies and characterization of Wilm's tumor YEATS mutations start to shed light on our understanding of the ENL IDR functions, its roles in normal hematopoiesis and pathogenesis remain unstudied. Precisely, how and whether ENL IDR contributes to MLL-ENL leukemogenesis is still unknown. Our MLL-ENLΔYEATS construct lacks part of the ENL IDR and failed to give rise to leukemia *in vivo,* pointing to the hypothesis that the ENL IDR might contribute to MLL-ENL leukemogenesis. Could ENL IDR-mediated phase separation be a mechanism in expanding our understanding of SEC and P-TEFb hijacking in MLL-ENL leukemias? For instance, how does the ENL IDR work in concert with the ENL AHD in SEC complex assembly in amplifying pro-leukemic gene expression? While protein-protein interactions mediated by the AHD has been shown to be important in MLL-ENL leukemias, is an intact and functional IDR domain also required for MLL-ENL fusion protein function? In light of our recent understanding of the ENL IDR and phase separation, these new questions remain to be answered in MLL-ENL leukemias.

3.2.4 Eya1 Regulation: Differences between MLL-ENL and MLL-AF9

One interesting observation from our study is the revelation of differential YEATS domain inclusion in MLL-ENL vs MLL-AF9 fusion proteins. Using the largest curation of MLL-ENL and MLL-AF9 patient breakpoint data to date, we identified that most MLL-ENL retain the YEATS domain and MLL-AF9 fusions almost exclusively lack the YEATS

domain. Our study together with others have begun to parse apart the differences between ENL and AF9, two paralogs previously thought to be interchangeable.

ENL and AF9 share high homology, with sequence homology of ~88% in the YEATS domain, ~80% in the AHD, and with ~74% across the whole protein[42,144,212]. Interestingly, despite the high sequence homology and shared list of protein-protein interactions, these protein domains can behave differently. For example, work from the Shi lab interrogating epigenetic reader functions of ENL and AF9 revealed that the two YEATS domains have differential affinity towards acetylated histone lysines[172,311]. Work from Bushweller's group recently reported the striking differential affinity of the two AHD domains with BCOR and CBX8 while maintaining similar affinity to DOT1L and the SEC scaffold protein AF4[144]. These findings revealed that ENL and AF9 have different biochemical properties.

One observation from MLL-ENL and MLL-AF9 is AHD retention in the fusion proteins. Indeed, the ENL and AF9 AHDs are both required in *ex vivo* colony formation and *in vivo* leukemogenesis. Specifically, ENL AHD showed much higher affinity to BCOR and CBX8 compared to AF9 AHD[144]. Both BCOR and CBX8 have been implicated in gene repression through their participation in canonical and non-canonical PRC1. CBX8 is required in MLL-ENL and MLL-AF9 leukemias, while BCOR has been shown to be required in MLL-AF9 leukemia[266,290]. In particular, the BCOR and MLL-AF9 interaction is critical for MLL-AF9 *in vivo* leukemogenesis[266]. In this study, Schmidt and colleagues showed that an MLL-AF9 AHD mutant perturbing its interaction with BCOR nullifies the expression of *Eya1* but not *Hoxa9* and *Meis1*[266]. The transcriptomic profiling of MLL-AF9 BCOR mutants, together with our *Eya1* results, suggest that the regulation

of a subset of MLL-AF9/MLL-ENL targets may be differentially regulated in comparison to classical MLL-r targets like *Hoxa9* and *Meis1*[266].

Our study pointed out that *Eya1* regulation in MLL-ENL leukemia is regulated by the fusion protein's YEATS epigenetic reader function, while in MLL-AF9 leukemia *Eya1* is regulated by the AF9 AHD-BCOR interaction[266]. Through these observations, several questions specific to the fusion proteins arise. For instance, does perturbing the ENL AHD-BCOR interaction also abolish *Eya1* expression and phenocopy what is seen in MLL-AF9 leukemia? How does the ENL AHD work in concert/opposition with the ENL AHD to regulate *Eya1* expression in MLL-ENL leukemia? Conversely, how does MLL-AF9 maintain *Eya1* in the absence of a YEATS domain in the fusion protein?

EYA1 is a transcriptional factor important in multiple organogenesis pathways[330]. *Eya1*, together with *Eya2*, are both highly expressed in the hematopoietic stem cells compared to the hematopoietic progenitor cells[99,292]. However, Eya1's expression does not vary across different HSC populations[99,292]. Overexpression of *Eya1* is sufficient to immortalize HSPCs *ex vivo*, with Six1 co-expression further augmenting colony formation ability[315]. In lin- bone marrow cells, *Eya1* has been demonstrated to be a direct target of the MLL protein. Interestingly in Menin-deficient LSK cells, the expression of *Eya1* is only partially and non-significantly disrupted[9]. This finding led to the hypothesis that a subset of MLL direct targets is regulated independently from Menin. More research is needed to fully delineate the roles of *Eya1*/*Six1* in MLL-r leukemia. For instance, are *Eya1* and *Six1* essential in MLL-r leukemias, and what is the relationship between *Eya1/Six1* and classical MLL-r targets *Hoxa9/Meis1* in regulating the MLL-r leukemogenesis program? At the same time, perhaps we can

further elucidate the differences between MLL-AF9 and MLL-ENL fusions by studying the *Eya1* locus. Understanding the intricate biochemical and functional differences between the ENL and AF9 protein domains might inform us on personalized treatment designs for MLL-ENL and MLL-AF9 patients and provide insights as to why MLL-ENL and MLL-AF9 fusion proteins differentially retain the YEATS domain.

3.2.5 Wild Type ENL and AF9 in MLL-r and Other AMLs

The difference between ENL and AF9 as MLL fusion partners was briefly described in the previous section. Additionally, the importance of wild type AF9 and ENL was also described in the context of hematopoiesis and AMLs respectively. According to one report, wild type AF9 has been shown to be a critical regulator for HSC maintenance[28]. Our understanding of wild type ENL's roles in hematopoiesis remains limited, with two studies reporting minimal effects on LSK proliferation and differentiation after ENL knockdown[84,311]. Interestingly, these reports identified wild type ENL as a critical factor in several different AML cell lines, while wild type AF9 seemed to be dispensable in AML. In addition, it was revealed the YEATS domain epigenetic reader function plays a critical role in AML's dependence on ENL.

What is the importance of WT ENL and its epigenetic reader function in MLL-ENL leukemia? Are MLL-ENL leukemias sensitive to wild type ENL loss? To our knowledge, no MLL-ENL derived cell lines were used in the previous studies identifying wild type ENL as a critical factor in AMLs. A study from Yokoyama's group addressed this topic through a shRNA approach and reported minimal effect of ENL knockdown on mouse MLL-ENL transformed cells[340]. We analyzed the YEATS inhibitor sensitivity between both mouse (MLL-ENL vs MLL-ENLF59A) and human (HB11;19 and KOPN8) cell lines.

The YEATS inhibitor SGC-iMLLT presumably would only target wild type ENL in KOPN8 and MLL-ENLF59A cells while targeting both wild type ENL and MLL-ENL fusion proteins in HB11;19 and MLL-ENL cells. Our data concluded that while KOPN8 and MLL-ENLF59A showed sensitivity to SGC-iMLLT, HB11;19 and MLL-ENL displayed greater sensitivity to YEATS domain inhibition. All these data together added to our understanding that MLL-ENL leukemias are indeed sensitive to WT ENL loss.

Approximately 50% of t(11;19) translocations happen upstream of the ENL promoter[200]. These translocations are unique and collectively referred to as spliced fusion. They are able to produce MLL-ENL fusion proteins without disrupting wild type ENL regulation presumably[200]. Does this mean that this subgroup of MLL-ENL leukemias produce the same level of wild type ENL protein compared to their untransformed counterpart? If so, is there a functional consequence of wild type ENL dosage base on MLL-ENL's dependence on wild type ENL? Clinically, does this mean that these patients would respond differently to YEATS inhibitors compared to patients with ENL breakpoints located within the first intron?

The two studies by Wan et al. and Erb et al. pioneered our understanding of WT ENL dependency in a broad spectrum of AML models[84,311]. Specifically, a wide panel of MLL-r acute leukemia cell lines were sensitive to ENL loss, including SEMK2 (MLL-AF4; ALL), OCI/AML-2 (MLL-AF6; AML), ML-2 (MLL-AF6; AML), MOLM-13 (MLL-AF9; AML), and NOMO-1 (MLL-AF9; AML)[84,311]. Other non-MLL-r leukemic cell lines such as SKM-1 (WT MLL-AML), U-937 (CALM-AF10; AML) and K562 (BCR-ABL; CML) were also sensitive to WT ENL loss[84,311]. Many lingering questions remain to be explored. For instance, are other common subtypes of AMLs also sensitive to wild type ENL loss?

Indeed, subsequent studies using a new YEATS inhibitor TDI-11055 showed that in addition to MLL-r AMLs, NPM-1 mutant AMLs are also sensitive to WT ENL YEATS inhibition[177]. Given the diverse oncogenic drivers in AMLs, more extensive characterization of different AMLs (such as core-binding factor AMLs, PML-RARA AMLs, CEBPA mutant AMLs, and TP53 mutant/complex karyotype AMLs; **Figure 1-3**) are needed to further our understanding of the role of WT ENL in AMLs.

Mechanistically, why are a broad spectrum of AMLs sensitive to ENL loss? Based on our understanding of MLL-r leukemias, targeting wild type ENL would presumably disrupt SEC and DotCom activities which are important in a broad spectrum of MLL-r AMLs. Two recent mechanistic reports on NPM-1c AMLs revealed that mutant NPM1 hijacks the SEC and MLL-menin complex, through nuclear biocondensate formation, to amplify expression of pro-leukemic targets such as *Hoxa9* and *Meis1*[298,316]. Using this model, we hypothesize that NPM1c leukemias are sensitive to ENL loss because of disruption in RNA pol II-dependent transcriptional regulatory complexes hijacked in NPM1c leukemias. The mechanistic understanding of AMLs' dependence on ENL is needed as we further identify AML subtypes sensitive to ENL loss.

3.2.6 Differential Regulation of MLL-ENL's Targets

The idea that different MLL-r proteins could regulate the same target through different mechanisms was briefly discussed in the previous section. To further expand upon this idea, different MLL-r fusions can drive different levels of expression of the same target. For example, *Meis1* is differentially activated by different MLL-r fusion proteins and its expression correlated with disease latency in mice[321]. Additionally, while

MLL fusion targeting depends on its N-terminal interaction with Menin and LEDGF, ChIP-seq results of different MLL fusion proteins indicate subsets of MLL-r targets are dependent on the fusion partner, pointing to subtle differences among the most common MLL fusion partners in MLL-r leukemogenesis[18,104,156]. Perhaps these differences could partially explain why different MLL-r fusions produce different disease latencies under clinical and experimental settings.

Another overarching question is how the same MLL-r fusion protein regulates different targets through different mechanisms. Discussed briefly above, we and others have shown that perturbating specific protein-protein interactions of MLL-r fusion proteins could have severe consequence on certain targets and not on others. Consistent with this idea, Slany's group has grouped MLL-ENL target genes into two distinct classes based on their dependence of SEC and DOT1L activity[104]. These observations raise the question of why specific target(s) are more or less dependent on particular function(s) of the MLL-r fusion proteins? Is this due to co-regulation by sequence-specific transcriptional factors? Or does a unique epigenetic landscape specific to each of these targets render them differentially sensitive to specific protein complexes? Further understanding of these target-specific mechanisms could build upon the MLL-r leukemogenesis model and aid in the design of personalized therapeutics.

3.2.7 Therapeutically Targeting MLL-r Leukemia

Our study identified the YEATS epigenetic reader on the MLL-ENL fusion as a vulnerability in MLL-ENL leukemias. How do our results tie into the larger picture of treating MLL-r leukemias? Therapeutic developments exploiting MLL-r leukemogenesis

mechanisms have been proposed. Pinometostat, a DOT1L inhibitor that blocks DOT1L enzymatic activity in a dosage dependent manner, only saw modest effects in a clinical trial[288]. Mechanisms of resistance to pinometostat, such as upregulation of efflux pumps, have been proposed in *in vitro* studies[29]. Menin inhibitors represent another example of therapeutics development in MLL-r leukemia. Two menin inhibitors, SNDX 5613 (Revumenib) and KO-539 (Ziftomenib), both target menin-MLL interactions and are currently in phase 1/2 clinical trials[12].

The DOT1L clinical trial results together with the resistance mechanism study point toward the necessity of using multiple agents in treating MLL-r leukemia. This idea has been demonstrated by Armstrong's group, where they showed that dual inhibition of Menin and DOT1L completely dislodged the MLL-AF9 fusion protein from its targets compared to single agent treatments[230]. Our study together with previous work provide strong rationale for YEATS inhibitors development in AMLs, especially those driven by oncogenic MLL-ENL fusion proteins containing the YEATS domain. Perhaps ENL YEATS inhibition offers another option for combinatory therapy for MLL-r AMLs. This idea could be tested in parallel with ongoing clinical trials, especially with the newly published YEATS inhibitor TDI-11055[177]. However, whether combinatory treatments elicit additional cytotoxicity compared to single agent treatment needs to be assessed.

3.2.8 Targeting Epigenetic Readers in AML LSCs

A major conclusion in our paper is that disrupting the YEATS epigenetic reader function in MLL-ENL greatly impacts leukemic stem cell (LSC) frequency. LSCs are a rare leukemic subpopulation believed to be able to give rise to a full-blown leukemia. This small fraction of leukemia cells has been associated with stemness characteristics,

including drug resistance, self-renewal, and quiescence[16,118]. Pioneering work from the Dick lab have established the gold standard for LSC properties in AML, including the ability to engraft and initiate leukemia *in vivo* and demonstrate self-renewal capacity when transplanted to a secondary recipient[23,161]. In this initial work, AML LSCs were designated as leukemia cells with the CD34+/CD38- immunotype, similar to normal HSCs[23]. The definition and characterization of LSCs remain an area of ongoing research, as understanding this subpopulation of leukemic cells could provide profound insights on AML therapy resistance and relapses.

Epigenetic proteins have long been associated with AML LSCs. LSD1, the first discovered histone demethylase for H3K4 and H3K9, has been associated with LSCs in AML[121]. Targeted LSD1 knockdown destroyed MLL-AF9 LSCs. This was demonstrated by the lack of leukemia in secondary transplant assays[121]. Currently, several compounds targeting LSD1 are in phase I/II clinical trials[89]. Some other examples of epigenetic proteins implicated in AML LSCs include DOT1L and the H3K9 methyltransferases SUV39H1 and G9A[44,165,219]. Interestingly, the role of SUV39H1 and G9A in regulating LSCs seem to be opposite, as loss of SUV39H1 and G9A increases and decreases LSC frequency respectively[44,165]. Perhaps, these studies point to the context-specific regulation of H3K9 methylation in driving the LSC program.

Our study identified the ENL epigenetic reading domain in the MLL-ENL fusion as a contributor to LSC frequency. On the other hand, the importance of wild type ENL in several different AMLs has been established. Interestingly, a recent study chemically targeting the ENL YEATS domain in the MV4;11 (MLL-AF4; AML) cell line revealed a loss of LSC gene signature[177]. These results represent a handful of studies linking

epigenetic reader proteins with LSCs in AML (another example being the identification of BRD4 in LSC maintenance[359]). Mechanistically, how does targeting the ENL YEATS domain in WT ENL or MLL-ENL impact LSC in AMLs? Would Eya1 overexpression in MLL-ENL YEATS mutants be enough to rescue the LSC defect? Does WT ENL YEATS inhibition impact a handful of MLL-r targets including Eya1? All of these mechanistic questions remain yet to be answered and would help progress our understanding on how ENL regulates AML LSCs.

3.2.9 Exploring Alternative Splicing in MLL-ENL Leukemia

Approximately 13% of AML patients have mutations in gene associated with spliceosome activity (CS-AML) (**Figure 1-3**)[70]. This group of leukemia is defined as having genetic mutations in regulators of chromatin and/or RNA-splicing machinery [70,225]. Many of these genes are splicing factors (*SF3B1, SRSF2, U2AF1,* and *ZRSR2*), members of the cohesion complex (*STAG2*), and members of chromatin modifying complexes (*ASXL1, BCOR*, and *EZH2*)[70,225]. Interestingly, *MLL*-PTD mutations (briefly described and discussed under **Chapter 1.3.6**) is also grouped under this category[70,225]. In a study done on a cohort of 413 AML patients, it was reported that CS-AML is associated with adverse prognosis[32].

The differences in splice isoform signatures in young vs aged HSPCs and HSPCs vs AML LSCs have already been established[51]. Interestingly, increasing evidence has established the link between epigenetics and splice isoform regulation. For instance, many epigenetic marks including H3K79me2 have been linked with alternative splicing[3,168]. In particular, H3K79me2 is enriched at skipped exons in human embryonic stem cells and well as differentially localized in normal vs cancer cells[3,168].

Given the role of the YEATS domain in regulating several epigenetic marks at a subset of MLL-ENL targets, it will be interesting to investigate whether the YEATS domain epigenetic reader function impacts alternative splicing. One potential analysis to address this question is by using the Multivariate Analysis of Transcript Splicing (rMATS) tool to detect potential alternative splicing events in the transcriptome of MLL-ENLΔYEATS cells vs the transcriptome of MLL-ENL cells[275].

3.3 Final Thoughts and Comments

Imatinib, commercially known as Gleevac, was designed to target the oncogenic BCR-ABL fusion protein after it was revealed that up to 95% of CMLs harbor this fusion protein[81,87]. It dramatically improved the prognosis of CML and demonstrated for the first time that oncogenic fusion proteins can be targeted through small molecule inhibitors[80,81]. Its success marked the milestone for the first personalized treatment not just in CML, but in cancer in general. Throughout the past decades, intensive research efforts on MLL-r leukemias have begun to unveil important leukemogenesis mechanisms in what appeared to be an extremely heterogeneous group of diseases. At the same time, ongoing efforts have yet to yield an FDA-approved personalized treatment in the clinic. Perhaps, the disconnect between bench and bedside mandates further scrutinization and fine combing through our knowledge and assumptions about these leukemias.

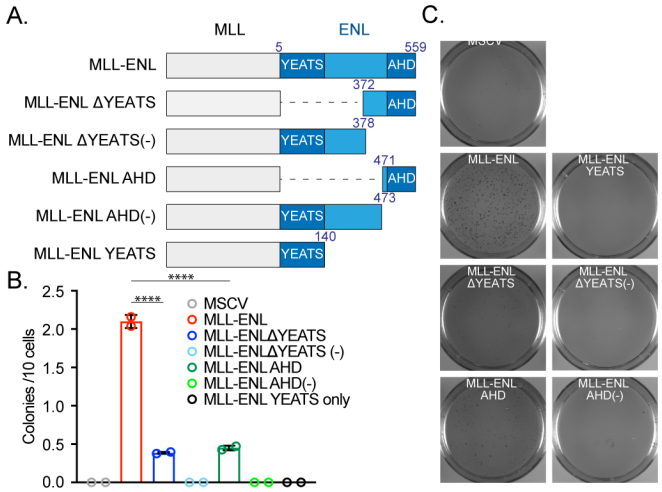
We started this project because we saw a high percentage of YEATS domains included in MLL-ENL breakpoint studies, yet the YEATS domain was believed to be functionally dispensable in MLL-ENL leukemogenesis. We explored this dichotomy in our work and found that not only is the YEATS domain retained in most MLL-ENL

fusion, but it has a functional oncogenic output. As our knowledge on MLL-r leukemia continues to grow and expand, new therapeutic ideas and big picture proposals are constantly tested both at the bench and in the clinic. We hope that this study provides a piece of the puzzle in finetuning our theoretical knowledge of MLL-ENL leukemogenesis mechanisms in patients that may improve the clinical prognosis of this aggressive subtype of acute leukemia.

Appendix 1: MLL-ENL Constructs and Their Transformative Properties *Ex Vivo*

This project was part of an effort to understand the minimal ENL domain necessary for MLL-ENL mediated transformation *ex vivo*. Briefly, several constructs were used in this experiment: MLL-ENL, MLL-ENLΔYEATS, MLL-ENLΔYEATS(-) (complement construct of MLL-ENLΔYEATS), MLL-ENL AHD (removal of the YEATS domain and IDR), MLL-ENL AHD (-) (complement construct of MLL-ENL AHD), and MLL-ENL YEATS (YEATS domain only). In order to narrow down the constructs for our *in vivo* assay (**Figure 2C, F**), I first performed *ex vivo* colony assay using the constructs listed in **Figure A-1** to assess the transformative potential of the different MLL-ENL domains.

Figure A-1: MLL-ENL Constructs and Their *Ex Vivo* Transformative Potential



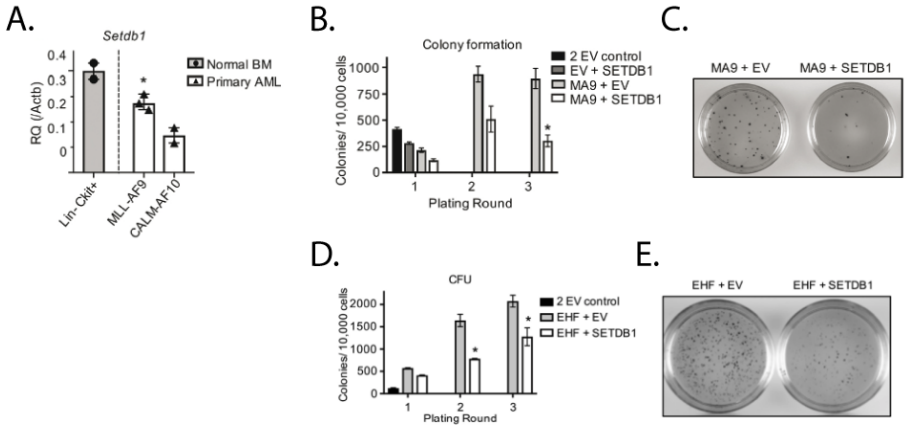
(A) Nomenclature of different MLL-ENL constructs generated to test how the ENL YEATS domain and AHD contribute to ex vivo colony formation assay. (B) Third round colony formation result using the constructs delineated in (A). Colony formation assay protocol was detailed in chapter 2. Figure presents 1 biological experimental result with 2 technical replicates for each construct. One way ANOVA with Dunnett's multiple comparisons test was performed to assess the statistical significance. (C) Representative INT staining of 3rd round colonies from cells transformed with the different constructs.

From this experiment, I made several conclusions. First, the ENL AHD is necessary for MLL-ENL mediated transformation *ex vivo.* This was not surprising to us, as the ENL AHD interacts with DOT1L and DOT1L's role in MLL-r leukemia has been well-established. Second, the YEATS domain and the IDR are not necessary for MLL-ENL mediated *ex vivo* transformation. Our MLL-ENLΔYEATS colony formation assay results showed similar results compared to previous studies. Additionally, MLL-ENL with the minimal ENL AHD is enough for *ex vivo* transformation. However, based on our *in vivo* data (**Figure 2-3C**), I would retrospectively predict that MLL-ENL AHD is not enough for MLL-ENL mediated *in vivo* leukemogenesis. These major conclusions informed us during my thesis work on MLL-ENL constructs to be used in the *in vivo* experiments.

Appendix 2: The Role of the H3K9 Methyltransferase SETDB1 in AML

This study was a collaboration with Dr. James Ropa in the Muntean Lab. In this study, Ropa et al. demonstrated the role of histone H3K9 methyltransferase SETDB1 in acute myeloid leukemias. They demonstrated that SETDB1 expression is downregulated and correlates with disease survival in AML patients. Specifically, SETDB1 overexpression drives AML differentiation and regulates a specific subset of pro-leukemic target genes in AMLs. I contributed to this project by characterizing SETDB1's expression in the hematopoietic stem and progenitor cells (HSPCs) and AMLs and analyzing the effect of SETDB1 overexpression in MLL-r/non-MLL-r AMLs and immortalized HSPCs.

Figure A-2: SETDB1 Expression and its Effect in AML Transformation *Ex Vivo*



(A) SETDB1 expression in Lin-cKit+ HSPCs vs mouse AML cell lines. (B) Effect of SETDB1 overexpression on MLL-AF9 colony formation ex vivo over three rounds of replating. (C) Representative images of INT-stained colonies in (B). (D) Effect of SETDB1 overexpression on E2A-HLF colony formation ex vivo over three rounds of replating. (E) Representative images of INT-stained colonies in (D). Panels in the figure taken from Ropa et al., Haematologica, 2019.[256]

Through these experiments, I found that SETDB1 expression is downregulated in MLL-r AMLs and non-MLL-r AMLs when compared to normal HSPCs (**Figure A-2A**). Additionally, forced SETDB1 expression in cells transformed by the MLL-AF9 or the E2A-HLF oncogenes decreased colony formation *ex vivo.* All of these data together pointed towards a model where SETDB1 exerts its suppressor effect in several different acute leukemias.

Appendix 3: The Role of CDC73 in HSPCs

This study was a collaboration with Dr. Nirmalya Saha in the Muntean Lab. In this study, Saha et al. demonstrated the importance of CDC73 in HSPCs as well as its leukemic-specific functions. Given the importance of PAF1c in MLL-r leukemias, its role in normal hematopoiesis remained unknown. Using a cre-lox system to knockout the PAF1c essential component Cdc73, Saha et al. found that CDC73 is important in the maintenance of the hematopoietic system. Cdc73 knockout is lethal and induces bone marrow failure. Additionally, Cdc73 exhibits a leukemic-specific regulation as opposed to its role in regulating the normal hematopoietic system. All of these findings help inform on whether the PAF1c, an essential component in MLL-r leukemogenesis, is potentially a druggable target. I contributed to this project by helping with the characterization of Cdc73 knockout in secondary leukemia development. My work contributed to the further characterization of Cdc73's requirement in both MLL-AF9 leukemia.

Figure A-3: The Importance of Cdc73 in MLL-AF9 Secondary Leukemia *In Vivo*

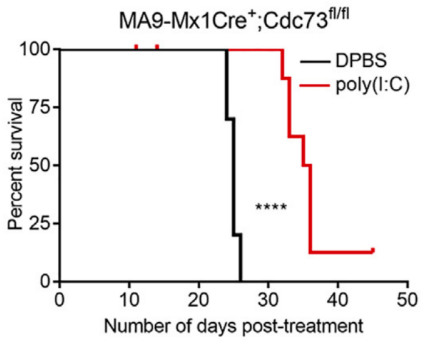


Figure shows a Kaplan-Meier curve of MLL-AF9 secondary leukemia development in mice receiving DPBS or poly(I:C) treatment. Poly(I:C) treatment activates the cre recombinase expression, which in turn excises Cdc73 in the leukemic cells. Figure taken from Saha et al. Stem Cell Reports, 2019.[262]

Appendix 4: Bioinformatics: Differential Expression Analysis

Under this appendix, I have included the list of significant differentially expressed genes identified in our RNA seq experiment. It was originally published in Hu et al. 2023 in the supplementary tables[131]. The methodology is detailed in ***Chapter 2.2.6 RNA-Seq Analysis***. A summary plot of these significant differentially expressed genes is included under the illustration in **Figure 2-10A**. Specifically, **Table A-1** includes all the significantly downregulated genes (total: 533 genes) and **Table A-2** includes all the significantly upregulated genes (total: 290 genes) in MLL-ENLΔYEATS cells compared to the MLL-ENL cells. Genes in **Table A-1** and **Table A-2** are ranked by the degree of differential expression, represented by the values in the log2FoldChange column. Each row under the two tables represents one significantly altered genes, with ENSEMBL ID, gene name, degree of differential expression (log2FoldChange) and the adjusted p values (padj). The result shows that there are significantly upregulated and downregulated genes in ΔYEATS cells, suggesting that YEATS domain deletion leads to global transcriptomic changes.

Table A-1: Downregulated Genes in ΔYEATS Cells

gene_id	external_gene_name	log2FoldChange	padj
ENSMUSG00000038070	Cntln	-12.788513	5.94E-15
ENSMUSG00000025932	Eya1	-12.525928	2.38E-14
ENSMUSG00000063157	Csn2	-11.355736	1.44E-11
ENSMUSG00000067768	Xlr4b	-11.275473	2.14E-11
ENSMUSG00000034780	B3galt1	-10.937278	1.35E-10
ENSMUSG00000033488	Cryzl2	-10.296377	3.18E-09

ENSMUSG00000028218	Fam92a	-10.13239	6.85E-09
ENSMUSG00000063445	Nmral1	-10.075212	1.04E-17
ENSMUSG00000051579	Tceal8	-9.6212648	7.56E-08
ENSMUSG00000024924	Vldlr	-9.6003609	9.65E-08
ENSMUSG00000021708	Rasgrf2	-9.4456448	8.01E-08
ENSMUSG00000043391	2510009E07Rik	-9.3249488	2.90E-07
ENSMUSG00000073125	Xlr3b	-9.2639534	9.20E-22
ENSMUSG00000067924	Rtl8b	-9.2610651	4.12E-07
ENSMUSG00000049881	2810025M15Rik	-9.2177013	6.13E-07
ENSMUSG00000046593	Tmem215	-9.060496	9.15E-07
ENSMUSG00000059852	Kcng2	-8.819842	2.77E-06
ENSMUSG00000033826	Dnah8	-8.7279449	1.82E-06
ENSMUSG00000020396	Nefh	-8.6736018	2.15E-06
ENSMUSG00000039116	Adgrg6	-8.5303324	9.25E-06
ENSMUSG00000022246	Rai14	-8.5184742	5.17E-12
ENSMUSG00000033287	Kctd17	-8.4953453	7.02E-29
ENSMUSG00000035183	Slc24a5	-8.4661841	1.23E-05
ENSMUSG00000110631	Gm42047	-8.4367217	5.92E-174
ENSMUSG00000036528	Ppfibp2	-8.4164186	1.49E-05
ENSMUSG00000076617	Ighm	-8.4049251	1.55E-90
ENSMUSG00000049230	Gm9833	-8.3615334	1.97E-05
ENSMUSG00000042258	Isl1	-8.2997918	2.44E-05
ENSMUSG00000072964	Bhlhb9	-8.2857087	2.61E-05
ENSMUSG00000040594	Ranbp17	-8.2131077	3.89E-05
ENSMUSG00000022756	Slc7a4	-8.1968825	3.79E-05
ENSMUSG00000032327	Stra6	-8.1085156	9.64E-16
ENSMUSG00000070056	Mfhas1	-8.0601478	6.42E-05
ENSMUSG00000032062	2310030G06Rik	-7.977508	2.79E-05
ENSMUSG00000055313	Pgbd1	-7.9468559	0.00010099
ENSMUSG00000067925	Rtl8a	-7.9220328	0.00012751
ENSMUSG00000024597	Slc12a2	-7.7089778	0.00025389
ENSMUSG00000020053	Igf1	-7.6724179	0.00029199
ENSMUSG00000042498	Radx	-7.517355	0.00053672
ENSMUSG00000038764	Ptpn3	-7.501127	0.00056894
ENSMUSG00000032387	Rbpms2	-7.4687256	0.00017287
ENSMUSG00000024747	Aldh1a7	-7.4456845	1.03E-16
ENSMUSG00000091387	Gcnt4	-7.4039416	5.28E-34
ENSMUSG00000042770	Hebp1	-7.4001381	1.56E-20
ENSMUSG00000048332	Lhfp	-7.3054047	2.45E-23

ENSMUSG00000060487	Samd5	-7.2689407	0.00033983
ENSMUSG00000039246	Lyplal1	-7.1862735	0.00044949
ENSMUSG00000039239	Tgfb2	-7.1336882	9.65E-08
ENSMUSG00000031362	Xlr4c	-7.1142361	0.00255729
ENSMUSG00000103585	Pcdhgb4	-7.0983196	0.00263757
ENSMUSG00000098176	Ccdc166	-7.065447	1.63E-07
ENSMUSG00000030207	Fam234b	-7.0574744	1.17E-14
ENSMUSG00000050147	F2rl3	-6.9914172	2.46E-07
ENSMUSG00000078546	Zfp995	-6.9855464	0.00087541
ENSMUSG00000097027	Gm26559	-6.9705886	0.00429048
ENSMUSG00000053310	Nrgn	-6.957207	0.00454228
ENSMUSG00000055980	Irs1	-6.942421	0.00438073
ENSMUSG00000116308	Gm49482	-6.9242538	0.00512631
ENSMUSG00000068130	Zfp442	-6.9136441	0.00471601
ENSMUSG00000073421	H2-Ab1	-6.8313732	7.42E-07
ENSMUSG00000074652	Myh7b	-6.7692538	0.00755346
ENSMUSG00000070565	Rasal2	-6.7422617	1.29E-09
ENSMUSG00000104847	Gm43177	-6.7350584	0.00859686
ENSMUSG00000053749	Gm9920	-6.733346	0.00854486
ENSMUSG00000060923	Acyp2	-6.6497043	0.0109977
ENSMUSG00000041889	Shisa4	-6.6072484	2.97E-06
ENSMUSG00000066363	Serpina3f	-6.6050587	0.01273601
ENSMUSG00000112478	Gm47761	-6.586687	1.52E-18
ENSMUSG00000048616	Nog	-6.5705159	0.01440527
ENSMUSG00000055643	Ubqln5	-6.5628392	0.01444836
ENSMUSG00000038884	Shfl	-6.4415726	0.00504497
ENSMUSG00000041959	S100a10	-6.4408867	0.00508095
ENSMUSG00000021732	Fgf10	-6.3826888	1.67E-28
ENSMUSG00000015355	Cd48	-6.3511089	3.17E-08
ENSMUSG00000026303	Mlph	-6.3465817	6.57E-25
ENSMUSG00000027030	Stk39	-6.3357107	6.12E-11
ENSMUSG00000021719	Rgs7bp	-6.3286797	0.0291085
ENSMUSG00000051550	Zfp579	-6.295868	0.0080452
ENSMUSG00000051212	Gpr183	-6.2679661	0.03610335
ENSMUSG00000034071	Zfp551	-6.2655823	0.03721216
ENSMUSG00000040653	Ppp1r14c	-6.2290339	1.44E-91
ENSMUSG00000027931	Npr1	-6.2126349	9.59E-08
ENSMUSG00000109857	Gm32817	-6.1877114	0.01010096
ENSMUSG00000030443	Zfp583	-6.1588311	0.04841837

ENSMUSG00000022223	Sdr39u1	-6.0747865	0.01381141
ENSMUSG00000029090	Adgra3	-5.9624242	3.41E-47
ENSMUSG00000039316	Rftn1	-5.9469658	3.45E-13
ENSMUSG00000040681	Hmgn1	-5.9155015	7.90E-15
ENSMUSG00000047842	Diras2	-5.9052629	0.02255796
ENSMUSG00000067928	Zfp760	-5.9033102	0.00015943
ENSMUSG00000078921	Tgtp2	-5.7727995	0.03164678
ENSMUSG00000103088	Pcdhgb6	-5.7609784	3.93E-06
ENSMUSG00000015243	Abca1	-5.7552396	2.90E-13
ENSMUSG00000022893	Adamts1	-5.7524324	3.39E-27
ENSMUSG00000041481	Serpina3g	-5.7416244	4.12E-19
ENSMUSG00000032011	Thy1	-5.729081	7.05E-08
ENSMUSG00000037370	Enpp1	-5.7289624	5.79E-10
ENSMUSG00000086537	Gnasas1	-5.7280545	0.00038516
ENSMUSG00000035164	Zc3h12c	-5.7168396	2.23E-19
ENSMUSG00000044254	Pcsk9	-5.7008601	4.99E-31
ENSMUSG00000075270	Pde11a	-5.698612	0.00043277
ENSMUSG00000019478	Rab4a	-5.6914909	0.00040957
ENSMUSG00000079057	Cyp4v3	-5.625848	0.0006348
ENSMUSG00000025491	Ifitm1	-5.6153552	3.10E-87
ENSMUSG00000005474	Myl10	-5.6091838	2.27E-30
ENSMUSG00000110481	Gm45705	-5.548098	0.001046
ENSMUSG00000041272	Tox	-5.5400908	1.13E-30
ENSMUSG00000031642	Sh3rf1	-5.5227176	8.48E-14
ENSMUSG00000045942	BC049762	-5.5204307	3.69E-20
ENSMUSG00000115317	Gm32618	-5.5187693	0.00100505
ENSMUSG00000021792	Prxl2a	-5.5161183	2.54E-36
ENSMUSG00000075410	Prcd	-5.4537862	0.0013903
ENSMUSG00000107728	Gm38910	-5.4059632	2.08E-11
ENSMUSG00000086503	Xist	-5.3891697	1.05E-82
ENSMUSG00000025268	Maged2	-5.3885866	4.18E-14
ENSMUSG00000022037	Clu	-5.3538415	1.95E-84
ENSMUSG00000087396	4933407K13Rik	-5.3491168	2.13E-06
ENSMUSG00000043252	Tmem64	-5.3448968	1.29E-16
ENSMUSG00000029287	Tgfbr3	-5.340387	1.21E-50
ENSMUSG00000039410	Prdm16	-5.3230431	2.47E-38
ENSMUSG00000051147	Nat2	-5.3168348	1.09E-13
ENSMUSG00000040146	Rgl3	-5.2663746	0.00307884
ENSMUSG00000029648	Flt1	-5.247926	1.54E-104

ENSMUSG00000002504	Slc9a3r2	-5.2392989	3.34E-10
ENSMUSG00000112302	Gm48226	-5.232255	4.54E-06
ENSMUSG00000043419	Rnf227	-5.2091649	0.00432565
ENSMUSG00000070803	Cited4	-5.1967145	5.36E-20
ENSMUSG00000037820	Tgm2	-5.1960787	4.47E-103
ENSMUSG00000024074	Crim1	-5.1480958	8.27E-12
ENSMUSG00000050132	Sarm1	-5.1358609	1.91E-08
ENSMUSG00000050108	Bpifc	-5.1188045	1.05E-14
ENSMUSG00000041801	Phlda3	-5.1107935	0.0002535
ENSMUSG00000101939	Gm28438	-5.0975516	1.87E-59
ENSMUSG00000082962	Gm10601	-5.0973104	1.23E-18
ENSMUSG00000020086	H2afy2	-5.0805529	8.16E-24
ENSMUSG00000052415	Tchh	-5.0608265	1.33E-10
ENSMUSG00000106334	Gm43549	-5.0406611	0.01045847
ENSMUSG00000036667	Tcaf1	-5.0338314	3.55E-31
ENSMUSG00000040327	Cul9	-4.9832471	4.62E-25
ENSMUSG00000104806	Gm42566	-4.973964	0.00090434
ENSMUSG00000036368	Rmdn2	-4.966598	4.28E-06
ENSMUSG00000075271	Ttc30a1	-4.9663407	0.01153617
ENSMUSG00000078439	Smim24	-4.9606655	1.05E-20
ENSMUSG00000024033	Rsph1	-4.9593646	0.00069585
ENSMUSG00000006154	Eps8l1	-4.9510414	6.86E-25
ENSMUSG00000097440	Gm6277	-4.9402071	1.73E-32
ENSMUSG00000027238	Frmd5	-4.9191015	4.56E-06
ENSMUSG00000028184	Adgrl2	-4.9150017	7.41E-05
ENSMUSG00000052302	Tbc1d30	-4.8523853	3.02E-24
ENSMUSG00000027201	Myef2	-4.824895	3.18E-39
ENSMUSG00000073968	Trim68	-4.8245337	5.50E-16
ENSMUSG00000043439	Epop	-4.8153246	9.59E-48
ENSMUSG00000035873	Pawr	-4.8057912	5.87E-40
ENSMUSG00000038702	Dsel	-4.7782726	3.37E-21
ENSMUSG00000047654	Tssk6	-4.7516874	2.38E-14
ENSMUSG00000018672	Copz2	-4.7243784	0.00024125
ENSMUSG00000037362	Ccn3	-4.7101011	1.50E-32
ENSMUSG00000032258	Lca5	-4.6789822	0.00055474
ENSMUSG00000021676	Iqgap2	-4.6581762	2.55E-38
ENSMUSG00000004837	Grap	-4.6472402	3.91E-18
ENSMUSG00000028007	Snx7	-4.6249094	9.57E-27
ENSMUSG00000069727	Zfp975	-4.6244585	7.43E-07

ENSMUSG00000015981	Stk32c	-4.6196149	1.68E-29
ENSMUSG00000034055	Phka1	-4.5968903	1.23E-18
ENSMUSG00000048126	Col6a3	-4.5890708	1.49E-54
ENSMUSG00000031875	Cmtm3	-4.5872426	1.63E-26
ENSMUSG00000052776	Oas1a	-4.585499	2.51E-34
ENSMUSG00000034848	Ttc21b	-4.5797119	1.96E-27
ENSMUSG00000061544	Zfp229	-4.5705111	1.38E-06
ENSMUSG00000057530	Ece1	-4.5398758	8.18E-24
ENSMUSG00000026153	Fam135a	-4.5380941	0.00078488
ENSMUSG00000090019	Gimap1	-4.4988438	0.00012794
ENSMUSG00000030525	Chrna7	-4.4765776	0.00021628
ENSMUSG00000073988	Ttpa	-4.4258714	6.96E-06
ENSMUSG00000032737	Inppl1	-4.4019477	9.58E-31
ENSMUSG00000023830	Igf2r	-4.3831413	4.34E-39
ENSMUSG00000032878	Ccdc85a	-4.3673892	2.63E-45
ENSMUSG00000049904	Tmem17	-4.3619223	0.00044189
ENSMUSG00000047454	Gphn	-4.3549827	2.06E-28
ENSMUSG00000079834	Tmlhe	-4.3342639	2.55E-12
ENSMUSG00000076431	Sox4	-4.3274343	9.54E-26
ENSMUSG00000063383	Zfp947	-4.3155975	0.01889857
ENSMUSG00000071723	Gspt2	-4.3061285	0.00013808
ENSMUSG00000079685	Ulbp1	-4.300323	4.00E-62
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ENSMUSG00000078247	Airn	-4.2372631	0.00562528
ENSMUSG00000026994	Galnt3	-4.2097046	5.87E-40
ENSMUSG00000079450	Cldn34c1	-4.1936788	1.49E-05
ENSMUSG00000037101	Ttc29	-4.1865201	1.73E-21
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ENSMUSG00000032718	Mansc1	-4.1798018	1.27E-34
ENSMUSG00000090523	Gypc	-4.1731241	2.58E-39
ENSMUSG00000005672	Kit	-4.1666831	2.27E-44
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ENSMUSG00000004558	Ndrg2	-4.1598376	2.32E-43
ENSMUSG00000020321	Mdh1	-4.1550584	5.05E-60
ENSMUSG00000055322	Tns1	-4.1478582	9.39E-05
ENSMUSG00000025494	Sigirr	-4.1296538	1.59E-08
ENSMUSG00000072244	Trim6	-4.1177092	3.60E-11

ENSMUSG00000036158	Prickle1	-4.0631275	3.90E-11
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ENSMUSG00000044816	D630023F18Rik	-4.0199775	6.38E-08
ENSMUSG00000089857	Zfp882	-4.017213	6.21E-05
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ENSMUSG00000025425	St8sia5	-3.9905814	2.29E-18
ENSMUSG00000026770	Il2ra	-3.9817091	0.00011682
ENSMUSG00000018217	Pmp22	-3.9790099	4.88E-73
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ENSMUSG00000045639	Zfp629	-3.9551963	1.34E-14
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ENSMUSG00000050910	Cdr2l	-3.9133255	4.64E-11
ENSMUSG00000075256	Cerkl	-3.8848433	1.33E-06
ENSMUSG00000021983	Atp8a2	-3.8604464	6.02E-08
ENSMUSG00000037606	Osbpl5	-3.8421116	2.90E-34
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ENSMUSG00000039611	Tmem246	-3.8091408	3.01E-09
ENSMUSG00000078877	Gm14295	-3.8011517	3.72E-08
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ENSMUSG00000047293	Gpr15	-3.752944	0.00070815
ENSMUSG00000097418	Mir155hg	-3.7494202	1.26E-07
ENSMUSG00000085006	BC021767	-3.7355936	1.53E-07
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ENSMUSG00000075370	Igll1	-3.7102907	6.45E-10
ENSMUSG00000030064	Frmd4b	-3.7027243	0.00423437
ENSMUSG00000032369	Plscr1	-3.6901832	1.65E-14
ENSMUSG00000047604	Frat2	-3.6771236	9.48E-61
ENSMUSG00000003746	Man1a	-3.6634269	1.31E-30
ENSMUSG00000026959	Grin1	-3.6613768	0.02508739
ENSMUSG00000117333	Gm16386	-3.6369055	0.02893479
ENSMUSG00000009585	Apobec3	-3.6359406	1.28E-25
ENSMUSG00000047496	Rnf152	-3.6287902	9.23E-12
ENSMUSG00000041119	Pde9a	-3.6156274	0.03164678
ENSMUSG00000004612	Nkg7	-3.6005725	4.57E-07

ENSMUSG00000010175	Prox1	-3.6004343	0.00744869
ENSMUSG00000060594	Layn	-3.5946129	0.0001752
ENSMUSG00000023828	Slc22a3	-3.5914633	3.35E-43
ENSMUSG00000031209	Heph	-3.5760728	2.90E-05
ENSMUSG00000022257	Laptm4b	-3.5723864	9.05E-07
ENSMUSG00000058056	Palld	-3.5540475	0.00316289
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ENSMUSG00000024681	Ms4a3	-3.5204834	3.82E-49
ENSMUSG00000004933	Matk	-3.5047805	1.98E-31
ENSMUSG00000026576	Atp1b1	-3.4904136	4.18E-07
ENSMUSG00000062931	Zfp938	-3.4896959	0.00231405
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ENSMUSG00000024140	Epas1	-3.4442186	2.83E-07
ENSMUSG00000024665	Fads2	-3.4430809	5.29E-52
ENSMUSG00000020319	Wdpcp	-3.4427907	0.00880967
ENSMUSG00000043263	Ifi209	-3.4423426	1.54E-17
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ENSMUSG00000067199	Frat1	-3.4327772	8.86E-31
ENSMUSG00000028926	Cdk14	-3.4251231	0.00096328
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ENSMUSG00000061731	Ext1	-3.4001634	0.00025312
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ENSMUSG00000038781	Stap2	-3.3470768	2.73E-35
ENSMUSG00000024909	Efemp2	-3.345605	0.00360699
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ENSMUSG00000097727	F630040K05Rik	-3.2911361	0.01125826
ENSMUSG00000038007	Acer2	-3.2712593	0.03663434
ENSMUSG00000003585	Sec14l2	-3.2689408	1.08E-07
ENSMUSG00000057596	Trim30d	-3.2663155	0.01062427
ENSMUSG00000047990	C2cd4a	-3.2652048	2.58E-29
ENSMUSG00000047264	Zfp358	-3.2476947	2.68E-06
ENSMUSG00000057123	Gja5	-3.2242987	0.00360699
ENSMUSG00000037095	Lrg1	-3.2223643	2.77E-14
ENSMUSG00000050530	Fam171a1	-3.2128173	9.63E-05
ENSMUSG00000021589	Rhobtb3	-3.2075496	3.52E-13
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ENSMUSG00000075273	Ttc30b	-3.1582723	2.30E-05
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ENSMUSG00000009350	Mpo	-3.1202753	9.11E-41
ENSMUSG00000072572	Slc39a2	-3.1122308	0.00623682
ENSMUSG00000021211	Akr1c12	-3.0830037	4.83E-18
ENSMUSG00000022419	Deptor	-3.0814131	2.58E-28
ENSMUSG00000038668	Lpar1	-3.0811352	5.23E-11
ENSMUSG00000039883	Lrrc17	-3.0615045	0.03657495
ENSMUSG00000034997	Htr2a	-3.0462938	1.67E-05
ENSMUSG00000028950	Tas1r1	-3.0416011	2.97E-05
ENSMUSG00000032890	Rims3	-3.0084169	2.10E-05
ENSMUSG00000019768	Esr1	-2.9835525	8.28E-12
ENSMUSG00000000416	Cttnbp2	-2.9810017	0.0013572
ENSMUSG00000008489	Elavl2	-2.9807154	1.56E-39
ENSMUSG00000030921	Trim30a	-2.9634767	9.45E-14
ENSMUSG00000001053	N4bp3	-2.9505416	9.86E-10
ENSMUSG00000027075	Slc43a1	-2.9467697	0.00835988
ENSMUSG00000053846	Lipg	-2.9419946	4.00E-19
ENSMUSG00000036466	Megf11	-2.9179008	0.00364225
ENSMUSG00000036298	Slc2a13	-2.9098267	0.04977152
ENSMUSG00000042289	Hsd3b7	-2.9001694	1.03E-19
ENSMUSG00000028776	Tinagl1	-2.8921275	9.31E-16
ENSMUSG00000038872	Zfhx3	-2.8671632	8.63E-07
ENSMUSG00000050295	Foxc1	-2.8608619	0.01606194
ENSMUSG00000057321	Usp17ld	-2.8538915	3.19E-05
ENSMUSG00000003992	Ssbp2	-2.839115	1.08E-07
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ENSMUSG00000040957	Cables1	-2.7780244	1.23E-24
ENSMUSG00000021552	Gkap1	-2.7726226	2.67E-08
ENSMUSG00000024063	Lbh	-2.7708464	8.60E-06
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ENSMUSG00000064043	Trerf1	-2.7522614	2.97E-20
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ENSMUSG00000033880	Lgals3bp	-2.7454313	4.69E-09
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ENSMUSG00000023764	Sfi1	-2.6704481	3.24E-15
ENSMUSG00000036053	Fmnl2	-2.6682179	5.98E-09
ENSMUSG00000022995	Enah	-2.6610509	1.28E-27
ENSMUSG00000110737	4930444F02Rik	-2.643207	2.81E-08
ENSMUSG00000089809	Rasgef1b	-2.6265938	8.32E-07
ENSMUSG00000055737	Ghr	-2.6261648	7.38E-20
ENSMUSG00000033684	Qsox1	-2.6200035	2.24E-25
ENSMUSG00000037071	Scd1	-2.6193072	4.45E-25
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ENSMUSG00000089715	Cbx6	-2.5743222	1.27E-13
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ENSMUSG00000022438	Parvb	-2.5406868	2.72E-07
ENSMUSG00000024782	Ak3	-2.5225527	2.59E-09
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ENSMUSG00000032232	Cgnl1	-2.4891698	2.83E-27
ENSMUSG00000035062	Zc4h2	-2.4861848	0.00570326
ENSMUSG00000029490	Mfsd7a	-2.4768567	1.39E-08
ENSMUSG00000064080	Fbln2	-2.476477	0.0004361
ENSMUSG00000038692	Hoxb4	-2.4681638	0.00586314
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ENSMUSG00000040591	1110051M20Rik	-2.3664537	0.00075158
ENSMUSG00000020102	Slc16a7	-2.3628679	0.00016112
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ENSMUSG00000027533	Fabp5	-2.3513103	3.76E-21

ENSMUSG00000040152	Thbs1	-2.3402615	4.30E-12
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ENSMUSG00000073643	Wdfy1	-2.2920393	4.92E-16
ENSMUSG00000035513	Ntng2	-2.2900988	4.65E-09
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ENSMUSG00000021876	Rnase4	-2.2345168	0.00894942
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ENSMUSG00000056973	Ces1d	-2.2176889	1.13E-12
ENSMUSG00000028838	Extl1	-2.2154465	0.00027452
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ENSMUSG00000054720	Lrrc8c	-2.2050884	0.01677839
ENSMUSG00000031613	Hpgd	-2.2042941	6.72E-21
ENSMUSG00000047669	Msl3l2	-2.1945401	1.80E-09
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ENSMUSG00000038147	Cd84	-2.1748353	2.54E-09
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ENSMUSG00000028758	Kif17	-2.1681637	0.01437188
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ENSMUSG00000025466	Fuom	-2.1229507	0.01898624
ENSMUSG00000020186	Csrp2	-2.0850699	2.84E-07
ENSMUSG00000010660	Plcd1	-2.0703757	0.0203326

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ENSMUSG00000043154	Ppp2r3a	-2.0586424	6.51E-12
ENSMUSG00000042104	Uggt2	-2.0512971	0.00227347
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ENSMUSG00000031503	Col4a2	-1.9879336	0.00144018
ENSMUSG00000024053	Emilin2	-1.9744034	6.72E-15
ENSMUSG00000045349	Sh2d5	-1.9691884	1.46E-07
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ENSMUSG00000073060	Zxda	-1.932583	9.55E-05
ENSMUSG00000032554	Trf	-1.9293034	2.03E-10
ENSMUSG00000017176	Nt5c3b	-1.9199204	0.00078488
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ENSMUSG00000026170	Cyp27a1	-1.9062528	0.00372225
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ENSMUSG00000005045	Chd5	-1.77759	0.00129083
ENSMUSG00000056144	Trim34a	-1.773829	0.0095836
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ENSMUSG00000026946	Nmi	-1.7397502	0.00492273
ENSMUSG00000021156	Zmynd11	-1.7342132	1.84E-07
ENSMUSG00000028730	Cfap57	-1.7340471	4.22E-05
ENSMUSG00000034485	Uaca	-1.7327013	3.62E-06
ENSMUSG00000112030	Gm35189	-1.7273217	0.00281997
ENSMUSG00000034957	Cebpa	-1.7270636	3.15E-10
ENSMUSG00000031129	Slc9a9	-1.7255326	0.00662378
ENSMUSG00000034738	Nostrin	-1.7219769	0.00316289
ENSMUSG00000073902	Gm1966	-1.7129285	8.53E-05
ENSMUSG00000035189	Ano4	-1.7063597	0.00024641
ENSMUSG00000055862	Izumo4	-1.7061644	0.02212726
ENSMUSG00000018474	Chd3	-1.7036493	0.00472268
ENSMUSG00000028076	Cd1d1	-1.6992886	1.08E-05
ENSMUSG00000031523	Dlc1	-1.6918883	0.00193673
ENSMUSG00000009376	Met	-1.6869828	0.01042489
ENSMUSG00000022824	Muc13	-1.6835225	2.47E-09
ENSMUSG00000004631	Sgce	-1.6779986	0.01153424
ENSMUSG00000042417	Ccno	-1.6767455	0.000272
ENSMUSG00000030303	Far2	-1.6685055	9.59E-07
ENSMUSG00000047996	Prrg1	-1.6617755	0.0021365
ENSMUSG00000117465	Gm49980	-1.6546222	0.02908106
ENSMUSG00000033713	Foxn3	-1.6322511	1.23E-05
ENSMUSG00000000938	Hoxa10	-1.6311024	1.35E-08
ENSMUSG00000023259	Slc26a6	-1.6306714	0.00256313
ENSMUSG00000031633	Slc25a4	-1.6252973	3.82E-07
ENSMUSG00000042302	Ehbp1	-1.6136243	0.00040957
ENSMUSG00000004707	Ly9	-1.6102769	0.00014667
ENSMUSG00000064179	Tnnt1	-1.6068188	0.01992363
ENSMUSG00000027360	Hdc	-1.6062434	0.01455384
ENSMUSG00000048200	Cracr2b	-1.5893769	0.00072654
ENSMUSG00000022836	Mylk	-1.5845468	5.57E-07
ENSMUSG00000020023	Tmcc3	-1.5828657	0.02629188
ENSMUSG00000032010	Usp2	-1.5660906	0.009921
ENSMUSG00000032418	Me1	-1.5534558	1.43E-05
ENSMUSG00000074355	Gm10676	-1.5459501	0.01237041
ENSMUSG00000032411	Tfdp2	-1.5364919	0.00022689
ENSMUSG00000047037	Nipa1	-1.5355612	0.0065947
ENSMUSG00000049625	Tifab	-1.5283674	5.94E-06
ENSMUSG00000028799	Zfp362	-1.5179024	0.00013527

ENSMUSG00000089872	Rps6kc1	-1.4975171	0.00103578
ENSMUSG00000051098	Mblac2	-1.4964904	0.01433632
ENSMUSG00000024446	Rpp21	-1.4876627	0.03372431
ENSMUSG00000032035	Ets1	-1.4769478	1.93E-05
ENSMUSG00000037697	Ddhd1	-1.4694751	9.85E-05
ENSMUSG00000055493	Epm2a	-1.4628467	0.02312148
ENSMUSG00000102051	Ly6a2	-1.4623389	0.00100505
ENSMUSG00000052212	Cd177	-1.4621341	0.00011718
ENSMUSG00000026879	Gsn	-1.4447029	1.71E-05
ENSMUSG00000039007	Cpq	-1.4388661	0.0035804
ENSMUSG00000035232	Pdk3	-1.4382267	0.0003387
ENSMUSG00000052949	Rnf157	-1.4371497	0.00536432
ENSMUSG00000040548	Tex2	-1.4261452	4.52E-05
ENSMUSG00000031762	Mt2	-1.4248848	0.0070256
ENSMUSG00000073600	Prob1	-1.4239684	0.0185082
ENSMUSG00000034612	Chst11	-1.4198487	0.00021639
ENSMUSG00000024799	Tm7sf2	-1.4180621	0.00078488
ENSMUSG00000029767	Calu	-1.4135942	6.26E-05
ENSMUSG00000028289	Epha7	-1.4089956	0.01125861
ENSMUSG00000073910	Mob3b	-1.4070564	0.00360854
ENSMUSG00000044080	S100a1	-1.4066035	0.00556748
ENSMUSG00000034522	Zfp395	-1.4004751	0.0004985
ENSMUSG00000029094	Afap1	-1.3940649	0.00012557
ENSMUSG00000032064	Dixdc1	-1.3892786	0.00904602
ENSMUSG00000052396	Mogat2	-1.38904	0.00183907
ENSMUSG00000016637	Ift27	-1.3865484	0.01014891
ENSMUSG00000021262	Evl	-1.3734083	0.02777755
ENSMUSG00000020961	Ston2	-1.3695628	0.00703638
ENSMUSG00000032484	Ngp	-1.3678666	0.00207421
ENSMUSG00000026585	Kifap3	-1.3645378	0.01866857
ENSMUSG00000062995	Ica1	-1.3636656	0.00086664
ENSMUSG00000042613	Pbxip1	-1.3601463	0.01433632
ENSMUSG00000035561	Aldh1b1	-1.3555562	0.00798623
ENSMUSG00000053702	Nebl	-1.3550176	0.00010604
ENSMUSG00000003199	Mpnd	-1.3457096	0.010685
ENSMUSG00000039377	Hlx	-1.3403253	0.01662952
ENSMUSG00000047735	Samd9l	-1.3267642	0.03465368
ENSMUSG00000021662	Arhgef28	-1.3172252	0.02980746
ENSMUSG00000048440	Cyp4f16	-1.3096457	0.0055075

ENSMUSG00000027189	Trim44	-1.2995518	0.00100857
ENSMUSG00000068129	Cst7	-1.2750682	0.00618884
ENSMUSG00000029925	Tbxas1	-1.2738377	0.01014891
ENSMUSG00000049295	Zfp219	-1.2608702	0.04063155
ENSMUSG00000032666	1700025G04Rik	-1.2598858	0.02912797
ENSMUSG00000029705	Cux1	-1.2521108	0.01153617
ENSMUSG00000027540	Ptpn1	-1.2508218	0.00129479
ENSMUSG00000041695	Kcnj2	-1.2396073	0.02234306
ENSMUSG00000005043	Sgsh	-1.2308859	0.01810017
ENSMUSG00000010142	Tnfrsf13b	-1.2255935	0.00782313
ENSMUSG00000030805	Stx4a	-1.2121094	0.04673366
ENSMUSG00000045039	Megf8	-1.2086213	0.01520324
ENSMUSG00000030560	Ctsc	-1.1998882	0.03856745
ENSMUSG00000024030	Abcg1	-1.1893881	0.03663434
ENSMUSG00000038210	Hoxa11	-1.1696842	0.01573711
ENSMUSG00000003528	Slc25a1	-1.1654496	0.02262158
ENSMUSG00000024978	Gpam	-1.1603506	0.03691258
ENSMUSG00000027474	Ccm2l	-1.1395432	0.02729051
ENSMUSG00000015937	H2afy	-1.1348168	0.01992363
ENSMUSG00000024014	Pim1	-1.1215066	0.02335213

Table A-2: Upregulated Genes in MLL-ENLΔYEATS Cells

gene_id	external_gene_name	log2FoldChange	padj
ENSMUSG00000012519	Mlkl	1.19422402	0.04279376
ENSMUSG00000040964	Arhgef10l	1.20927698	0.02792646
ENSMUSG00000026568	Mpc2	1.21894673	0.02255796
ENSMUSG00000029238	Clock	1.23654447	0.0426729
ENSMUSG00000040204	Pclaf	1.23895	0.00207421
ENSMUSG00000046245	Pilra	1.24258363	0.00798623
ENSMUSG00000063193	Cd300lb	1.24638155	0.03772533
ENSMUSG00000020659	Cbll1	1.25165014	0.01437188
ENSMUSG00000072825	Cep170b	1.25740987	0.04779694
ENSMUSG00000026437	Cdk18	1.26000074	0.02164036
ENSMUSG00000029915	Clec5a	1.26326285	0.00177761
ENSMUSG00000029426	Scarb2	1.28928985	0.00113129
ENSMUSG00000046805	Mpeg1	1.29987273	0.02777755
ENSMUSG00000001739	Cldn15	1.30762645	0.01570564
ENSMUSG00000042082	Arsb	1.30822759	0.00736859

ENSMUSG00000040511	Pvr	1.31884742	0.01199644
ENSMUSG00000025780	Itih5	1.33157128	0.00530876
ENSMUSG00000038034	Igsf8	1.33363339	0.00462149
ENSMUSG00000031442	Mcf2l	1.33456591	0.01431278
ENSMUSG00000022094	Slc39a14	1.33523204	0.04141428
ENSMUSG00000024098	Twsg1	1.34201215	0.01077679
ENSMUSG00000020654	Adcy3	1.35100045	0.0476161
ENSMUSG00000005413	Hmox1	1.36575984	0.00736033
ENSMUSG00000061436	Hipk2	1.36810134	0.00117587
ENSMUSG00000009630	Ppp2cb	1.37014183	0.00019383
ENSMUSG00000018927	Ccl6	1.38131186	0.00497005
ENSMUSG00000072568	Lratd2	1.3916222	0.00356514
ENSMUSG00000097493	9930014A18Rik	1.40193072	0.03826117
ENSMUSG00000038679	Trps1	1.40990528	0.02490851
ENSMUSG00000019960	Dusp6	1.4156003	6.04E-05
ENSMUSG00000038331	Satb2	1.42088477	0.0002535
ENSMUSG00000054715	Zscan22	1.42638967	0.03750511
ENSMUSG00000029156	Sgcb	1.42642836	0.00503744
ENSMUSG00000022849	Hspbap1	1.44423632	0.01140688
ENSMUSG00000041754	Trem3	1.4528706	0.00143448
ENSMUSG00000043740	B430306N03Rik	1.45417791	0.00538172
ENSMUSG00000063077	Kif1b	1.46255465	7.19E-06
ENSMUSG00000028680	Plk3	1.47709992	0.01816071
ENSMUSG00000050335	Lgals3	1.48059967	2.40E-05
ENSMUSG00000028414	Fktn	1.48082021	0.00414993
ENSMUSG00000040339	Fam102b	1.48331674	0.00048997
ENSMUSG00000023495	Pcbp4	1.49920124	0.03185079
ENSMUSG00000038301	Snx10	1.51220104	0.01376664
ENSMUSG00000028551	Cdkn2c	1.52012015	2.42E-06
ENSMUSG00000033565	Rbfox2	1.52138515	1.07E-05
ENSMUSG00000022026	Olfm4	1.53052413	1.63E-06
ENSMUSG00000054702	Ap1s3	1.5343583	0.00025019
ENSMUSG00000046223	Plaur	1.54935119	2.58E-05
ENSMUSG00000026822	Lcn2	1.55614177	6.38E-05
ENSMUSG00000067916	Zfp991	1.56267479	2.40E-05
ENSMUSG00000038253	Hoxa5	1.56437949	1.07E-05
ENSMUSG00000015944	Castor2	1.57464621	0.00326026
ENSMUSG00000039497	Dse	1.5982838	0.02888998
ENSMUSG00000090124	Ugt1a7c	1.5991733	9.85E-05

ENSMUSG00000036273	Lrrk2	1.60160268	3.48E-05
ENSMUSG00000022479	Vdr	1.6040375	0.03877818
ENSMUSG00000032122	Slc37a2	1.60505414	0.02446884
ENSMUSG00000031749	St3gal2	1.60753137	9.04E-05
ENSMUSG00000020231	Dip2a	1.65827468	0.00867738
ENSMUSG00000019850	Tnfaip3	1.6812624	0.02662657
ENSMUSG00000030122	Ptms	1.69378767	0.01910789
ENSMUSG00000040722	Scamp5	1.698931	0.00093841
ENSMUSG00000025330	Padi4	1.70373482	9.49E-07
ENSMUSG00000027368	Dusp2	1.70422217	0.03802765
ENSMUSG00000032514	Ttc21a	1.70806047	0.01384675
ENSMUSG00000038295	Atg9b	1.71237585	1.37E-07
ENSMUSG00000079644	Gm1110	1.71743374	1.31E-05
ENSMUSG00000023249	Parp3	1.73556188	7.76E-06
ENSMUSG00000072066	6720489N17Rik	1.74296855	0.04148198
ENSMUSG00000021822	Plau	1.75367458	0.01010096
ENSMUSG00000024533	Spire1	1.75630748	4.62E-05
ENSMUSG00000072214	Sept5	1.75886253	1.40E-06
ENSMUSG00000043017	Ptgir	1.77195322	1.08E-07
ENSMUSG00000108060	4921529L05Rik	1.77888647	9.05E-07
ENSMUSG00000004328	Hif3a	1.78232478	5.05E-05
ENSMUSG00000029771	Irf5	1.78272383	0.0042979
ENSMUSG00000028542	Slc6a9	1.78933817	0.00511816
ENSMUSG00000026447	Pik3c2b	1.82949378	0.0145302
ENSMUSG00000044583	Tlr7	1.83435543	0.00356514
ENSMUSG00000052688	Rab7b	1.83469143	0.00113165
ENSMUSG00000030474	Siglece	1.8407423	0.01473621
ENSMUSG00000021556	Golm1	1.87752443	7.61E-09
ENSMUSG00000042286	Stab1	1.877706	3.00E-05
ENSMUSG00000046675	Tmem251	1.89049456	0.01050803
ENSMUSG00000031824	6430548M08Rik	1.90125831	2.21E-05
ENSMUSG00000044827	Tlr1	1.91639886	0.00012516
ENSMUSG00000042367	Gjb3	1.92377601	4.71E-07
ENSMUSG00000050390	C77080	1.96044613	0.00508757
ENSMUSG00000066026	Dhrs3	1.98584086	6.44E-05
ENSMUSG00000044037	Als2cl	2.00266826	0.00140461
ENSMUSG00000040809	Chil3	2.00348262	5.24E-12
ENSMUSG00000023800	Tiam2	2.00458931	0.00443275
ENSMUSG00000033577	Myo6	2.02159694	4.88E-10

ENSMUSG00000084883	Ccdc85c	2.03596477	2.75E-06
ENSMUSG00000047443	Erfe	2.04027689	0.000272
ENSMUSG00000040751	Lat2	2.04420366	6.99E-06
ENSMUSG00000074825	Itpripl1	2.0664571	9.52E-06
ENSMUSG00000069793	Slfn9	2.07004564	1.05E-10
ENSMUSG00000039157	Fam102a	2.1049747	0.02049425
ENSMUSG00000022270	Retreg1	2.14207867	4.13E-13
ENSMUSG00000018459	Slc13a3	2.14512482	0.00129479
ENSMUSG00000056054	S100a8	2.15904769	9.44E-13
ENSMUSG00000021978	Extl3	2.16505389	3.80E-07
ENSMUSG00000052013	Btla	2.17368563	2.26E-12
ENSMUSG00000067336	Bmpr2	2.17469429	3.76E-10
ENSMUSG00000039716	Dock3	2.18015842	0.00012314
ENSMUSG00000062980	Cped1	2.18556724	0.02655221
ENSMUSG00000027199	Gatm	2.19207516	1.61E-15
ENSMUSG00000024659	Anxa1	2.22516891	4.76E-11
ENSMUSG00000039976	Tbc1d16	2.22739168	0.00011459
ENSMUSG00000055148	Klf2	2.23216077	1.37E-08
ENSMUSG00000032724	Abtb2	2.23644914	0.00069585
ENSMUSG00000026315	Serpinb8	2.23715678	5.45E-05
ENSMUSG00000021751	Acox2	2.2411866	0.00040957
ENSMUSG00000027994	Mcub	2.24942748	0.00529738
ENSMUSG00000020230	Prmt2	2.25089826	0.02294379
ENSMUSG00000030170	Wnt5b	2.27777602	0.00562528
ENSMUSG00000042826	Fgf11	2.27804728	0.00082736
ENSMUSG00000056071	S100a9	2.29030559	9.53E-21
ENSMUSG00000030882	Dnhd1	2.30597394	0.00482545
ENSMUSG00000039934	Gsap	2.31837399	0.00026429
ENSMUSG00000026821	Ralgds	2.31888105	8.01E-08
ENSMUSG00000031995	St14	2.3220604	5.47E-11
ENSMUSG00000059657	Stfa2l1	2.32674178	0.03856745
ENSMUSG00000037736	Limch1	2.32834185	0.00305922
ENSMUSG00000018500	Adora2b	2.33494645	0.03130667
ENSMUSG00000025804	Ccr1	2.34964087	1.49E-11
ENSMUSG00000031379	Pir	2.37314198	0.00138041
ENSMUSG00000041420	Meis3	2.38418958	0.01340557
ENSMUSG00000015846	Rxra	2.38471016	1.16E-12
ENSMUSG00000003420	Fcgrt	2.3931937	0.00017983
ENSMUSG00000034659	Tmem109	2.40572048	1.18E-10

ENSMUSG00000022014	Epsti1	2.40741465	0.00102521
ENSMUSG00000033032	Afap1l1	2.42341374	3.70E-20
ENSMUSG00000025006	Sorbs1	2.42436966	0.04534999
ENSMUSG00000069833	Ahnak	2.43423343	0.02212726
ENSMUSG00000025197	Cyp2c23	2.44123392	0.02865414
ENSMUSG00000030657	Xylt1	2.44542489	6.38E-08
ENSMUSG00000047409	Ctdspl	2.46476216	1.90E-06
ENSMUSG00000034765	Dusp5	2.47554732	7.58E-10
ENSMUSG00000023034	Nr4a1	2.48028631	9.85E-05
ENSMUSG00000026177	Slc11a1	2.4811145	0.00085467
ENSMUSG00000032547	Ryk	2.48685871	3.81E-06
ENSMUSG00000053219	Raet1e	2.50139787	0.04461339
ENSMUSG00000040528	Milr1	2.51230507	8.81E-21
ENSMUSG00000031530	Dusp4	2.51618968	0.03008051
ENSMUSG00000026509	Capn2	2.51648351	9.97E-15
ENSMUSG00000018001	Cyth3	2.52070311	2.61E-06
ENSMUSG00000037185	Krt80	2.52913486	3.91E-05
ENSMUSG00000017737	Mmp9	2.54022598	5.03E-05
ENSMUSG00000040451	Sgms1	2.55913083	0.0053473
ENSMUSG00000000440	Pparg	2.56639593	4.60E-08
ENSMUSG00000029924	Slc37a3	2.57695504	0.02212726
ENSMUSG00000038463	Olfml2b	2.59593484	4.32E-20
ENSMUSG00000079419	Ms4a6c	2.60140513	4.04E-06
ENSMUSG00000031377	Bmx	2.65466965	0.00035042
ENSMUSG00000039200	Atf7ip2	2.6672404	0.00022689
ENSMUSG00000026817	Ak1	2.69062066	3.85E-07
ENSMUSG00000020656	Grhl1	2.69233124	0.00522611
ENSMUSG00000107182	Gm43268	2.69324375	0.01101053
ENSMUSG00000049130	C5ar1	2.69888361	2.58E-11
ENSMUSG00000071561	Cstdc5	2.70784388	2.16E-26
ENSMUSG00000079597	Cstdc4	2.70973899	3.07E-21
ENSMUSG00000000058	Cav2	2.73691233	1.14E-12
ENSMUSG00000013089	Etv5	2.78675175	2.87E-23
ENSMUSG00000035451	Foxa1	2.81271778	0.00050184
ENSMUSG00000063245	Zfp993	2.82542959	2.74E-07
ENSMUSG00000056737	Capg	2.89308459	4.40E-24
ENSMUSG00000021127	Zfp36l1	2.89425004	1.06E-15
ENSMUSG00000021250	Fos	2.94263197	4.35E-08
ENSMUSG00000026796	Fam129b	2.9567026	2.03E-23

ENSMUSG00000057315	Arhgap24	2.97583095	1.46E-10
ENSMUSG00000037664	Cdkn1c	2.98096876	2.12E-11
ENSMUSG00000020865	Abcc3	2.98862517	6.67E-08
ENSMUSG00000017756	Slc12a7	3.00206933	2.63E-21
ENSMUSG00000042607	Asb4	3.05727811	0.01972899
ENSMUSG00000063804	Lin28b	3.08834174	8.05E-05
ENSMUSG00000045312	Lhfpl2	3.12949735	8.78E-41
ENSMUSG00000048779	P2ry6	3.13330404	0.03680342
ENSMUSG00000029406	Pitpnm2	3.14421562	4.31E-07
ENSMUSG00000024912	Fosl1	3.14488981	1.86E-08
ENSMUSG00000070348	Ccnd1	3.20460056	1.41E-06
ENSMUSG00000093765	Gm20658	3.21009674	5.93E-05
ENSMUSG00000020017	Hal	3.222502	1.69E-06
ENSMUSG00000026321	Tnfrsf11a	3.25587771	0.0030681
ENSMUSG00000030187	Klra2	3.25636388	8.60E-06
ENSMUSG00000006219	Fblim1	3.29545598	3.56E-05
ENSMUSG00000078452	Raet1d	3.30446306	0.01622774
ENSMUSG00000041324	Inhba	3.30940786	9.33E-16
ENSMUSG00000029304	Spp1	3.33598065	6.80E-48
ENSMUSG00000071562	Stfa1	3.35359901	2.14E-42
ENSMUSG00000052920	Prkg1	3.35785201	0.01153792
ENSMUSG00000017724	Etv4	3.3893277	8.38E-20
ENSMUSG00000039760	Il22ra2	3.39360136	2.88E-40
ENSMUSG00000032204	Aqp9	3.42182852	1.05E-09
ENSMUSG00000074852	Hpse2	3.44601169	4.62E-42
ENSMUSG00000106968	C78283	3.51045214	0.00096711
ENSMUSG00000030431	Tmem238	3.52271224	1.43E-23
ENSMUSG00000020717	Pecam1	3.53537178	5.01E-43
ENSMUSG00000030380	Mzf1	3.57678268	1.20E-29
ENSMUSG00000031026	Trim66	3.58986053	9.33E-16
ENSMUSG00000038732	Mboat1	3.66463575	9.57E-24
ENSMUSG00000036617	Etl4	3.66594795	2.57E-05
ENSMUSG00000069255	Dusp22	3.68842443	0.0010174
ENSMUSG00000034220	Gpc1	3.69062688	3.25E-42
ENSMUSG00000030352	Tspan9	3.73398219	0.00040957
ENSMUSG00000063458	Lrmda	3.76442882	5.65E-12
ENSMUSG00000021796	Bmpr1a	3.78552689	2.50E-37
ENSMUSG00000022123	Scel	3.80043249	0.03576652
ENSMUSG00000029663	Gngt1	3.8914799	0.000644

ENSMUSG00000073418	C4b	3.94700446	0.00131559
ENSMUSG00000050379	Sept6	3.96222285	1.14E-24
ENSMUSG00000001768	Rin2	3.99705824	4.28E-17
ENSMUSG00000072949	Acot1	4.05049841	8.26E-13
ENSMUSG00000118330	Gm30042	4.07531854	5.16E-08
ENSMUSG00000031465	Angpt2	4.0902049	0.00846515
ENSMUSG00000055976	Cldn23	4.0950903	1.82E-06
ENSMUSG00000015053	Gata2	4.11242943	1.40E-18
ENSMUSG00000029082	Bst1	4.1402839	3.54E-12
ENSMUSG00000072812	Ahnak2	4.22161424	0.02702321
ENSMUSG00000114923	Gm49345	4.22525372	0.00131903
ENSMUSG00000045917	Tmem268	4.23714411	1.27E-08
ENSMUSG00000027776	Il12a	4.25610704	3.55E-11
ENSMUSG00000027777	Schip1	4.26836107	2.00E-13
ENSMUSG00000028063	Lmna	4.29036107	4.16E-59
ENSMUSG00000053617	Sh3pxd2a	4.30189353	8.28E-52
ENSMUSG00000029108	Pcdh7	4.39308506	3.94E-44
ENSMUSG00000097216	4932441J04Rik	4.43266634	0.00034005
ENSMUSG00000059810	Rgs3	4.45963444	3.52E-06
ENSMUSG00000054200	Ffar4	4.49067791	0.00672211
ENSMUSG00000024985	Tcf7l2	4.54547697	3.42E-08
ENSMUSG00000024737	Slc15a3	4.56616267	0.00049379
ENSMUSG00000072596	Ear2	4.6841719	0.02990378
ENSMUSG00000052364	B630019K06Rik	4.75669418	0.00015898
ENSMUSG00000025473	Adam8	4.77700575	2.32E-50
ENSMUSG00000036545	Adamts2	4.85641905	1.28E-15
ENSMUSG00000027333	Smox	4.86262906	2.59E-09
ENSMUSG00000045573	Penk	4.94596256	1.70E-92
ENSMUSG00000019312	Grb7	4.99106852	0.00910613
ENSMUSG00000035413	Tmem98	5.08944751	0.00599053
ENSMUSG00000030084	Plxna1	5.15903768	4.10E-20
ENSMUSG00000019866	Crybg1	5.18996791	3.06E-25
ENSMUSG00000022602	Arc	5.20332132	0.00394744
ENSMUSG00000027171	Prrg4	5.20932891	0.00011609
ENSMUSG00000030789	Itgax	5.23224824	0.00010435
ENSMUSG00000096719	Mrgpra2b	5.28107175	1.16E-13
ENSMUSG00000054905	Stfa3	5.4630338	0.00113165
ENSMUSG00000029468	P2rx7	5.58058349	4.78E-11
ENSMUSG00000032281	Acsbg1	5.66742651	6.29E-08

ENSMUSG00000085996	A830012C17Rik	5.67316234	7.58E-10
ENSMUSG00000037419	Endod1	5.88262148	7.62E-51
ENSMUSG00000005547	Cyp2a5	5.97171169	4.66E-07
ENSMUSG00000031799	Tpm4	6.07830309	2.79E-96
ENSMUSG00000054136	Adm2	6.08016015	4.29E-12
ENSMUSG00000115242	Gm49018	6.17070362	0.04144646
ENSMUSG00000029651	Mtus2	6.21873389	0.03736074
ENSMUSG00000052270	Fpr2	6.22294012	0.03586554
ENSMUSG00000104371	Gm37513	6.27031196	0.03117786
ENSMUSG00000102660	Gm38378	6.27031196	0.03117786
ENSMUSG00000108345	4933435G04Rik	6.27233905	0.03111304
ENSMUSG00000055320	Tead1	6.31498565	0.00645084
ENSMUSG00000006344	Ggt5	6.3563144	1.04E-05
ENSMUSG00000059994	Fcrl1	6.36157812	0.02446884
ENSMUSG00000048337	Npy4r	6.3625329	0.02409166
ENSMUSG00000074634	Tmem267	6.47862444	1.99E-14
ENSMUSG00000008845	Cd163	6.57759666	0.01313735
ENSMUSG00000024268	Celf4	6.60478917	2.26E-12
ENSMUSG00000092165	Gm5624	6.68941087	0.00910613
ENSMUSG00000074635	3110070M22Rik	6.72384383	0.00798623
ENSMUSG00000057762	Gm6169	6.88932166	0.00466739
ENSMUSG00000040136	Abcc8	6.98172423	7.27E-48
ENSMUSG00000078087	Rps12l1	7.29542405	0.00108041
ENSMUSG00000095620	Csta2	7.31375543	0.00026172
ENSMUSG00000097694	G730013B05Rik	7.64137399	8.54E-05
ENSMUSG00000113959	Gm46339	7.66647795	0.00027443
ENSMUSG00000094942	Gm3604	7.68343107	7.33E-05
ENSMUSG00000085395	Gm13056	7.68560268	0.0002535
ENSMUSG00000048612	Myof	7.87172889	0.00012386
ENSMUSG00000031146	Plp2	7.9693415	8.07E-94
ENSMUSG00000037709	Fam13a	8.10375577	5.10E-05
ENSMUSG00000069306	Hist1h4m	8.33107995	1.98E-05
ENSMUSG00000072774	Zfp951	9.36443877	2.24E-07
ENSMUSG00000018339	Gpx3	9.78769864	1.17E-16
ENSMUSG00000006445	Epha2	9.80120353	3.21E-08
ENSMUSG00000035299	Mid1	9.98310313	1.30E-08
ENSMUSG00000063171	Rps4l	10.0091559	1.17E-08
ENSMUSG00000036743	Psma8	11.5806739	4.23E-12
ENSMUSG00000093954	Gm16867	12.323796	7.38E-14

ENSMUSG00000095562	Gm21887	13.7106411	2.01E-17
ENSMUSG00000096768	Gm47283	14.0926366	1.81E-18

Appendix 5: Bioinformatics: PCA and Volcano Plot

Under this appendix, I have included the results of a principal component analysis (PCA) and volcano plot from our differential expression analysis. Part of the methodologies for these analyses is detailed in ***Chapter 2.2.6 RNA-Seq Analysis***. Specifically, the PCA plot in **Figure A-4** was generated by first transforming the counts in DESeqDataSet (dds) through the variance stabilizing transformation (VST) method and then plot the result using the plotPCA function within the DESeq2 package. The volcano plot in **Figure A-5** was generated by taking the differential expression analysis results from DESeq2 and using the EnhancedVolcano package[22]. The PCA plot further confirms the identity of our samples as the replicates from each cell line cluster together. The volcano plot provides another summary visualization of our differential expression analysis in addition to the MA plot shown in **Figure 2-10A**.

Figure A-4: Princial Component Analysis of RNA Seq Samples

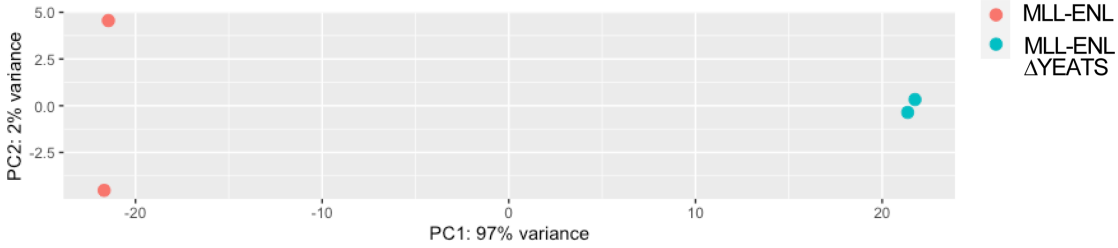

Figure depicts our PCA results from the 4 samples used in our RNA seq experiment. The two technical replicates from each of the two cell lines cluster close with each other, with the majority of the variance explained by the first principal component (PC1; 97%) followed by the second principal component (PC2; 2%).

Figure A-5: Volcano Plot Result of RNA Seq

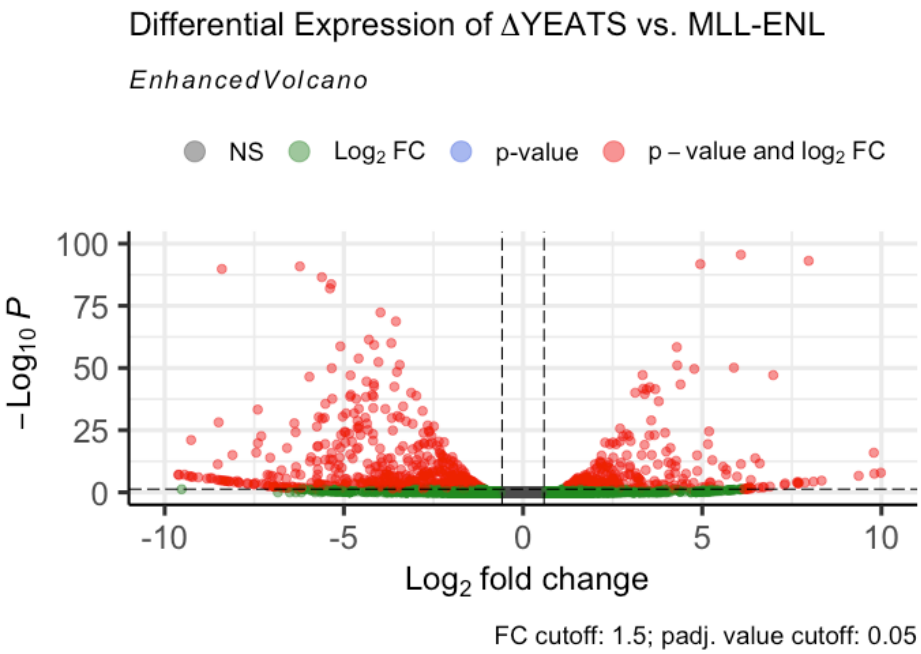

Figure depicts a volcano plot demonstrating our differential expression analysis results. The volcano plot contains information on the fold change and significance of each gene tested but exclude the transcript count information and hence different from the MA plot in Figure 2-10A. Specifically in this plot, we have significance (p-value; adjusted p-value cutoff is >0.05) and transformed fold change (Log2FC, FC cutoff is >1.5). Red dots represent genes that pass both criteria, grey dots represent genes that met neither criteria (NS), green dots represent genes that met the fold change cutoff but not the significance cutoff, and blue dots represent genes that met the significance cutoff but not the fold change cutoff. Significant differentially expressed genes are represented with red dots, with genes downregulated in MLL-ENLΔYEATS cells on the left half (< -log2(1.5)) and genes upregulated in MLL-ENLΔYEATS cells on the right half (> log2(1.5)).

Appendix 6: Bioinformatics: Gene Set Enrichment Analysis

Under this appendix, I have included the list of significant enriched pathways in our GSEA analysis. This data was originally published in Hu et al. 2023 in the supplementary tables[131]. The methodology of this analysis was detailed in ***Chapter 2.2.6 RNA-Seq Analysis***. The GSEA plots for a selected panel of pathways are included under **Figure 2-10C**. Specifically, we used the pathways curated under the MSigDB C2 subcollection CGP: Chemical and Genetic Perturbations for our analysis. **Table A-3** includes all the significantly enriched pathways in MLL-ENL cells (total: 131 pathways) and **Table A-4** includes all the significantly enriched pathways in MLL-ENLΔYEATS cells (total: 68 pathways). In both tables, each row represents one pathway with adjusted p value (padj), normalized enrichment score (NES), and the size of the gene set (size). Pathways are ranked by the NES value. We have identified many pathways enriched or depleted in MLL-ENLΔYEATS cells through the analysis, which links functional meanings with YEATS domain deletion in MLL-ENL leukemia.

Table A-3: Significantly Enriched Pathways in MLL-ENL Cells

pathway	padj	NES	size
MOSERLE_IFNA_RESPONSE	0.000138	2.205	40
DAUER_STAT3_TARGETS_DN	0.000138	2.179	57
KRASNOSELSKAYA_ILF3_TARGETS_UP	0.000137	2.109	69
LEE_AGING_MUSCLE_DN	0.000779	2.047	63
SEITZ_NEOPLASTIC_TRANSFORMATION_BY_8P_DELETION_UP	0.000915	2.027	70
HORTON_SREBF_TARGETS	0.001925	2.025	34
GERHOLD_ADIPOGENESIS_UP	0.001660	2.019	53

BROWNE_INTERFERON_RESPONSIVE_GENES	0.000320	2.013	82
BOYLAN_MULTIPLE_MYELOMA_PCA1_UP	0.000225	2.001	99
DER_IFN_ALPHA_RESPONSE_UP	0.000308	1.996	107
ZHANG_INTERFERON_RESPONSE	0.002310	1.984	28
ZHANG_ANTIVIRAL_RESPONSE_TO_RIBAVIRIN_UP	0.004538	1.977	34
LI_CISPLATIN_RESISTANCE_UP	0.005328	1.962	29
RADAEVA_RESPONSE_TO_IFNA1_UP	0.003299	1.962	60
MOOTHA_GLUCONEOGENESIS	0.004006	1.955	28
PAPASPYRIDONOS_UNSTABLE_ATEROSCLEROTIC_PLAQUE_DN	0.005428	1.944	37
MIKKELSEN_MEF_LCP_WITH_H3K27ME3	0.005836	1.940	38
BOYAULT_LIVER_CANCER_SUBCLASS_G3_DN	0.005202	1.938	41
TING_SILENCED_BY_DICER	0.005978	1.934	38
SCHMIDT_POR_TARGETS_IN_LIMB_BUD_UP	0.005428	1.925	31
WANG_IMMORTALIZED_BY_HOXA9_AND_MEIS1_UP	0.004795	1.921	47
SEMENZA_HIF1_TARGETS	0.011178	1.919	39
COLIN_PILOCYTIC_ASTROCYTOMA_VS_GLIOBLASTOMA_UP	0.010496	1.902	34
TSAI_RESPONSE_TO_RADIATION_THERAPY	0.016338	1.879	36
ICHIBA_GRAFT_VERSUS_HOST_DISEASE_D7_DN	0.015944	1.866	30
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_UP	0.000371	1.860	191
WANG_MLL_TARGETS	0.000137	1.840	235
JOHANSSON_BRAIN_CANCER_EARLY_VS_LATE_DN	0.013084	1.837	56
EBAUER_MYOGENIC_TARGETS_OF_PAX3_FOXO1_FUSION	0.013111	1.831	46
SHIPP_DLBCL_VS_FOLLICULAR_LYMPHOMA_DN	0.013111	1.820	67
HUANG_GATA2_TARGETS_UP	0.000433	1.820	189
FURUKAWA_DUSP6_TARGETS_PCI35_UP	0.013431	1.817	62
DER_IFN_BETA_RESPONSE_UP	0.003244	1.815	138
DACOSTA_ERCC3_ALLELE_XPCS_VS_TTD_DN	0.017798	1.814	43
MCCLUNG_DELTA_FOSB_TARGETS_2WK	0.014015	1.814	47
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_UP	0.001925	1.813	152
BRACHAT_RESPONSE_TO_METHOTREXATE_DN	0.017798	1.807	45
HOSHIDA_LIVER_CANCER_SUBCLASS_S3	0.000137	1.806	264
YOSHIOKA_LIVER_CANCER_EARLY_RECURRENCE_UP	0.019158	1.805	38
KIM_RESPONSE_TO_TSA_AND_DECITABINE_UP	0.004680	1.805	117
POMEROY_MEDULLOBLASTOMA_DESMOPLASIC_VS_CLASSIC_DN	0.014335	1.796	64
JACKSON_DNMT1_TARGETS_DN	0.026891	1.794	29
LE_EGR2_TARGETS_DN	0.004006	1.789	101
IKEDA_MIR30_TARGETS_DN	0.030793	1.788	30
MEISSNER_BRAIN_HCP_WITH_H3_UNMETHYLATED	0.038586	1.787	27
BROWN_MYELOID_CELL_DEVELOPMENT_DN	0.002984	1.784	143

LEE_LIVER_CANCER_E2F1_DN	0.018092	1.773	59
MODY_HIPPOCAMPUS_NEONATAL	0.021373	1.769	46
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_GRANULOCYTE_UP	0.018092	1.762	64
FARMER_BREAST_CANCER_CLUSTER_4	0.033925	1.754	28
LEIN_CHOROID_PLEXUS_MARKERS	0.011530	1.752	78
VERHAAK_AML_WITH_NPM1_MUTATED_DN	0.000459	1.749	252
JAATINEN_HEMATOPOIETIC_STEM_CELL_UP	0.000137	1.746	347
HERNANDEZ_ABERRANT_MITOSIS_BY_DOCETACEL_2NM_UP	0.005978	1.743	101
GILDEA_METASTASIS	0.033404	1.742	38
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_UP	0.002744	1.742	199
KLEIN_TARGETS_OF_BCR_ABL1_FUSION	0.038586	1.741	43
KIM_GERMINAL_CENTER_T_HELPER_UP	0.013431	1.738	84
TSENG_ADIPOGENIC_POTENTIAL_DN	0.039927	1.738	44
SPIRA_SMOKERS_LUNG_CANCER_DN	0.022824	1.738	63
DER_IFN_GAMMA_RESPONSE_UP	0.008785	1.735	95
YANG_BREAST_CANCER_ESR1_LASER_UP	0.038586	1.733	35
NIELSEN_GIST_AND_SYNOVIAL_SARCOMA_UP	0.039215	1.727	35
KIM_LRRC3B_TARGETS	0.039490	1.723	35
SHETH_LIVER_CANCER_VS_TXNIP_LOSS_PAM3	0.019642	1.723	72
BERENJENO_TRANSFORMED_BY_RHOA_DN	0.000137	1.714	464
DELYS_THYROID_CANCER_DN	0.005428	1.713	191
YAMAZAKI_TCEB3_TARGETS_UP	0.004006	1.712	187
BOYLAN_MULTIPLE_MYELOMA_C_D_DN	0.000921	1.709	261
ZHONG_SECRETOME_OF_LUNG_CANCER_AND_MACROPHAGE	0.011756	1.705	97
SENESE_HDAC1_TARGETS_DN	0.001244	1.701	262
ZHENG_GLIOBLASTOMA_PLASTICITY_DN	0.038527	1.699	62
OUELLET_CULTURED_OVARIAN_CANCER_INVASIVE_VS_LMP_DN	0.045303	1.697	38
WONG_ENDMETRIUM_CANCER_DN	0.030095	1.684	66
BRIDEAU_IMPRINTED_GENES	0.045303	1.681	47
STAMBOLSKY_TARGETS_OF_MUTATED_TP53_DN	0.038001	1.678	65
YAO_HOXA10_TARGETS_VIA_PROGESTERONE_UP	0.036456	1.673	68
FOSTER_KDM1A_TARGETS_UP	0.005259	1.669	204
LIU_PROSTATE_CANCER_DN	0.000320	1.660	403
QI_HYPOXIA	0.013240	1.648	158
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_0	0.036456	1.647	73
KINSEY_TARGETS_OF_EWSR1_FLII_FUSION_DN	0.000371	1.645	383
PURBEY_TARGETS_OF_CTBP1_AND_SATB1_UP	0.016338	1.644	121
WANG_SMARCE1_TARGETS_UP	0.003085	1.641	258
RUIZ_TNC_TARGETS_UP	0.011756	1.640	166

LI_INDUCED_T_TO_NATURAL_KILLER_DN	0.019078	1.635	130
HELLEBREKERS_SILENCED_DURING_TUMOR_ANGIOGENESIS	0.033836	1.633	77
HADDAD_T_LYMPHOCYTE_AND_NK_PROGENITOR_UP	0.021933	1.630	123
PETROVA_ENDOTHELIUM_LYMPHATIC_VS_BLOOD_DN	0.014273	1.627	183
HESS_TARGETS_OF_HOXA9_AND_MEIS1_UP	0.037109	1.617	78
PLASARI_TGFB1_TARGETS_10HR_DN	0.005085	1.615	236
CHIANG_LIVER_CANCER_SUBCLASS_CTNNB1_DN	0.029557	1.615	157
SENESE_HDAC2_TARGETS_DN	0.031833	1.614	106
SHETH_LIVER_CANCER_VS_TXNIP_LOSS_PAM4	0.013850	1.613	219
MIYAGAWA_TARGETS_OF_EWSR1_ETS_FUSIONS_DN	0.007122	1.605	213
CHANG_CORE_SERUM_RESPONSE_DN	0.011256	1.600	228
BOQUEST_STEM_CELL_UP	0.012335	1.600	205
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_16D_UP	0.021291	1.595	143
WOOD_EBV_EBNA1_TARGETS_UP	0.034515	1.591	122
JI_RESPONSE_TO_FSH_UP	0.021291	1.578	149
LIM_MAMMARY_LUMINAL_MATURE_DN	0.039927	1.576	103
MEISSNER_NPC_HCP_WITH_H3K4ME3_AND_H3K27ME3	0.047609	1.568	96
MIYAGAWA_TARGETS_OF_EWSR1_ETS_FUSIONS_UP	0.006690	1.567	282
ICHIBA_GRAFT_VERSUS_HOST_DISEASE_D7_UP	0.042712	1.540	128
CHIARADONNA_NEOPLASTIC_TRANSFORMATION_CDC25_UP	0.041866	1.538	145
WINTER_HYPOXIA_METAGENE	0.013890	1.530	252
ONDER_CDH1_TARGETS_2_UP	0.016122	1.528	243
MASSARWEH_TAMOXIFEN_RESISTANCE_DN	0.014842	1.527	240
PICCALUGA_ANGIOIMMUNOBLASTIC_LYMPHOMA_UP	0.022313	1.517	200
SMIRNOV_CIRCULATING_ENDOTHELIOCYTES_IN_CANCER_UP	0.035803	1.511	198
ZHOU_INFLAMMATORY_RESPONSE_FIMA_DN	0.013770	1.505	332
VECCHI_GASTRIC_CANCER_EARLY_DN	0.016325	1.501	268
SMID_BREAST_CANCER_NORMAL_LIKE_UP	0.011824	1.485	418
MEISSNER_NPC_HCP_WITH_H3_UNMETHYLATED	0.012122	1.484	407
EBAUER_TARGETS_OF_PAX3_FOXO1_FUSION_UP	0.039927	1.482	232
LIM_MAMMARY_STEM_CELL_UP	0.013890	1.468	407
MCBRYAN_PUBERTAL_BREAST_3_4WK_UP	0.038887	1.463	216
KAAB_HEART_ATRIUM_VS_VENTRICLE_UP	0.019078	1.462	319
ACEVEDO_LIVER_CANCER_WITH_H3K27ME3_UP	0.030102	1.454	240
ROZANOV_MMP14_TARGETS_UP	0.031921	1.451	296
CHANDRAN_METASTASIS_DN	0.027827	1.446	265
BOQUEST_STEM_CELL_CULTURED_VS_FRESH_UP	0.027827	1.436	425
KUMAR_TARGETS_OF_MLL_AF9_FUSION	0.015944	1.433	416
SMID_BREAST_CANCER_LUMINAL_B_DN	0.026891	1.428	423

HATADA_METHYLATED_IN_LUNG_CANCER_UP	0.026909	1.427	398
RIGGI_EWING_SARCOMA_PROGENITOR_UP	0.019000	1.418	469
LINDGREN_BLADDER_CANCER_CLUSTER_2B	0.027827	1.407	436
PASQUALUCCI_LYMPHOMA_BY_GC_STAGE_UP	0.039356	1.404	324
LINDGREN_BLADDER_CANCER_CLUSTER_1_DN	0.030793	1.402	436
MONNIER_POSTRADIATION_TUMOR_ESCAPE_DN	0.039927	1.389	451
CHARAFE_BREAST_CANCER_LUMINAL_VS_BASAL_DN	0.034156	1.355	476

Table A-4: Significantly Enriched Pathways in MLL-ENLΔYEATS Cells

pathway	padj	NES	size
ZHOU_INFLAMMATORY_RESPONSE_FIMA_UP	0.034389	-1.291	445
FISCHER_DIRECT_P53_TARGETS_META_ANALYSIS	0.039215	-1.292	376
ZHENG_GLIOBLASTOMA_PLASTICITY_UP	0.039927	-1.312	294
FISCHER_G2_M_CELL_CYCLE	0.036399	-1.337	309
MCLACHLAN_DENTAL_CARIES_UP	0.047609	-1.355	238
SMIRNOV_RESPONSE_TO_IR_6HR_UP	0.044522	-1.358	220
ONDER_CDH1_TARGETS_2_DN	0.013612	-1.380	408
BOYAULT_LIVER_CANCER_SUBCLASS_G3_UP	0.021555	-1.382	264
DURCHDEWALD_SKIN_CARCINOGENESIS_DN	0.016325	-1.386	318
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_UP	0.022824	-1.393	259
MANALO_HYPOXIA_DN	0.013249	-1.404	343
GOLDRATH_ANTIGEN_RESPONSE	0.004201	-1.438	414
JAATINEN_HEMATOPOIETIC_STEM_CELL_DN	0.021234	-1.457	221
DAZARD_RESPONSE_TO_UV_SCC_UP	0.036399	-1.458	175
GOTZMANN_EPITHELIAL_TO_MESENCHYMAL_TRANSITION_DN	0.016540	-1.472	250
DELYS_THYROID_CANCER_UP	0.001788	-1.484	445
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_HSC_DN	0.016338	-1.486	217
LI_INDUCED_T_TO_NATURAL_KILLER_UP	0.001788	-1.496	383
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	0.019642	-1.505	191
JAEGER_METASTASIS_DN	0.015194	-1.514	192
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	0.022313	-1.534	176
KIM_WT1_TARGETS_UP	0.005428	-1.539	240
BASSO_CD40_SIGNALING_UP	0.034619	-1.568	109
BARIS_THYROID_CANCER_DN	0.043737	-1.592	78
WANG_ESOPHAGUS_CANCER_VS_NORMAL_DN	0.031921	-1.598	123
HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN	0.029414	-1.616	112
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_BLUE_UP	0.005978	-1.662	154
WILCOX_RESPONSE_TO_PROGESTERONE_DN	0.033399	-1.665	81

PHONG_TNF_RESPONSE_VIA_P38_PARTIAL	0.001588	-1.675	190
ZHANG_TLX_TARGETS_DN	0.016819	-1.680	104
WANG_METHYLATED_IN_BREAST_CANCER	0.035927	-1.700	53
CHIARADONNA_NEOPLASTIC_TRANSFORMATION_KRAS_CDC25_UP	0.039490	-1.705	56
NAGASHIMA_NRG1_SIGNALING_UP	0.001925	-1.710	171
KUNINGER_IGF1_VS_PDGFB_TARGETS_DN	0.042828	-1.718	46
KOBAYASHI_EGFR_SIGNALING_24HR_DN	0.000150	-1.719	306
LIAN_LIPA_TARGETS_3M	0.025231	-1.724	55
GALINDO_IMMUNE_RESPONSE_TO_ENTEROTOXIN	0.013305	-1.728	107
ISHIDA_E2F_TARGETS	0.031558	-1.742	56
IVANOVSKA_MIR106B_TARGETS	0.008314	-1.758	105
VALK_AML_CLUSTER_6	0.049264	-1.762	29
GAL_LEUKEMIC_STEM_CELL_DN	0.000225	-1.765	270
SPIRA_SMOKERS_LUNG_CANCER_UP	0.039490	-1.765	42
KUROZUMI_RESPONSE_TO_ONCOCYTIC_VIRUS	0.021555	-1.783	37
PEPPER_CHRONIC_LYMPHOCYTIC_LEUKEMIA_UP	0.021373	-1.791	50
ZWANG_CLASS_2_TRANSIENTLY_INDUCED_BY_EGF	0.039220	-1.791	34
PHONG_TNF_TARGETS_UP	0.013770	-1.825	59
BROWNE_HCMV_INFECTION_2HR_DN	0.017798	-1.836	56
AMIT_EGF_RESPONSE_240_HELA	0.005978	-1.841	75
SUBTIL_PROGESTIN_TARGETS	0.012122	-1.843	55
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_DN	0.001808	-1.843	115
SHIN_B_CELL_LYMPHOMA_CLUSTER_7	0.015280	-1.873	31
ZHAN_MULTIPLE_MYELOMA_PR_UP	0.013111	-1.882	45
BROWN_MYELOID_CELL_DEVELOPMENT_UP	0.000137	-1.882	207
WILENSKY_RESPONSE_TO_DARAPLADIB	0.018770	-1.892	29
REICHERT_MITOSIS_LIN9_TARGETS	0.013850	-1.893	31
LIAN_LIPA_TARGETS_6M	0.005202	-1.906	65
JOHANSSON_GLIOMAGENESIS_BY_PDGFB_UP	0.004795	-1.922	70
FARMER_BREAST_CANCER_CLUSTER_2	0.007242	-1.955	35
MOLENAAR_TARGETS_OF_CCND1_AND_CDK4_DN	0.007168	-1.959	61
RASHI_NFKB1_TARGETS	0.010072	-1.966	25
GRAHAM_CML_DIVIDING_VS_NORMAL_DIVIDING_UP	0.007242	-1.980	31
VALK_AML_CLUSTER_5	0.005428	-2.009	41
GENTILE_UV_RESPONSE_CLUSTER_D4	0.000326	-2.097	75
FONTAINE_PAPILLARY_THYROID_CARCINOMA_UP	0.000682	-2.097	66
WHITFIELD_CELL_CYCLE_LITERATURE	0.001198	-2.109	48
WANG_BARRETTS_ESOPHAGUS_AND_ESOPHAGUS_CANCER_DN	0.000921	-2.158	31
NAGASHIMA_EGF_SIGNALING_UP	0.000137	-2.261	47

KANG_DOXORUBICIN_RESISTANCE_UP	0.000137	-2.274	65

Appendix 7: Bioinformatics: Gene Ontology Analysis

Under this appendix, I have included the result of our gene ontology (GO) analysis. A portion of this data was originally published in Hu et al. 2023[131]. The methodology of this analysis was detailed in ***Chapter 2.2.6 RNA-Seq Analysis***. A portion of this analysis was included under **Figure 2-10B** with only Molecular Function (MF) GO terms. Specifically in **Figure A-6**, GO terms from three aspects are presented: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). GO terms under each aspect were ranked by adjusted p values and only the top 5 most significant GO terms are presented in figure. Similar to our GSEA results, our GO analysis links functional meanings with YEATS domain deletion in MLL-ENL leukemia.

Figure A-6: Gene Ontology (GO) Analysis of the BP, CC and MF Aspects

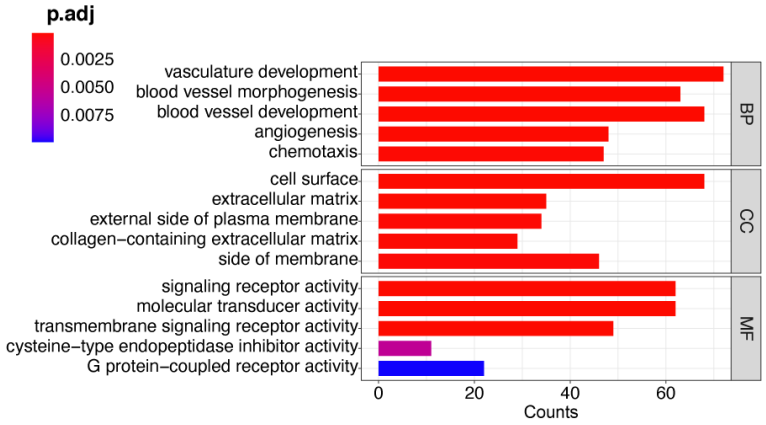

Figure illustrates the results of our GO analysis from the Biological Process (BP), Cellular Components (CC) and Molecular Function (MF) aspects. Adjusted p values (p.adj) were presented through the color gradient and the counts information represents the number of overlapped genes between the differentially expressed genes and the genes under each GO term.

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