Elucidating the proteomic interactions of the mixed lineage leukemia mutant MLL-PTD

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Abstract

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with a poor prognosis. A mutation in the mixed lineage leukemia 1 (*MLL1*) gene that results in partial tandem duplication (PTD) of the N-terminal region is found in 5-10% of cytogenetically normal AML. MLL-PTD is a gain of function mutation and causes increased self-renewal and proliferation of hematopoietic stem and progenitor cells. However, the mechanism through which MLL-PTD induces leukemic transformation is currently unknown. We aimed to identify cofactors that differentially interact with MLL-PTD versus MLL-WT such that inhibitors of these interactions could be tested as a therapeutic strategy for AML. In order to facilitate a comparative study between the wild type and mutant MLL, we transfected HEK293T cells with FLAG and HA-tagged MLL-WT or MLL-PTD. Expression of the tagged MLL was validated by western blot and RT-qPCR. We performed mass spectrometry analysis on samples from both a FLAG and HA immunoprecipitation experiment and identified candidate proteins for therapeutic intervention. Inhibitors of two of these interactions, the lysine acetyltransferase KAT2A and the oncogenic co-factor MEN1, selectively kill leukemic cells with MLL rearrangements. The MLL-PTD cell line EOL-1 is the most sensitive to these small molecule inhibitors. Finally, we show, for the first time, evidence suggesting MLL-PTD retains its ability to undergo proper cleavage in the cytoplasm and interact with WRAD complex members. Therefore, we have uncovered some of the key, previously unknown, protein partners of the leukemogenic protein MLL-PTD.

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AML	Acute Myeloid Leukemia
ALL	Acute Lymphoblastic Leukemia
ASH2L	Absent, Small, or Homeotic Discs Protein 2 Like
ChIP	Chromatin Immunoprecipitation
CML	Chronic Myeloid Leukemia
CN	Cytogenetically Normal
COMPASS	COMplex of Proteins ASsociated with Set1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DOT1L	Disrupter of Telomeric Silencing Type 1-Like
DPY30	Dumpy Wings 30
DTT	Dithiothreitol
FLT3	Fms Related Tyrosine Kinase 3
FP	Fusion Protein
GCN5	General Control Nonderepressible 5
GO	Gene Ontology
H3K4	Histone 3, Lysine 4
HA-tag	Human Influenza Hemagglutinin Tag
HOX	Homeobox
HPLC	High Performance Liquid Chromatography
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
IC50	Half Maximal Inhibitory Concentration
IF	Immunofluorescence
IMDM	Iscove's Modified Dulbecco's Medium
INF	Infinite
IP	Immunoprecipitation
KAT2A	Lysine Acetyltransferase 2A
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMT2A	Lysine Methyltransferase 2A
LC-MS/MS	Liquid Chromatography-Mass Spectrometry (Tandem)
LEDGF	Lens Epithelium Derived Growth Factor
LFQ	Label-Free Quantification
MBM	Menin Binding Motif
MDS	Myelodysplastic Syndrome
MEN1	Multiple Endocrine Neoplasia Type 1

MLL	Mixed Lineage Leukemia
MLL^C	MLL C-terminus
MLL^N	MLL N-terminus
MRD	Minimal Residual Disease
NCS	Newborn Calf Serum
OHRI	Ottawa Hospital Research Institute
PBS	Phosphate Buffer Saline
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PHD	Plant Homeodomain
PIC	Protease Inhibitor Cocktail
PTD	Partial Tandem Duplication
PWWP	Pro-Trp-Trp-Pro Domain
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RBBP5	Retinoblastoma Binding Protein 5
RLSC	Robust LOESS Signal Correction
mRNA	Messenger Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
s-AML	Secondary AML
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STAGA	SPT3-TAFII31-GCN5-L Acetyltransferase
SET	Su(var)3-9, Enhancer-of-Zeste and Trithorax
t-AML	Therapy Related AML
TrxG	Trithorax Group
WDR5	WD-40 Repeat Protein 5
WRAD	WDR5, RBBP5, ASH2L and DPY30 Complex
WT	Wild Type

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1. Introduction

1.1 Hematopoiesis

Hematopoiesis refers to the formation of the cellular components of the blood. In humans, the first large nucleated blood cells arise in the yolk sac around day 18 in order to supply the rapidly growing embryo with oxygen (Kelemen et al., 1979). This primitive hematopoiesis gives way to definitive hematopoiesis when a population of stem cells propagate in the intraembryonic aorta-gonads-mesonephros (AGM). During development, the primary site of hematopoiesis shifts from the AGM to the liver and spleen. Shortly before birth, the primary source of definitive hematopoietic cells is a specialized niche residing in the bone marrow (Mikkola and Orkin, 2006). The bone marrow of the central skeleton remains the primary site of hematopoiesis during human adult life.

Approximately 140 trillion blood cells are produced each year, making it one of the most dynamic systems in the body (Dancey et al., 1976). A pool of hematopoietic stem cells (HSC) are necessary to replenish the short-lived cells of the blood. HSCs retain both the long-term ability to self-renew and the ability to differentiate into all blood cell lineages (Becker et al., 1963; Siminovitch et al., 1963; Weissman and Shizuru, 2008). Hematopoiesis is a hierarchical process with the HSC at the apex. They undergo a successive loss of individual lineage potentials and become restricted to multi-, bi- and finally unipotent progenitors committed to only one cell fate. Each ordered series of lineage restrictions occurs at a binary branch point, thereby, resulting in a tree-like differentiation/commitment hierarchy. This is referred to as the ‘classical’ model of

hematopoiesis (Akashi et al., 2000; Kondo et al., 1997; Figure 1A). HSCs and hematopoietic progenitors are commonly defined by their ability to express the cell surface marker CD34 (Baum et al., 1992). HSCs initially restrict their ability to self-renew and become multipotent progenitors (MPP). The first major lineage split in the hematopoietic tree is between the lymphoid and the myeloid lineages. Myeloid cells include erythrocytes which transport oxygen and carbon dioxide, megakaryocytes which produce the platelets responsible for blood clotting and granulocytes which are involved in innate immune defence. The lymphoid lineage includes the immune cells of the acquired system, which includes B and T cells, in addition to natural killer cells.

Hematopoietic stem and progenitor cell populations have been typically defined by their ability to produce cell lineages through both *in vitro* and *in vivo* experiments as well as immunophenotypically by the expression of specific cell surface markers. However, these methods have limitations. Immunophenotypically defined populations are rarely homogeneous and cut-off values can be somewhat arbitrary. Furthermore, disturbances such as stress, ageing and inflammation can cause changes in marker expression (Kovtonyuk et al., 2016). Recent technological advancements which provide resolution at the single cell level, such as single cell RNA-seq and differentiation studies, have sought to provide increased resolution of HSC lineage progression. Over the last eight years, there have been many seminal papers that have revised the classical model of hematopoiesis (Figure 1A). HSCs are revealed to be a much more heterogeneous population than previously described and are capable of lineage biases (Carrelha et al., 2018; Sanjuan-Pla et al., 2013; Yamamoto et al., 2013). In contrast to the classical

model, lineage restriction is shown to occur much earlier, such as in the MPP or even in the HSC compartments, although, this observation remains highly controversial. (Karamitros et al., 2018; Mercier and Scadden, 2015; Notta et al., 2016; Paul et al., 2015; Perié et al., 2015; Velten et al., 2017). Populations such as CMPs already display uni-lineage transcriptional profiles (Figure 1B). Moreover, most recent evidence suggests that stem and progenitor cells are no longer discreet populations but rather acquire lineage commitment through a continuous and gradual series of changes to their transcriptional profiles (Macaulay et al., 2016; Nestorowa et al., 2016; Pina et al., 2012; Velten et al., 2017). Progenitor populations are therefore considered a transitory state and not discreet cell types. Following a Waddington-like model, each progenitor lineage is separated by a "hill" (Waddington, 1957). Initially, these hills are small and can easily be overcome allowing for flexibility to become other cells of the blood and accommodate changing conditions. However, each hill rises higher and higher to mirror the loss of multilineage potential making lineage switching nearly impossible (Figure 1C) (Haas et al., 2018). All told, our understanding of the hematopoietic hierarchy is undergoing constant revision with much work still required to address controversies in the field. To facilitate the ease of discussion for this thesis, we will be using the classical model of hematopoiesis to explain disease progression, in particular, to define cells of the myeloid lineage (Figure 1A).

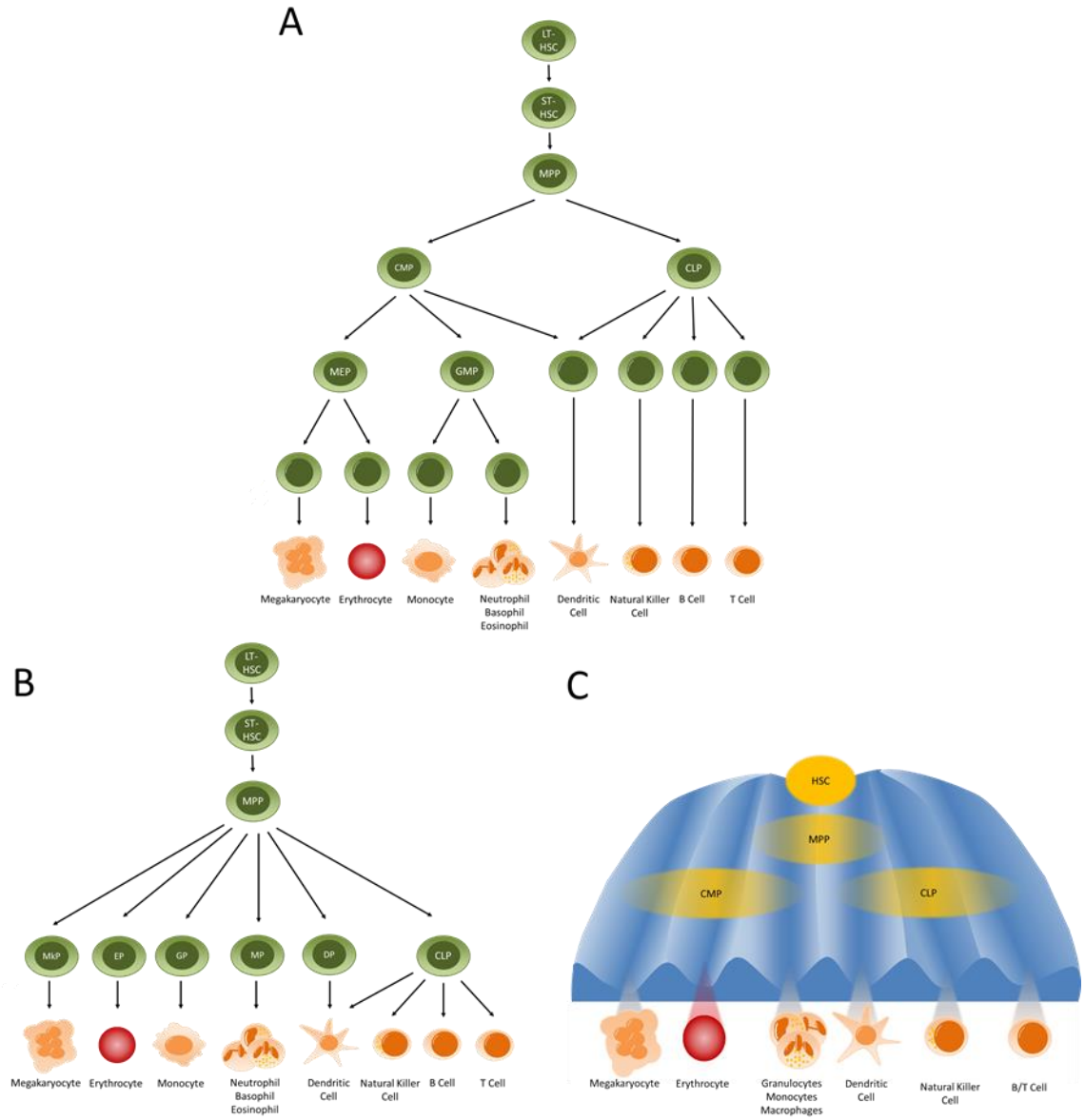

Figure 1. Models of Hematopoiesis. The classical model of hematopoiesis (A) shows a homogeneous population of hematopoietic stem cells (HSC) which lose their ability to self renew to become multipotent progenitors (MPP). The first binary branchpoint produces common myeloid (CMP) and common lymphoid progenitors (CLP) capable of further differentiation to bipotent (megakaryocyte-erythrocyte progenitor MEP; granulocyte-monocyte progenitor GMP) before becoming unipotent progenitors capable of forming the cells of the blood. The early split model (B) shows early lineage separation directly resulting in unipotent progenitors. Recent research using single cell transcriptomics has produced a Waddington-like model (C) whereby cells undergo a continuum of differentiation and gradually acquire their lineage potentials. This model postulates that there are no discreet progenitors. Adapted from (Haas et al., 2018).

1.2 Acute Myeloid Leukemia

Leukemia is a neoplasm arising from hematopoietic dysregulation which results in blocked differentiation of progenitor cells and an accumulation of these cells in the bone marrow (Fialkow et al., 1981). Leukemia can broadly be classified into four categories based on the hematopoietic lineages affected (lymphoid or myeloid) and the rapidity of disease progression (acute or chronic). Acute myeloid leukemia (AML) is characterized by abnormal proliferation and maturation of hematopoietic cells of the myeloid lineage and is the most common acute leukemia that affects adults (Oliver et al., 2014). The incidence of the disease increases exponentially with age with a median onset age of 67 (American Cancer Society, 2014). AML is a genetically heterogeneous disease with multiple morphological subtypes and distinct cytogenetic associations (Lowenberg and Burnett, 2005). AML is commonly classified using one of two systems. The French-American-British (FAB) classification system was established in 1976 and divides AML into eight groups (M0-7) based on morphology and stage of maturation of the leukemic cells (Bennett et al., 1976, 1985b, 1985a, 1991). In 2001, the World Health Organization created a new system of classification, which was last updated in 2016, and has essentially modernized and replaced the FAB system. It classifies AML into six categories based on morphology but also takes into account recent research on clinical manifestations, genetics and immunophenotyping (Arber et al., 2016; Swerdlow et al., 2016).

AML patients suffer from anemia, frequent infections, fever, fatigue and easy bruising. Symptoms are caused by both bone marrow failure, as cancerous cell accumulation

prevents the proliferation of healthy cells, as well as the infiltration of malignant cells to other organs such as the liver, spleen, lymph nodes, skin and brain (Hoffbrand and Moss, 2011). Most AML patients are currently treated using one or more rounds of induction chemotherapy, primarily using cytarabine and an anthracycline (daunorubicin, idarubicin or mitoxantrone). These drugs are given simultaneously and continuously for 7 and 3 days, respectively, in order to achieve clinical remission (Crowther et al., 1970; Dohner et al., 2017). Because these drugs target any rapidly proliferating cells, the treatments are poorly tolerated. Patients with comorbidities, especially those with cardiovascular complications or who are elderly, are often treated with only low doses of chemotherapy with the goal of prolonging survival but not curing the disease. Even those patients who achieve complete remission (CR), defined as less than 5% of leukemic blasts in the bone marrow, will need to continue consolidation chemotherapy in order to remove any residual disease. While 58% of patients enter remission with standard chemotherapy, an alarmingly high 69% will relapse (Verma et al., 2010). Consolidation can include additional chemotherapy or an allogeneic hematopoietic stem cell transplant (allo-HSCT) (Dohner et al., 2017). However, allo-HSCT is also incredibly taxing on the body, as the person's own immune system is destroyed, and there is a high risk of complications including infection and graft rejection. This standard course of treatment has remained largely unchanged over the last 40 years. The current five-year survival rate is only ~20-25% which suggests that additional therapeutic interventions are needed for AML patients (American Cancer Society, 2018; Oliver et al., 2014).

Over the last decade, there have been numerous advancements allowing for the discovery of new antileukemic drugs. AML is entering the age of personalized medicine whereby specific drugs will target each patient's specific mutational profile and risk classification. For example, clinical trials are underway to evaluate inhibitors of common AML mutations FLT3 and IDH1 (De Kouchkovsky and Abdul-Hay, 2016). In addition, the standard of care now includes treating elderly patients who are ineligible for intensive chemotherapy with the hypomethylating agents decitabine and azacitidine (Dohner et al., 2017).

AML is primarily thought to follow the "two-hit hypothesis" of disease development. In this model, one mutation from both class I and class II are required for leukemogenesis (Gilliland and Griffin, 2002). Class I mutations enhance the proliferation of hematopoietic progenitor cells by activating signalling pathways. Genes involved in these pathways include *FLT3*, *KIT*, *NRAS*, *RAS-MAPK*, *PI3K* and *JAK/STAT*. Class II mutations, which include *NPM1*, *RUNX1* and *CEBPA*, usually initiate the onset of AML by blocking myeloid differentiation and altering the transcription of key targets (Gilliland, 2001). In addition, a new class of mutations has emerged which are involved in epigenetic regulation. Class III mutations are capable of misregulating both differentiation and proliferation. These mutations affect many epigenetic regulators such as MLL, DNMT2A, TET2, ASXL1 and EZH2 (Shih et al., 2012). Therefore, the onset of AML is dependent on the interaction of various mutations.

1.3 Epigenetic Regulation

Each cell in the human body contains approximately two meters of DNA (Emanuel et al., 2009). In order to compact this length into the size of a 10-100μM eukaryotic cell, DNA winds tightly around a protein core. The basic repeating unit, called a nucleosome, is composed of ~146bp of DNA wrapped around a histone octamer normally composed of two subunits each of H2A, H2B, H3 and H4 (Luger et al., 1997). Furthermore, each cell in the body possesses largely the same template DNA. Despite this, cells in multicellular organisms have a plethora of lineages and identities. Therefore, each cell type requires a level of heritable regulation which can alter the control and expression of various genes, depending on the needs and function of the individual cell, without causing changes to the DNA itself. This level of regulation is termed epigenetics which uses the Greek prefix 'epi' to mean 'above'. Each cell has a unique epigenome which is the sum of its histone modifications and DNA methylation. The epigenome is regulated by writers which deposit the marks, erasers which remove them and readers which can bind to the modifications. This regulation transforms DNA into silenced, poised and active domains (Allis and Jenuwein, 2016).

The tails of histone proteins undergo post-translational modifications including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ribosylation of various lysine, arginine, serine and threonine residues. The sum of these modifications is called the histone code and together they regulate regions of euchromatic or heterochromatic states. Heterochromatin refers to tightly compacted DNA associated with silenced gene expression. It can be further subdivided into constitutive

heterochromatin, which is permanently silenced, and facultative heterochromatin, where genes are rarely expressed. In contrast, euchromatin refers to decondensed chromatin which has the potential to be actively transcribed (Allis and Jenuwein, 2016).

Histone post-translational modifications can have either activating or repressive effects depending on the type and location of the modification. For example, methylation at the lysine 4 residue of histone H3 (H3K4) is activating while methylation on the same histone at lysine 27 is considered repressive. Histone modifications are generally thought to alter genetic regulation in one of three ways. The first is that they alter the structure of chromatin itself, secondly by disrupting the binding of proteins associated with chromatin or histones and third is through recruiting the binding of chromatin reader proteins (Allis and Jenuwein, 2016). Because of their ability to alter gene expression, it is no surprise that epigenetic factors are often the driver mutations of cancers (Dawson and Kouzarides, 2012).

1.4 Mixed Lineage Leukemia 1 (MLL1)

The ability of epigenetic modifications to cause heritable changes to mammalian gene expression has led to an important area of research. Some of the first identified protein complexes capable of such changes to gene regulation are the antagonistic polycomb (PcG) and trithorax protein groups (TrxG). These proteins were first discovered in Drosophila and were deemed critical to the maintenance, but not initiation, of *hox* gene expression (Lewis, 1978). They are highly conserved and are present in unicellular eukaryotic organisms, plants and fungi (Schuettengruber et al., 2017). Since their

discovery, these two heterogeneous groups of proteins have been found to form multiple complexes. This allows them to regulate a number of different processes, from the cell cycle to embryonic development and X chromosome inactivation (Schuettengruber et al., 2011).

The mixed lineage leukemia (*MLL*) gene is a trithorax homologue identified in humans (Ziemin-van der Poel et al., 1991). MLL maintains the expression of target genes through the addition of 'activating' chromatin modifications, specifically, mono-, di- and trimethylation of lysine 4 on histone 3's tail (Shinsky et al., 2015; Wang et al., 2009). These covalent modifications are required for the epigenetic regulation of many important cellular processes (Meeks and Shilatifard, 2017). There are three pairs of H3K4 methyltransferase proteins in mammals: SET1 A/B, MLL1/2 and MLL3/4. SET1A and B maintain global levels of H3K4 methylation, with a preference for trimethylation, while the MLL proteins have gene specificity and exhibit mostly mono- and dimethylation activities (Shinsky et al., 2015; Wu et al., 2008). MLL3/4 binds enhancers and promotes their activation through both H3K4 monomethylation and the recruitment of the acetyltransferase p300 (Dorighi et al., 2017; Hu et al., 2013; Lee et al., 2013; Wang et al., 2016). MLL1 and MLL2 share much homology. Both bind to gene promoters and enhancers and make up the same COMPASS-like complex (COMplex of Proteins ASsociated with Set1), which includes the WRAD sub-complex, menin and HCF1 (Yokoyama et al., 2004). Despite these similarities, they are mutually exclusive in the COMPASS-like complex (i.e. only one or the other can be present) and are largely thought to have non-overlapping roles (Dou et al., 2005). For example, they are thought

to have largely unshared target genes (Bach et al., 2009). In addition, both MLL1 and MLL2 knockout mice are embryonic lethal suggesting non-redundant roles (Glaser et al., 2006; Yu et al., 1995).

MLL's role in hematopoiesis has been demonstrated through genetic studies in mice. Homozygous $MLL1^{-/-}$ knockout mice are embryonic lethal and display abnormalities with hematopoiesis, downregulated *Hox* gene expression and neural crest patterning defects (Hess et al., 1997; Yagi et al., 1998; Yu et al., 1995). $MLL^{-/+}$ mice, while not lethal, have homeotic developmental defects, growth retardation and are anemic (Yagi et al., 1998; Yu et al., 1995). Furthermore, conditional hematopoietic-specific knockout adult mice show that MLL is crucial for adult hematopoietic stem cell maintenance. These animals are anemic, thrombocytopenic and have reduced hematopoietic progenitors in the bone marrow as well as low survival rates (Gan et al., 2010). The effects on hematopoiesis by MLL are hypothesized to occur mainly through deregulated maintenance of *Hox* gene expression. Furthermore, MLL1 has been demonstrated to regulate transcription factors such as GATA3, MYC and genes important in cell cycle regulation such as cyclins A, B and E and CDK inhibitors (Hess et al., 1997; Milne et al., 2005a; Takeda et al., 2006; Yagi et al., 1998; Yamashita et al., 2006; Yu et al., 1995). MLL has also been shown to be essential in the maintenance of quiescence in hematopoietic stem cells and for promoting progenitor proliferation (Jude et al., 2007; McMahon et al., 2007).

MLL is a large multidomain protein (Figure 2A). Its N-terminal region contains two motifs that binds to the oncogenic cofactor menin (MEN1) (Grembecka et al., 2010). MEN1 mediates the binding between MLL and lens epithelium-derived growth factor (LEDGF) (Yokoyama and Cleary, 2008). Downstream of the menin binding motif (MBM) are three AT-hooks which bind to the minor groove of adenine and thymine rich regions (Reeves and Nissen, 1990). MLL's MT domain, which contains a DNA binding CxxC zinc finger motif, is capable of binding unmethylated CpG dinucleotides and interacts with histone deacetylases and polycomb proteins (Birke et al., 2002; Xia et al., 2003). CpG dinucleotides are particularly enriched at promoter regions and unmethylated islands are associated with gene expression (Deaton and Bird, 2011). These two N-terminal DNA binding domains are believed to stabilize MLL1 on chromatin through multivalent interactions (Rao and Dou, 2015). The protein also contains four plant homeotic domains (PHDs) whose functions are still not completely understood. PHD1 and 4 are reported to help mediate interactions between the N and C terminal subunits while PHD2 helps to mediate the degradation of MLL. PHD3 is a chromatin reader and interacts with trimethylated H3K4 in addition to Cyp33, an MLL corepressor (Wang et al., 2010b). Sandwiched between PHD3 and PHD4 is a bromodomain which interacts with acetylated lysine residues (Ali et al., 2014). Both trimethylated H3K4 and acetylated lysines are highly enriched around transcription start sites and stabilize MLL binding to the loci (Rao and Dou, 2015; Wang et al., 2010b). The protein further contains a transcriptional activation domain (TAD) which recruits factors such as histone acetyltransferase CBP/p300 and hMOF (Dou et al., 2005; Ernst et al., 2001). The C-terminus contains the catalytic SET domain responsible for the

protein's H3K4 methyltransferase activity. This domain also has the ability to bind the SWI/SNF family of chromatin remodelling enzymes (Rozenblatt-Rosen et al., 1998).

MLL is transcribed as a single unit but undergoes cleavage in the cytoplasm by taspase1 into a large MLL^N (320kDa) subunit and a smaller MLL^C (180kDa) subunit (Yokoyama et al., 2002). These re-associate through the phenylalanine- and tyrosine-rich N (FYRN) and C (FYRC) terminal region domains to form a stable non-covalent MLL heterodimer (Hsieh et al., 2003). MLL undergoes biphasic regulation during the cell cycle and is degraded by the ubiquitin ligase complexes SCF and APC during S and M phases respectfully (Liu et al., 2007). During hematopoietic differentiation, MLL is degraded by ASB2, a member of the ESC E3 ubiquitin ligase complex. When there is a knockdown of ASB2 in myeloid lineage leukemic cells, there is delayed cell differentiation and increased expression of MLL target gene *HOXA9* (Wang et al., 2012).

All SET containing H3K4 methyltransferases associate with a highly conserved core group of four proteins, called WRAD, consisting of WD repeat protein 5 (WDR5), retinoblastoma-binding protein 5 (RBBP5), absent small or homeotic discs protein 2 like (ASH2L) and dumpy wings 30 (DPY30) (Figure 2B) (Patel et al., 2009). MLL's catalytic activity is weak and inefficient due to the unfavourable conformation it adopts when alone (Southall et al., 2009). WRAD is, therefore, necessary to increase its catalytic activity. The first three proteins form a core entity which enhance MLL's methyltransferase activity 50-500 fold (Rao and Dou, 2015). WDR5 interacts with MLL through its conserved WIN domain (Dou et al., 2006). While the stable heterodimer

formed by ASH2L and RBBP5 is capable of binding directly to MLL, its interaction is stabilized by WDR5 (Li et al., 2016). Further binding of dimeric DPY30 increases MLL's activity twofold (Dou et al., 2006). MLL has over a dozen known protein-binding partners but interacts with two proteins not associated with other members of the SET methyltransferase family. These are menin, which is a tumour suppressor, as well as HCF1 or HCF2, which are transcriptional coregulators and are involved in cell cycle regulation (Rao and Dou, 2015).

1.5 MLL Mutations

The mixed lineage leukemia 1 gene (*MLL1*, also called *HRX*, *ALL-1* and *KMT2A*) on chromosome 11, band q23, is one of the most frequently mutated genes in cancer. It was first identified in 1991 as a recurrent locus of chromosomal translocation in acute leukemias expressing both lymphoid and myeloid surface antigens (Ziemin-van der Poel et al., 1991). MLL mutations are present in 10% of AML and are particularly common in infant acute leukemias and those with trisomy 11 (Biondi et al., 2000; Appendix A).

MLL mutations in leukemia arise in one of three ways: reciprocal chromosomal translocations, internal partial tandem duplications (PTD) and, very rarely, amplification of unrearranged MLL (Dharmarajan and Cosgrove, 2011). Other MLL mutations including frameshift, nonsense and missense mutations are found in multiple solid tumour cancers including those of the uterus, colon, and lung (Grossman et al., 2016). However, the relevance of these mutations for the development of cancer is unknown. The most common MLL mutation in pediatric patients, found in 70-80% of infant cases,

is a balanced translocation of 11q23 (Winters and Bernt, 2017). This results in the formation of a chimeric oncoprotein where the 5' end of MLL (first 1400 amino acids) is fused with another gene. These gain of function mutations drive acute myeloid and lymphoid leukemic transformations in the murine model (Milne, 2017). Eighty-five distinct translocation partners for MLL have been identified to date including multiple transcriptional activators and epigenetic enzymes (Meyer et al., 2009). However, the most common six partners make up approximately 85% of all translocation mutations. These are AF4, AF6, AF9, AF10, ENL and ELL (Meyer et al., 2013). MLL translocation mutants are thought to be oncogenic in two distinct ways. The first is through the recruitment of protein complexes normally associated with the fusion protein to loci under the regulation of MLL. For example, AF4, AF5, ENL, AF9 and ELL can bind the histone 3 lysine 79 (H3K79) methyltransferase DOT1L, bromodomain-containing protein 4 (BRD4) and TIP60 to form the super elongation complex capable of recruiting polymerase II transcription elongation factor b (P-TEFb) kinase (Li et al., 1998; Ma and Staudt, 1996). This complex is then recruited to MLL's targets including the *HOXA* loci (Milne et al., 2005b). Secondly, MLL fusions can form leukemogenic nuclear complexes. This is most elegantly demonstrated by the fact MLL fused with lacZ, which normally expresses the non-cancerous protein β-galactosidase, causes leukemic transformation in mice (Dobson et al., 2000). This mechanism is thought to be through the formation of stable dimers and oligomers (Martin et al., 2003; So et al., 2003). Importantly, MLL-fusion leukemogenic mutants lose their cell cycle regulation and are resistant to degradation by the SCF and APC E3 ubiquitin ligases (Liu et al., 2007).

Additionally, they lose ASB2-mediated degradation during hematopoietic differentiation (Wang et al., 2012).

1.6 Partial Tandem Duplication (PTD) of MLL

Following the discovery of MLL fusion mutations, southern blot analysis revealed MLL rearrangements in pediatric and adult patients lacking cytogenetic evidence of 11q23 translocations (Caligiuri et al., 1994; Chen et al., 1993; Cimino et al., 1993). These mutations were identified as in-frame repetitions of N-terminus exons (Schichman et al., 1994). The most common duplication occurs between exons 2 through 6 or 8 in a 5' to 3' orientation (Schichman et al., 1994; Shih et al., 2006; Steudel et al., 2003). This causes a duplication of the two DNA binding domains: the CXXC and AT-hooks. Therefore, unlike MLL translocation mutants, MLL-PTD retains all of its C-terminal domains. It has been suggested that the partial tandem duplication mutation occurs as a result of mispaired Alu-mediated homologous recombination (Strout et al., 1998).

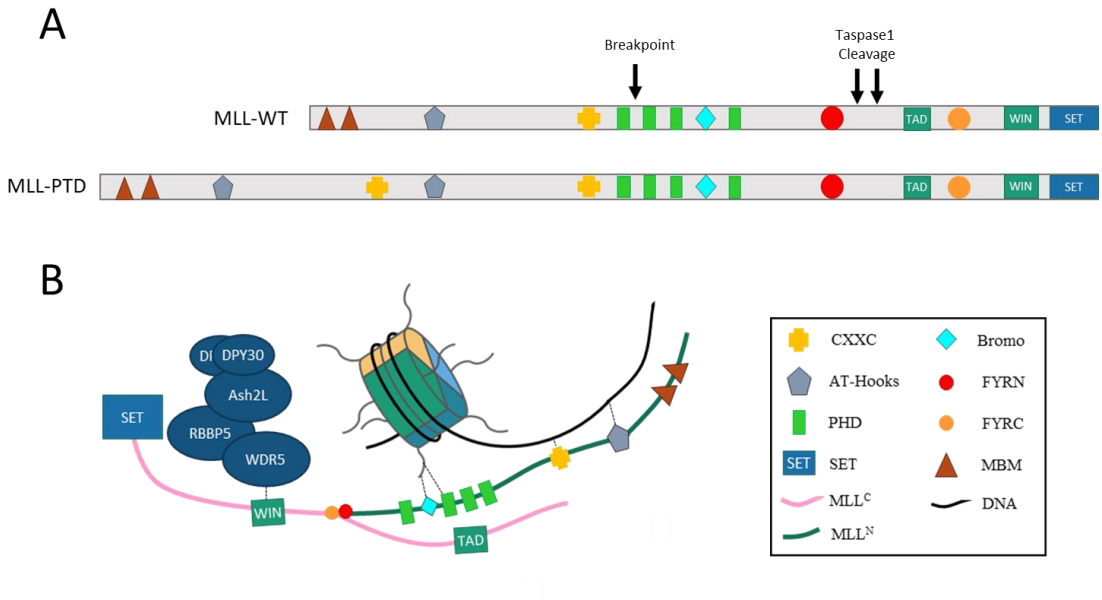

Figure 2. Structure of MLL-WT and MLL-PTD (A), including its domains and motifs, and a schematic representation of the interaction between MLL-WT and the WRAD complex and nucleosome (B). Dashed lines show interactions. Figure modified from Rao and Dou and is not to scale (Rao and Dou, 2015).

Clinical Features

The incidence of MLL-PTD has been analyzed through multiple single and multi-centre studies since its discovery (summary in Appendix A). Overall, MLL-PTD is found in 4-6% of AML patients. MLL-PTD is most abundant in AML with a normal karyotype (6-12%) or trisomy 11 as the only aberration (25-37%). MLL-PTD mutations are more likely to be found in elderly patients (Kihara et al., 2014). The incidence of MLL-PTD mutations in AML increases significantly with age from 0.04 per 100,000 among patients 21-30 years to 0.74 per 100,000 in those aged 61-70. (Bacher et al., 2005). Patients with MLL-PTD mutations are significantly older than those harbouring translocation MLL mutations (Shih et al., 2006). In addition to AML, MLL-PTD mutations have been found in 2.7% of myelodysplasia (MDS) (Bacher et al., 2007). MDS is a disease akin to AML

and is characterized by pancytopenia caused by defective hematopoiesis. The distinction between the two diseases is based on a less than or greater than 20% of bone marrow cells composed of blasts for MDS and AML respectively (Arber et al., 2016; Swerdlow et al., 2016).

MLL-PTD AML patients are often categorized as having an unfavourable prognosis (Grossmann et al., 2012; Kihara et al., 2014). Overall, most studies have shown a trend of shorter event-free and overall survival as well as increased rates of clinical relapse in AML patients who harbour the MLL-PTD mutation compared to those without it (Dohner et al., 2002; Grossman et al., 2016; Haferlach et al., 2012; Kihara et al., 2014; Marcucci et al., 2005; Schlenk et al., 2008; Shimada et al., 2008). Following chemotherapy, early allo-HSCT helps improve the prognosis of patients with MLL-PTD (Schlenk et al., 2008). The MLL-PTD mutation in MDS is associated with significantly more rapid disease progression as well as decreased overall survival (Dicker et al., 2010).

MLL-PTD mutations are found in all FAB subtypes except M3 promyelocytic leukemia (see the summary of statistics in Appendix A). Patients with MLL-PTD mutations are most commonly diagnosed with M2 myeloblastic AML with maturation. MLL-PTD patients have significantly lower absolute leukocyte counts as well as higher numbers of CD34+ cells at diagnosis compared to patients who do not harbour the mutation. This suggests an immature phenotype (Steudel et al., 2003). Both MLL translocation mutations and MLL-PTD have been found exclusively in leukemias. However, unlike MLL-fusion mutants, MLL-PTD is not found in acute lymphoblastic leukemia (Drexler

et al., 2004; Sun et al., 2017). This suggests an exclusive role for MLL-PTD in myeloid leukemias.

MLL-PTD is suitable to detect minimal residual disease (MRD) in patients with karyotypically normal AML. MRD is defined as leukemia not detected by morphology but only by sensitive laboratory techniques such as PCR and flow cytometry. When analysed after induction therapy, MRD is an important prognostic marker for patient survival and helps inform treatment plans for consolidation therapy. AML patients who 2, 4 and 6 months post-therapy achieved a greater than 2 log-reduction in *MLL-PTD* expression by PCR had significantly higher overall survival compared to patients with less than 2 log fold change (Weisser et al., 2005). Patients who achieve clinical relapse (<5% blast in BM) but not molecular remission, based on *MLL-PTD* expression levels, relapse quickly (Weisser et al., 2005). MLL-PTD AML patients relapse significantly faster than those who do not carry this mutation indicating an important role for MLL-PTD as a detector of minimal residual disease (Ommen et al., 2014).

Molecular Characterization

Compared to the MLL-fusion proteins, little is known about the oncogenic mechanism of MLL-PTD. A mouse model published by Dorrance et al. provides the most detailed information, to date, on MLL-PTD's role in leukemogenesis. $MLL^{PTD/PTD}$ and $MLL^{PTD/-}$ knock-in mice are embryonic lethal (Dorrance et al., 2006, 2008). The viability of $MLL^{PTD/-}$ mice until P1 is longer than the E10.5 achieved by $MLL^{-/-}$ knockout mice (Dorrance et al., 2008; Yu et al., 1998). However, both $MLL^{WT/-}$ and $MLL^{PTD/WT}$ mice

have normal life expectancies (Dorrance et al., 2008). This suggests that although MLL-PTD has some compensatory ability during development, its expression alone is not sufficient for proper embryogenesis. Ultimately, expression of at least one MLL-WT allele is necessary for appropriate murine development. Heterozygote $MLL^{PTD/WT}$ knock-in mice are viable yet display phenotypic abnormalities. A ventral shift in the boundary of *Hoxa9* expression at E12.5 in the somitic mesoderm causes axial skeleton defects including a duplication of the first sacral vertebrae and a missing or rudimentary 13^{th} rib in these mice (Dorrance et al., 2006).

Furthermore, $MLL^{PTD/WT}$ mice have perturbed hematopoiesis (Dorrance et al., 2006). Lineage-specific CFU assays from splenocytes of $MLL^{PTD/WT}$ mice show significantly more progenitors from erythroid, myeloid and mixed phenotypes when compared to age and sex-matched $MLL^{WT/WT}$ littermate controls. When primary CFUs were replated, the total number of colonies per plate did not differ significantly between PTD and WT mice. However, the size of the colony was significantly larger than those from both MLL-WT and MLL-AF9 fusion mutant mice suggesting an increase in proliferation. Only the secondary colonies taken from MLL-PTD mice can form tertiary and quaternary colonies suggesting increased self-renewal. Hematopoietic stem and progenitor cells (HSPC) taken from $MLL^{PTD/WT}$ mice display a proliferative advantage in both colony replating assays and competitive transplantation when compared to WT HSPCs (Zhang et al., 2012). Lethally irradiated mice transplanted with $MLL^{PTD/WT}$ HSPCs show rescued hematopoiesis, however, there is a bias for the lymphoid lineage and a blockade in myeloid differentiation. HSPCs under steady state conditions have reduced absolute

numbers, however, when exposed to stress, they display elevated proliferation and reduced apoptosis when compared to HSPCs from WT animals (Zhang et al., 2012). Therefore, MLL-PTD is capable of expanding progenitor populations, causing lineage bias and conferring stem cell-like properties.

MLL regulates the maintenance of homeobox (*Hox)* gene expression during development (Yu et al., 1998). Homeobox genes are crucial for both anterior-posterior patterning of the axial skeleton and for hematopoietic development (Alharbi et al., 2013). Homeobox-containing transcription factors are a family of proteins which control cell fate during development. In mammals, the 39 *HOX* genes are arranged in four paralogous clusters (A, B, C and D) (Krumlauf, 1994). In addition to their role in cell fate determination, HOX proteins from the A and B clusters are critical for the normal development of hematopoiesis (Lawrence et al., 2005). The most well studied of these *HOX* genes in a hematopoietic context is *HOXA9*. It is highly expressed in hematopoietic stem cells and its expression decreases as the cells lose their multipotency and become more differentiated (Pineault et al., 2002). More than 50% of acute myeloid leukemias overexpress *HOXA9,* and this is thought to be a critical junction in AML development from a variety of mutations including MLL, NPM1 and NUP98 (Collins and Hess, 2016). The overexpression of *HOXA9* is believed to prevent progenitor cells from undergoing proper differentiation, thereby, resulting in one of the hallmarks of leukemia: an accumulation of hematopoietic progenitor cells that lack the ability to develop functional cells of the blood (Fialkow et al., 1981). Overexpression of *Hoxa9* in mice is sufficient to cause leukemia (Thorsteinsdottir et al., 2002).

Since MLL regulates *HOX* gene expression, $MLL^{PTD/WT}$ mice expectedly exhibit overexpression of *Hoxa7, Hoxa9* and *Hoxa10* in fetal liver and adult spleen, bone marrow and blood (Dorrance et al., 2006). The cis-regulatory elements of *Hoxa7* and 9 had increased H3 and H4 acetylation (a chromatin activation mark), decreased H3K9 methylation and increased H3K4 methylation in the mutant background animals. A lack of activating epigenetic changes in the promoter of *Hoxa10* suggests it is overexpressed through an unknown distinct mechanism from *Hoxa7* and *Hoxa9*. Importantly, $MLL^{WT/-}$ mice who lack one copy of the *Mll-wt* gene do not have significant epigenetic alterations or changes in *Hoxa* gene expression. Therefore, the observed changes in $MLL^{PTD/WT}$ mice is evidence that MLL-PTD is a gain of function mutation (Dorrance et al., 2008). Matching what was seen in the murine system, MLL-PTD patients have elevated *HOXA9* expression (Gao et al., 2016). In addition, knockdown of MLL-PTD in patient samples carrying the mutation inhibits AML-CFU blast colony formation and causes morphologic changes to the cells indicative of maturation (Whitman et al., 2005). This provides evidence in human cells that MLL-PTD is a gain of function mutation.

Despite these multiple phenotypic abnormalities, $MLL^{PTD/WT}$ mice and irradiated mice transplanted with $MLL^{PTD/WT}$ HSCs do not develop leukemia (Dorrance et al., 2006; Zhang et al., 2012). This supports the two-hit hypothesis which states that the development of one or more cooperating mutations is required for leukemogenesis (Shih et al., 2012). A study by Kao et al. reported that 90.8% of AML patients with MLL-PTD have at least one detected additional mutation. The most common secondary mutations

in MLL-PTD AML are genes involved in epigenetic regulation followed by transcription pathway genes and finally tumour suppressor genes (Kao et al., 2015). MLL-PTD mutations are significantly associated with mutations in FLT3, RUNX1, IDH1, STAG2, WT1, PTDNII, EZH2 and U2AF1 (Balgobind et al., 2010; Schnittger et al., 2010; Sun et al., 2017; Tang et al., 2009). It is mutually exclusive of other class II mutations including CEBPA, TET2 and NPM1 (Sun et al., 2017). MLL-PTD is considered an early cooperating mutation and is believed to arise after the mutations IDH1/2, DNMT3A, U2AF1 but before type I proliferative mutations such as FLT3 and RAS (Sun et al., 2017).

The most frequently mutated gene in MLL-PTD patients, found in 45-60% of MLL-PTD AML, is the oncogene Fms-Like Tyrosine Kinase 3 (*FLT3*) (Kao et al., 2015; Sun et al., 2017). When bound to its ligand, FLT3 undergoes dimerization and autophosphorylation. This starts the phosphorylation of signalling cascades involved in differentiation, proliferation, and survival of hematopoietic progenitor and stem cells (Stirewalt and Radich, 2003). FLT3 is downregulated as the hematopoietic cells become more differentiated and its expression is limited to CD34+ stem and progenitor cells (Gabbianelli et al., 1995; Rosnet et al., 1996). Interestingly, FLT3 is aberrantly expressed in 70-90% of AML patients (Carow et al., 1996; Rosnet et al., 1996). Mutations in the FLT3 receptor, commonly internal tandem duplications (ITD), cause constitutive signalling, thereby, resulting in a ligand-independent increase in cell survival and proliferation in addition to a block in myeloid differentiation (Hayakawa et al., 2000; Kiyoi et al., 1998; Mizuki et al., 2000; Zheng et al., 2002). Zorko et al. showed that

$MLL^{PTD/WT}$:$Flt3^{ITD/WT}$ double knock-in mice spontaneously develop AML with 100% penetrance. These mice develop leukocytosis, splenomegaly, thrombocytopenia and anemia and have 20% blasts in their bone marrow. There is a 25-fold higher expression of *Hoxa9* in bone marrow cells of double knock-in mice compared to either WT or single knock-ins (Zorko et al., 2012). Furthermore, the double mutants displayed increased levels of DNA methyltransferases and global DNA methylation (Bernot et al., 2013). This is supported by *in vitro* work as MLL-PTD cell lines have increased global DNA methylation compared to those carrying MLL-WT (Whitman et al., 2008).

In MLL-PTD patients, only one chromosome 11 is mutated while the other allele remains wild-type (Caligiuri et al., 1994). However, expression of the WT protein is repressed (Whitman et al., 2005). *MLL-WT* expression can be restored through treatment with DNA methyltransferase and or/ histone deacetylase inhibitors suggesting an epigenetic role in their downregulation (Whitman et al., 2005). In addition, MLL-WT activity is rescued after MLL-PTD repression using antisense oligonucleotides indicating autoregulation. Restoration of WT expression causes impaired proliferation and AML-CFU blast colony formation of MLL-PTD blasts *in vitro* (Whitman et al., 2005). In $MLL^{PTD/WT}$:$Flt3^{ITD/W}$ double knock-in mice, *Mll-wt* expression in young preleukemic mice is at equivalent levels to $MLL^{WT/WT}$ animals. However, by the time the animals develop leukemia, there is a 60% decrease in MLL-WT transcript copy number (Zorko et al., 2012). Taken together, this *in vitro* and *in vivo* work suggests that in addition to the MLL-PTD and secondary mutations, changes in the ratio of MLL-WT to MLL-PTD are necessary for AML leukemogenesis. This contrasts with data from the fusion mutations

which have enhanced leukemogenesis with wild-type expression of *MLL1* or its paralog *MLL2* (Chen et al., 2017).

Gene Expression

Gene expression profiles differ between MLL-PTD and MLL with translocation mutations and between MLL-PTD and AML with a normal karyotype (Ross et al., 2004). Liu et al. identified 21 genes differentially expressed between the two MLL main mutation types including a cluster of 9 *HOXB* genes (Liu et al., 2011). However, there remained some similarities between *MLL-PTD* and translocation mutant *MLL* leukemia gene expression such as overexpression of *HOXA* genes including *HOXA4*, 5, 7, 9 and 10.

MLL-PTD patients are significantly more likely to overexpress genes known to confer a poor prognosis compared to other AML patients. One such gene, *BAALC*, is upregulated in CD34+ populations of BM cells. MLL-PTD patients are more likely to have high *BAALC* expression which is an independent prognostic marker for poor outcomes in patients younger than 60. Similar to *HOXA9*, its downregulation is correlated with cell differentiation (Langer et al., 2008). Furthermore, MLL-PTD has significantly higher levels of unmethylated Wnt inhibitor WIF1 suggesting its overexpression (Hou et al., 2011). Indeed, overactivation of Wnt signalling causes increased survival and uncontrolled cell growth in hematopoiesis and leukemia (Reya et al., 2003; Wang et al., 2010a).

1.7 Rationale

AML has a 20-30% survival rate suggesting the need for additional therapeutic interventions for these patients. The partial tandem duplication of MLL is present in ~5% of AML and is associated with a worse prognosis than in patients who do not harbour the mutation. MLL-PTD is a gain of function mutation and causes increased self-renewal and proliferation of hematopoietic stem and progenitor cells. However, the molecular mechanism through which MLL-PTD induces leukemic transformation is currently unknown. Therefore, the aim of my project is to decipher the role of MLL-PTD in oncogenesis by comparing its cofactor binding targets to those of wild-type MLL (MLL-WT). This was achieved through mass spectrometry analysis of FLAG and HA-tagged MLL-PTD and comparing this to the cofactors of MLL-WT. This research identified the proteins that differentially bind to the mutant protein and therefore those proteins which contribute to the leukemogenesis of MLL-PTD. This provides new insight into the molecular mechanism of the mutated protein in addition to providing possible new therapeutic targets.

1.8 Hypothesis

We hypothesize that the PTD mutation of MLL1 will cause deregulated binding to cofactors, thereby, modifying gene expression to favour leukemia development and/or maintenance.

1.9 Objectives

The general goal of the project is to identify cofactors that differentially interact with MLL-PTD versus MLL such that inhibitors of these interactions could be tested as a therapeutic strategy for AML. Towards this goal, I have established the following objectives:

1) Compare cofactor binding between MLL-PTD and MLL-WT by mass spectrometry following immunoprecipitation using FLAG- and HA-tagged MLL overexpressed in 293T cells
2) Validate the targets found in Objective 1 using western blots and CoIPs
3) Test inhibitors of these targets for their effects on an MLL-PTD cell line

2. Materials and Methods

2.1 Generating MLL and MLL-PTD Expressing Plasmids

Constructs were made by laboratory technician Jianguo Wu. The pcDNA5/TO plasmid was purchased from Invitrogen. The MLL constructs were cloned in three parts because of their large size. The 3xFLAG tag was cloned using the HindIII and BamHI sites. Next, a ClaI restriction site was added to the multiple cloning site of the pcDNA5/TO using Xbal and Apal. A 500bp PCR product containing the 5' MLL fragment, bookended with Xhol and ClaI restriction digest sites at the 5' and 3' ends respectively, was cloned into the plasmid. Finally, we cloned the 12kb 3' HA tagged MLL-WT or MLL-PTD fragment into the Fsel and ClaI sites. We confirmed each step by sequencing. The final construct can express 3xFLAG-MLL-HA or 3xFLAG-MLL-PTD-HA mRNA (See plasmid maps in Appendix E).

2.2 Cell Culture

HEK293T cells were cultured in DMEM (GE Healthcare) supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin (Wisent). K562, MV4-11 and EOL-1 cells were cultured in RPMI 1640 with L-glutamine (GE Healthcare) supplemented with 10% FBS and 1% penicillin/streptomycin. HL-60 cells were cultured in IMDM (Gibco) supplemented with 20% FBS, 4mM L-glutamine (Sigma) and 1% penicillin/streptomycin. K562, EOL-1, MV4-11, 293T and HL-60 cell lines were purchased from the American Type Culture Collection.

2.3 Transfection of 293T Cells

HEK293T cells were seeded in antibiotic free media 24h prior to transfection using polyethyleneimine (PEI). Media was changed 1h prior to transfection. 150cm plates at 70-80% confluency were transfected with 319µL of PEI (1mg/mL), 90µL NaCl (1.5M) and equimolar amounts of plasmid, 29µg or 22.3µg for MLL-PTD and MLL-WT respectively, in a final volume of 1800µL. This solution was added dropwise to the cells. Media was changed 24h after transfection. Cells were harvested 48h post transfection.

2.4 RT-qPCR

Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc.) according to the manufacturer's instructions. First strand cDNA synthesis was performed using 2µg of DNA, 4U RNAseOUT (Invitrogen) and 0.5µg random primers (Invitrogen) in a final volume of 14µL. The tube was gently mixed and heated at 70^{o}C for 5min and immediately cooled on ice for 2min. To this was added 5µL 5X RT Buffer (Promega), 5µL 10mM dNTP mix (Thermo Fisher) and 1µL mMLV-RT enzyme (Promega) for a final volume of 25µL. cDNA synthesis was completed through heating at 42^{o}C for 50min, 70^{o}C for 15min and cooling to 4^{o}C. Quantitative PCR was performed using 5µL of 10x diluted cDNA, 0.8µL from a 5µM mix of forward and reverse primers and 10µL PerfeCTa SYBR Green Supermix (Quanta) in a reaction volume of 20µL. Samples were run on a Corbett Research Rotor Gene 6000 with the following conditions: 2min hold at 50^{o}C; 2min hold at 95^{o}C; 45 cycles of 95^{o}C for 10sec, 60^{o}C for 20sec and 72^{o}C for 34sec; melt 60^{o}C-95^{o}C. A list of primers can be found in Appendix B.

2.5 Nuclear Protein Extraction

Nuclear extracts were taken as follows. Cells were pelleted and washed twice with 1X cold PBS. Cells were lysed using swelling buffer (25mM Hepes K^+ phosphate pH 7.9, 1.5mM $MgCl_2$, 0.6mM KCl, glycerol (25% v/v) with freshly added 0.5mM DTT and 1X protease inhibitor cocktail). Cells were vortexed every 5min for 30min. Cell nuclei were pelleted and the supernatant containing cytoplasmic protein was removed. The nuclei were washed twice with cold 1X PBS. Buffer C (20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 0.6mM KCl, glycerol (25% v/v), 0.5mM DTT, 1X protease inhibitor cocktail) was added with a volume of 60% of the pellet volume. Nuclei were vortexed every 10min for 1h. The same volume of buffer D (60% of the pellet volume) was added. Samples were pelleted to remove debris and the supernatant containing nuclear protein was kept. Samples were snap frozen in liquid nitrogen and stored at -80^{o}C.

2.6 Co-Immunoprecipitation

Dynabeads magnetic beads (Invitrogen) were chemically cross-linked to antibody using dimethyl pimelimidate (Thermo Scientific). A ratio of 25µg of antibody per 100µL of beads was used. Antibodies raised in rabbit (HA, menin and IgG) were crosslinked to Dynabeads protein A while those raised in mouse (FLAG and IgG) were crosslinked to Dynabeads protein G.

The cross-linking protocol is as follows. Beads were washed twice with 5 bead volumes of potassium phosphate buffer (0.1M, pH 8.2) with 0.1% ovalbumin (Sigma). Antibody solution with a final potassium phosphate concentration of 0.1M was prepared and added

to the Dynabeads and rotated at RT for 30min. The supernatant was discarded and beads were washed with 5 bead volumes of potassium phosphate buffer (0.1M, pH 8.2) with 0.1% ovalbumin. Beads were washed twice with triethanolamine (0.2M, pH 8.2) before incubation with 10mg/mL dimethyl pimelimidate solution in triethanolamine. Beads were mixed immediately and incubated with rotation at room temperature for 30min. The supernatant was discarded and beads were washed with 50mM Tris (pH 7.4), three times with 1X PBS with 0.1% ovalbumin and once with IP100 buffer (25mM Tris pH 7.9, 5mM $MgCl_2$, 10% glycerol, 100mM KCl, 0.1% NP-40, 1X PIC). Finally, beads were washed with 100mM glycine (pH 3) to remove aggregated antibodies before washing twice with IP100 buffer. Beads were resuspended in the initial bead volume with IP100 buffer and stored at 4^{o}C before use. Crosslinking efficiency was validated through Coomassie staining of an acrylamide gel containing the supernatant of crosslinked and uncrosslinked beads boiled in 2X loading dye. The presence of a band in the uncrosslinked sample but not in the x-linked indicated a successful reaction.

Prior to immunoprecipitation (IP), NP-40 was added to the nuclear extract to a final concentration of 0.05%. This was vortexed and centrifuged at 14,000 rpm (18,400 x g) at 4^{o}C for 15min. The concentration of the supernatant was determined by Bradford assay. The nuclear extract was pre-cleared using either Dynabeads Protein A or G at 4^{o}C for 2h before being added to the antibody cross-linked beads overnight with rotation at 4^{o}C. The beads were washed four times with IP150 buffer with 10min of rotation at 4^{o}C between each wash. Proteins were eluted using 2X SDS loading dye (5% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM DTT) boiled at 95^{o}C for 5min prior to western

blot. For IPs prior to mass spec, proteins were eluted using 5% acetic acid (pH 3), heated at 25^{o}C for 15min, dried using a vacufuge (Eppendorf) and resuspended in 2X loading dye.

2.7 Mass Spectrometry

Following isolation of our samples by IP, we proceeded to run the extracts on a 4-15% gradient gel for fractionation (Bio-Rad). Silver staining was performed using the protocol described in Shevchenko et al. One-centimetre bands from the silver stained gel were excised and sent to the OHRI Proteomics Core Facility for LC-MS/MS analysis. Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Shevchenko et al., 1996). Peptide extracts were concentrated by Vacufuge (Eppendorf). LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Peptide separation was performed on a C18 2µ column (Thermo Scientific) at a constant flow rate of 300 nanoliters per minute separated using a 5-36% gradient of acetonitrile over 15min. Data dependent acquisition of MS^2 spectra was used from the most abundant ions in MS. Dynamic exclusion of two counts within 10sec triggers exclusion for 60sec was used. MASCOT software version 2.6 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The overserved spectra were matched against human sequences from SwissProt (version 2016-09) and against an in-house database of common contaminants. The results were exported to Scaffold (Proteome Software, USA) for further validation and viewing. In addition, MaxQuant software version 1.6.0.13 was used to process the raw files. All MaxQuant parameters

were left as default except that LFQ was turned on. The reference proteome is UniProt_Human_20170718, with contaminants option turned on.

Protein and peptide identities were determined using either the MASCOT software version 2.6, followed by total TIC analysis, or Andromeda followed by analysis using MaxQuant. The algorithms used in MASCOT and Andromeda differ in two main ways. The first is that MASCOT determines protein identity from MS2 plots while Andromeda uses MS1 and retention time (Cox et al., 2011; Perkins et al., 1999). Quantification by MS1 plots has been shown to be more accurate in protein identification and more consistent across samples (Yuan et al., 2013). Secondly, Andromeda uses stricter filtering criteria including a different peptide match and protein threshold. Therefore, MASCOT has the advantage of identifying more proteins whereas Andromeda focuses only on the identities that are statistically strong. MS data was viewed using the Scaffold software and further analysis was completed using Excel, DAVID, STRING and Cytoscape.

The mass spec data identified a total of 830 and 449 proteins from the HA IPs using MASCOT and Andromeda respectively. These values were 447 and 240 for the FLAG IPs. All proteins which were obviously contamination such as keratin and trypsin were eliminated. Next, all non-nuclear proteins were removed from analysis as MLL is found exclusively in the nucleus because of its nuclear localization signal (Jude et al., 2007; Yokoyama et al., 2011; Figure 4). Following this, proteins which were solely found in the untransfected control were removed as these are contamination. We next eliminated

proteins that were not more than 1.5x enriched in the MLL-WT and MLL-PTD samples compared to the untransfected control. However, analysis was also performed omitting this step in order to increase our list of candidate proteins and avoid erroneous exclusion. In both the FLAG and HA IPs, there was slightly more identified MLL-WT protein compared to MLL-PTD. Therefore, the data was normalized using MLL abundance to prevent conclusions about binding stoichiometry based simply on the fact there was more MLL-WT than MLL-PTD. For the HA IP, the intensity of all MLL-PTD identified proteins in MaxQuant was multiplied by a factor of 1.4. For the FLAG IP this value was 1.3. The final step in our data analysis was to identify all proteins that had a greater than 2x fold change between MLL-WT and MLL-PTD as these would be the candidate proteins we would focus on for further analysis. The number of proteins identified in each sample as having a greater than 2x fold change are summarized in Figure 7.

2.8 Western Blot Analysis

Bradford analysis was performed using Protein Assay Reagent (Bio-Rad). Proteins were run on 6% or 10% SDS-page gels. Gels were 'wet' transferred at 150V for 70min. Membranes were blocked in 5% milk for 1h and incubated with antibodies overnight at 4^{o}C with shaking. Membranes were washed three times with 1X PBS with agitation for 5min. They were next incubated with secondary antibody for 1h at RT with agitation. Western blots were revealed using chemiluminescence (GE Healthcare). Precast gradient gels (4-15%) were used for silver staining of IP elutions prior to mass spec. A list of antibodies can be found in Appendix C.

2.9 Drug and Proliferation Assays

A total of $3x10^5$ cells (HL-60, MV4-11) or $1x10^5$ cells (K562, EOL-1) were plated into 24-well plates in a volume of 1mL per well. Cells were treated with either DMSO, the indicated concentrations of MB-3 (0.001-50µM), EPZ004777 (0.001-50µM) or MI-503 (1-400µM). All drugs were dissolved in DMSO and were added to a final concentration of 0.2% (MI-503 and EPZ004777) or 0.4% (MB-3) DMSO. Proliferation was measured using the Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) every 1-3 days as indicated. Cells were reseeded to the starting concentration and resuspended in fresh media with drug every 4 days.

2.10 Flow Cytometry

Cells were seeded at a concentration of 200,000 cells/mL. Cells were reseeded to this concentration, and fresh media and either 0.2µM or 0.5µM MI-503 was added every 48h. At days 2, 4 and 8, a total of $2x10^6$ cells were removed from culture. Cells were washed twice with PBS and resuspended in 1mL 1X annexin binding buffer (BD Biosciences). From this, 100µL were incubated with 2µL each of FITC-AnnexinV (BD Pharmogen) and 7aad (BD Pharmogen) for 15min at room temperature away from light. Samples were analysed using a LSR Fortessa II (BD Biosciences). Single stain controls were used to determine gating and 10,000 events were collected per sample.

2.11 Statistical Analysis

One-way ANOVA with Tukey's Multiple Comparison Test was used to calculate significance among IC50 values. Differences were considered significant when the P-

values were less than 0.05 as indicated by asterisks *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001. Statistical analysis was performed using the GraphPad Prism software.

3. Results

3.1 Exogenous MLL and MLL-PTD are Localized to the Nucleus

We expect that MLL-PTD will display differences in its ability to interact with cofactors compared to its WT counterpart because of its additional domains and increased size. To assess this hypothesis, we performed immunoprecipitation followed by mass spectrometry using FLAG- and HA-tagged MLL-PTD. Firstly, we transfected the human embryonic kidney cell line HEK293T with plasmids constructed in our lab capable of expressing FLAG- and HA-tagged MLL-WT or MLL-PTD (Figure 3A). RT-qPCR was used to verify the efficacy of transfection. PTD and WT transfected cells show approximately similar exogenous MLL mRNA expression when detected using primers for the FLAG tag (Figure 3B). Furthermore, MLL-PTD transfected cells express the same exon junction unique to the partial tandem duplication found in EOL-1 cells, a patient-derived eosinophilic AML cell line (Figure 3C). We proceeded to verify protein expression by western blot. Nuclear extracts taken 48h post-transfection show considerable MLL^{C} overexpression compared to untransfected negative controls (Figure 4). We can verify that some of this is the exogenous MLL protein through visualization of the HA-tag (Figure 4). The HA tagged MLL^{C} subunit is exclusively found in the nucleus which suggests the proper association and shuttling by the MLL^{N} subunit.

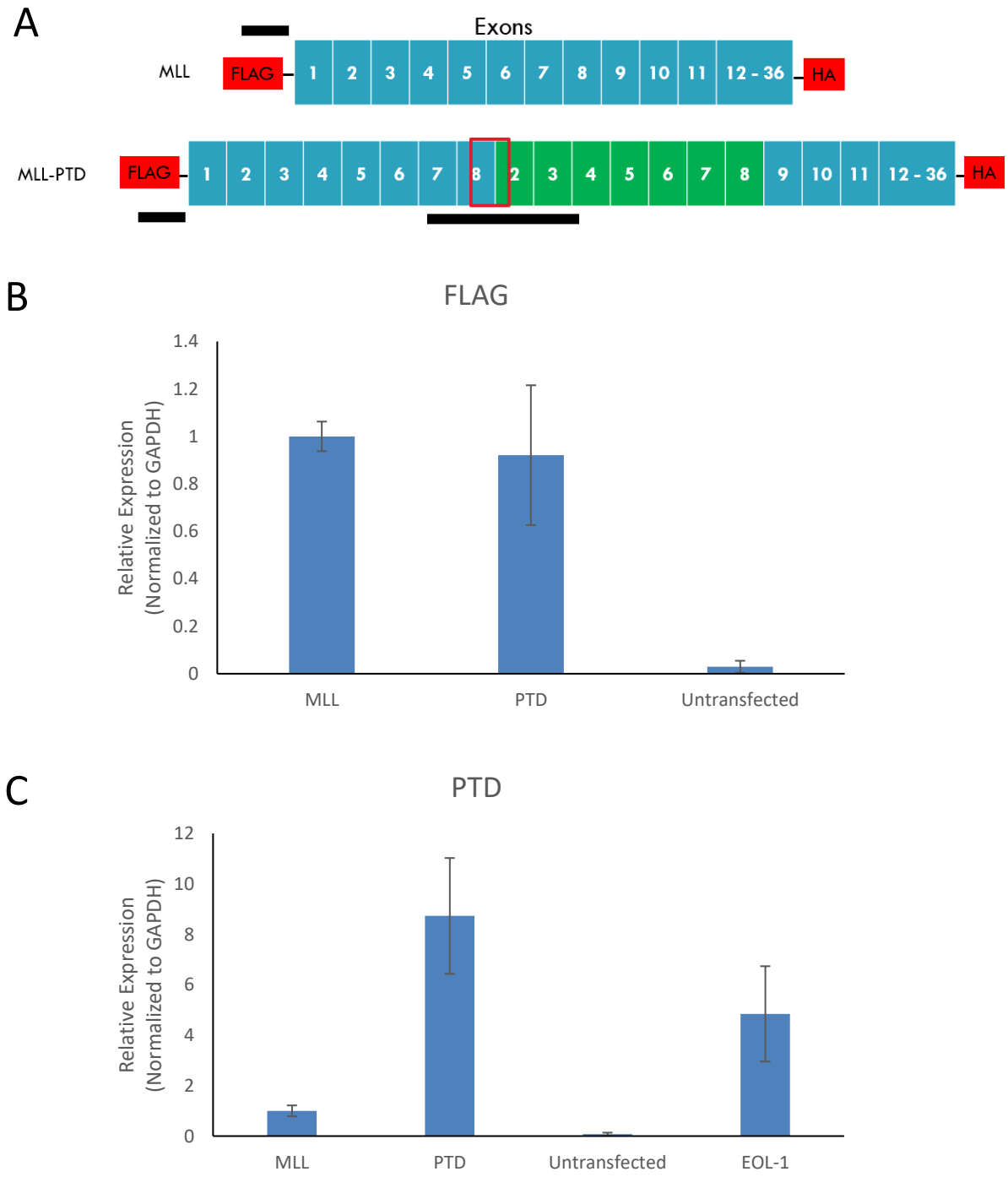

Figure 3. HEK293T cells express transfected MLL. Relative mRNA expression of FLAG and HA tagged *MLL-WT* or *MLL-PTD* (A) in transfected 293T cells, identified by RT-qPCR using primers specific for the FLAG tag (B) and the PTD exon junction (C). EOL-1 is a leukemic cell line containing the MLL-PTD mutation and serves as a positive control. The MLL-PTD exon 8-2 junction and the position of the primers, indicated by black bars, is shown in A. Error bars represent the standard deviation of N=2.

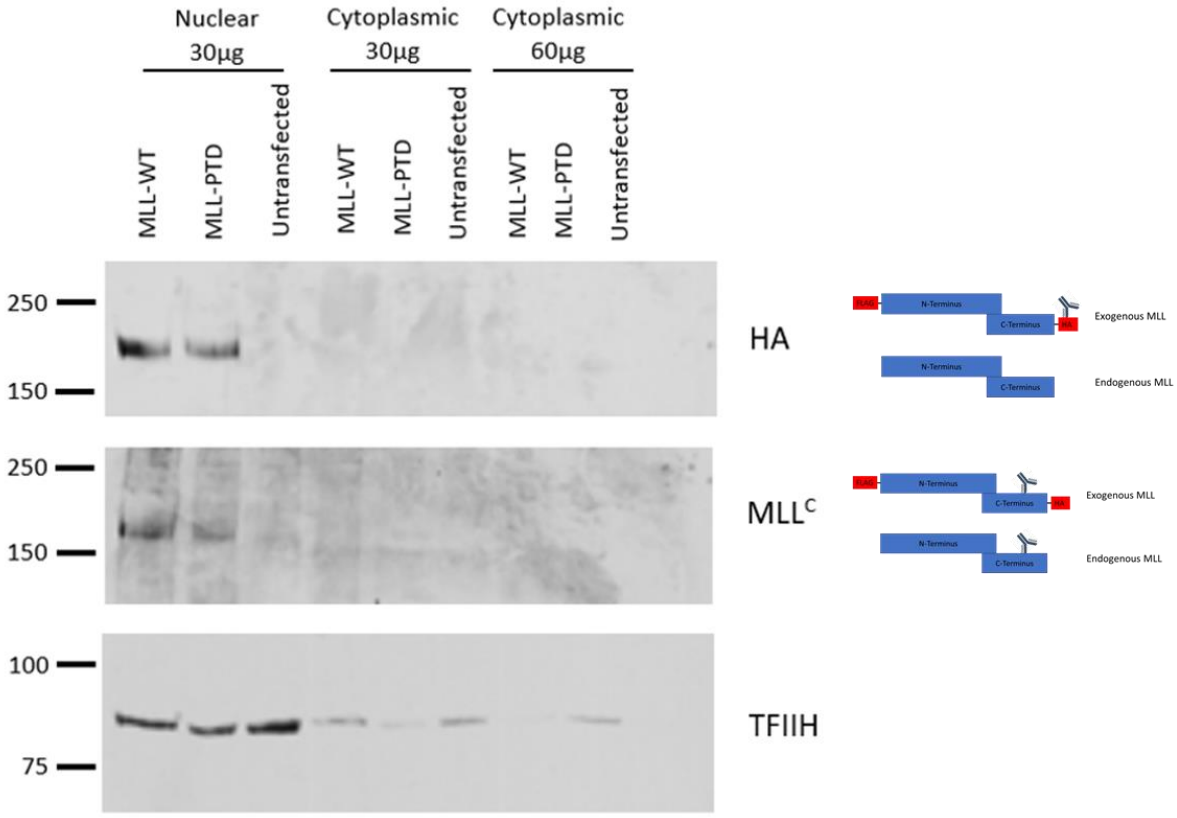

Figure 4. MLL is localized to the nucleus. Western blot showing nuclear and cytoplasmic fractionation of HA and FLAG tagged MLL-WT or MLL-PTD expression in transfected HEK293T cells. The total amount of protein loaded is indicated. TFIIH serves as both a loading control and to indicate the efficacy of the nuclear vs cytoplasmic fractionation. The HA tag is located on the exogenously expressed MLL^C subunit. The MLL^C antibody detects both endogenous and exogenous MLL. Representative result from N=3 experiments.

3.2 Mass Spectrometry Identifies Candidate Proteins for MLL-PTD Mediated Leukemogenesis

To assess cofactor binding, we performed immunoprecipitation using antibodies against the FLAG or HA tag in MLL-PTD, MLL-WT or untransfected cells. We could visualize an enrichment of the HA tag in the elutions of both the HA and FLAG IPs and its depletion in the supernatants (Figure 5). Following this confirmation of a successful IP, samples were run on a gradient gel and silver stained to visualize the proteins (Figure 6). Digested bands from the silver stained gels were sent for tandem mass spectrometry performed by the OHRI Proteomics Core Facility using the Orbitrap Fusion Lumos (Thermo Fisher). In total, the mass spec data identified 449 proteins from the HA IPs and 240 for the FLAG IPs (Figure 7).

Mass spectrometry identified the WRAD complex members WDR5, ASH2L and RBBP5 as interacting partners of both MLL-PTD and MLL-WT (Table 1). Uncomplexed MLL exhibits weak methyltransferase activity and relies on its association with this highly conserved complex (Dou et al., 2006). When validated by western blot, immunoprecipitation experiments show no difference between the interaction of MLL-PTD with WRAD complex members ASH2L and RBBP5 compared to MLL-WT (Figure 8). These results are noteworthy as they suggest, for the first time, that MLL-PTD retains its ability to bind the WRAD complex.

The IP-MS experiment identified several candidate proteins that required further validation. We chose to focus on two proteins that differentially interact with MLL-PTD: one known MLL-WT interacting protein and one novel candidate. Menin (MEN1) is a

unique cofactor of MLL1 and MLL2 but not of other SET domain containing methyltransferases (Dou et al., 2006). It binds to the N-terminus of MLL close to the start of the PTD duplication and is critical for leukemic transformation of MLL-fusion mutants (Huang et al., 2012; Yokoyama et al., 2005). MEN1 was shown in the mass spec data to have an enrichment in the MLL-PTD sample compared to MLL-WT. Immunoprecipitation of HA- and FLAG-tagged protein shows 1.5-fold MEN1 enrichment in the elution from the MLL-PTD IP compared to the WT when validated by western (Figure 8). This suggests an increased interaction with the mutant and is the first known evidence demonstrating that MLL-PTD keeps the WT's ability to interact with the oncogenic cofactor menin.

Proteomic analysis uncovered several novel MLL interactors. We chose to focus on the enzyme lysine acetyltransferase 2A (KAT2A) also called general control nonderepressible 5 (GCN5). KAT2A is the catalytic subunit of the STAGA complex (Martinez et al., 1998). All ten members of this complex were identified in the mass spec data and were at least 2x enriched in the MLL-PTD sample compared to the wild type (Table 1). KAT2A is a known interacting partner of the WRAD complex member WDR5, however, it has not previously been shown to interact with WDR5 when it is bound to MLL (Wang et al., 2008). The ability of KAT2A and STAGA complex members to associate with MLL is a novel finding.

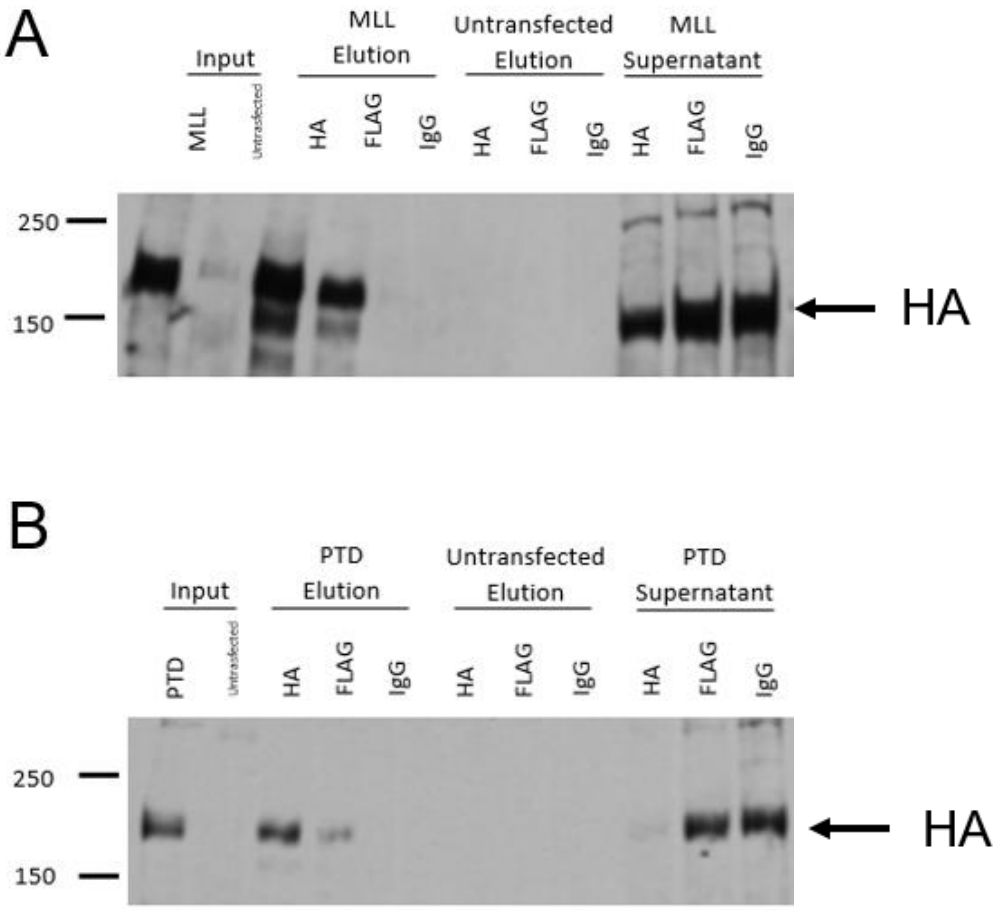

Figure 5. IPs successfully pull-down HA tagged MLL. Immunoprecipitation of nuclear extracts from 293T cells transfected with plasmids expressing FLAG and HA tagged MLL-WT (A) or MLL-PTD (B). Both the HA and FLAG IPs show an enrichment of HA expression in the elution and depletion in the supernatant. Representative result from N=3 experiments.

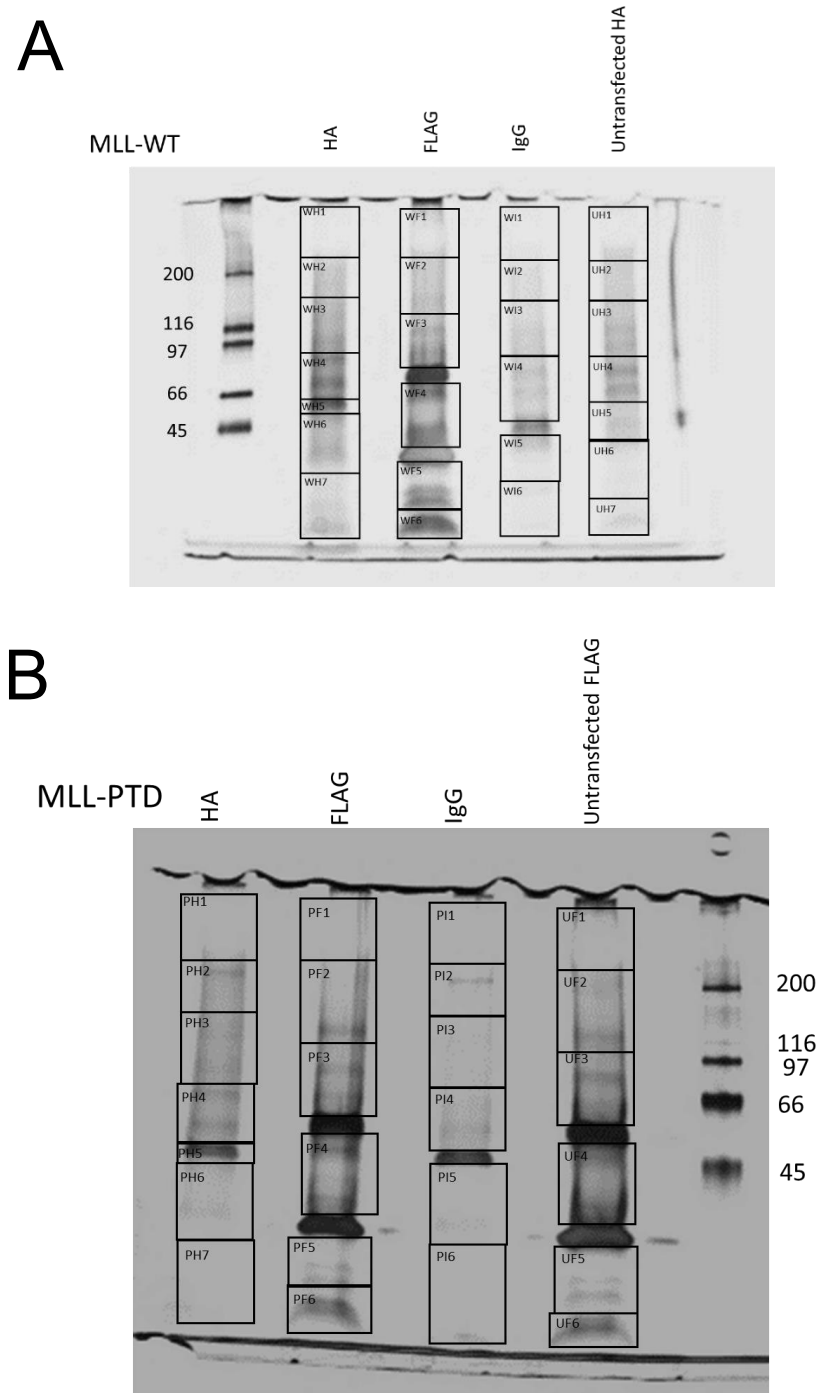

Figure 6. Silver Stain of the IPs. The elutions from the IPs of MLL-WT (A) and MLL-PTD (B) transfected cells were run on 4-20% gradient gels and silver stained. These bands, indicated by boxes, were excised and analysed by tandem mass spectrometry. The bands for antibody light and heavy chain were excluded from analysis.

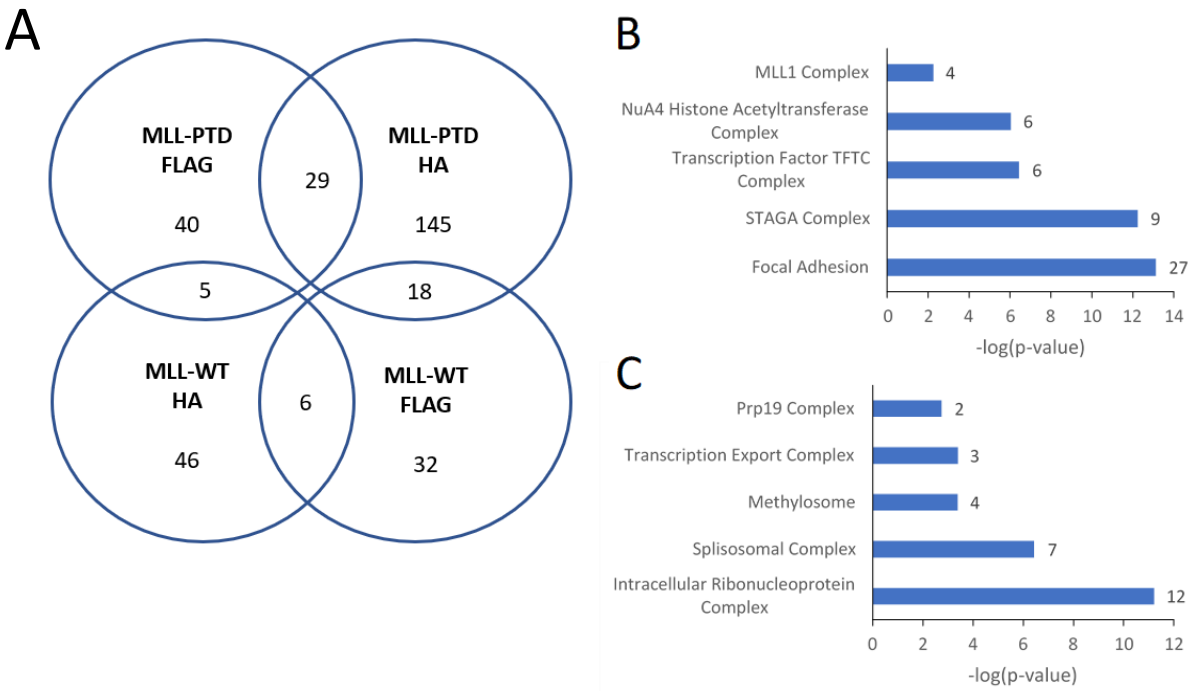

Figure 7. Summary of mass spec results. Numbers within the Venn diagram (A) represent the number of identified proteins which had a greater than 2-fold change between MLL-PTD and MLL-WT. There were 40 proteins that had at least 2x more abundance in the MLL-PTD IP from FLAG and 145 from HA. 29 proteins had a greater than 2-fold abundance in both the FLAG and HA IPs. The graphs show selected cellular component GO term results from the proteins at least 2x more abundant in MLL-PTD (B) and 2x more abundant in MLL-WT (C). The numbers beside the bars represent the total number of proteins from the sample with that GO term.

Table 1. Summary of candidate proteins. Normalized intensity is based on the ratio of the area under the MS1 peak curves. Fold change shows the relative difference between PTD and WT samples. INF represents an infinite fold change and is used when peptides were identified in the PTD sample but not the WT.

Complex	Protein	Molecular Weight (kDa)	Normalized Intensity		Fold Change
			WT	PTD	PTD/WT
STAGA	SUPT7L	46	0	2097152	INF
	KAT2A	94	524288	2581897	4.92
	TADA2B	48	851708.4	2247672	2.64
	TAF10	22	2097152	8990687	4.29
	TAF12	18	1204498	8388608	6.96
	TAF6L	68	602248.8	2965821	4.92
	TRRAP	436	0	1482910	INF
	TADA1	37	1482910	3651354	2.46
	TAF5L	66	978356	3913424	4.00
	TADA3	49	456419.2	1703417	3.73
WRAD	ASH2L	69	2408995	2767209	1.15
	RBBP5	59	5163794	2097152	0.41
	WDR5	37	2581897	1290948	0.50
-	MEN1	61	1589344	2097152	1.32

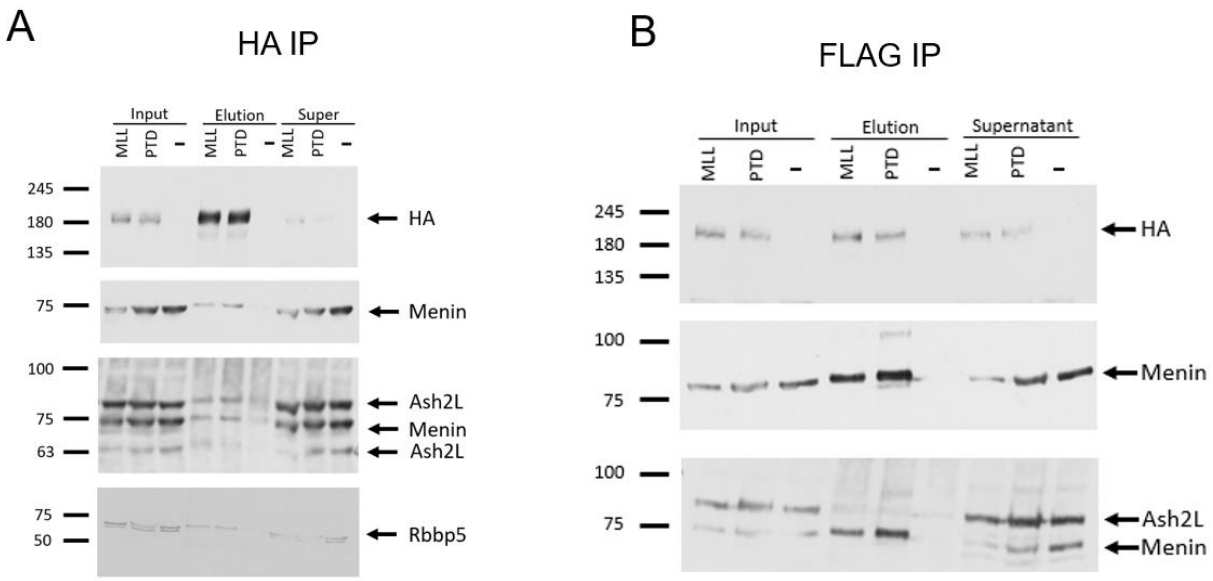

Figure 8. MLL-PTD interacts with the WRAD complex. Immunoprecipitation of nuclear extracts from 293T cells transfected with plasmids expressing FLAG and HA tagged MLL-WT or MLL-PTD. Both the HA (A) and FLAG (B) IPs show an enrichment of HA expression in the elution and depletion in the supernatant. HA abundance in the elution is equilibrated between MLL-WT and MLL-PTD. MLL's cofactors menin, RBBP5 and ASH2L are co-immunoprecipitated. '–' represents the untransfected negative control. 'Super' indicates the supernatant. Antibodies were revealed in the order shown (top to bottom). Representative result from N=3 experiments.

3.3 Small Molecule Inhibitors of Menin and KAT2A Impair MLL-PTD Cell Proliferation

Our proteomic data has uncovered candidate cofactors in MLL-PTD mediated oncogenesis. Following validation of these interactions by western, we tested these factors as potential targets for antileukemic drugs. In addition to providing rationale for preclinical efficacy, testing cells which harbour MLL-PTD with inhibitors of the interactions uncovered by mass spec will confirm their importance for leukemogenesis.

As our mass spec data suggested an enriched interaction between MLL-PTD and menin compared to MLL-WT, we tested the menin-MLL inhibitor MI-503 on a panel of four cell lines (Table 2). MI-503 prevents the interaction by binding to the MLL site on menin. EOL-1 expresses an MLL-PTD fusion mutation (Figure 3). HL-60 and MV4-11 serve as negative and positive controls respectively based on the published literature (Borkin et al., 2015). K562 cells were included as they have a combination of elevated *HOXA9* expression and MLL-WT. MI-503 has been studied for anti-leukemic abilities in MLL fusions. However, its efficiency in MLL-PTD leukemias has not been tested.

Table 2. MLL mutational status of four cell lines used for small molecule inhibition assays.

Cell Line	Patient Status	MLL Status	Other Genetic Abnormalities
EOL-1	AML	MLL-PTD	FIP1L1-PDGFRA
MV4-11	AML	MLL-AF4	FLT3-ITD
K562	CML	MLL-WT	BCR-ABL1
HL-60	AML	MLL-WT	Amplified MYC

Our results show that after only 48h of drug treatment, EOL-1 cells, which express MLL-PTD, have the lowest IC50 value at 0.331µM (Figure 9). EOl-1 cells, over the course of

the proliferation assay, continue to be the most sensitive to menin-MLL disruption. By day 12, their IC50 value is further reduced to 0.068μM. MV4-11 cells are less sensitive to the drug, however, there is no statistically significant difference among their IC50 values and those of EOL-1 at any of the tested time points. MV4-11 have IC50 values of 0.714μM and 0.1846μM at days 2 and 12 respectively. K562 cells are significantly less responsive to menin-MLL inhibition compared to EOL-1 by day 12. HL-60, our negative control, initially have low sensitivity to the drug however by day 12, they have a similar IC50 value to K562. Therefore, all cell lines show a time and dose dependent response to MI-503 (Figure 9). EOL-1 cells are the most sensitive to disruption of the MLL-menin interaction. This is followed, in order of sensitivity, by MV4-11, K562 and finally, HL-60.

In order to further investigate the effects of menin-MLL inhibition, we analysed apoptosis using annexin and 7aad staining after 2, 4, and 8 days of treatment with MI-503. In EOL-1 cells, there was an increase in apoptotic and necrotic cells compared to the DMSO control 8 days after treatment with 0.5μM of MI-503 (Figure 10). MV4-11 cells also observed a time and dose dependent increase in apoptosis however, it is not as much as is seen in EOL-1. Only 29.6% and 50.1% of cells remained viable for EOL-1 and MV4-11, respectfully, after 8 days of drug treatment (Figure 10D). There was no change in apoptosis or necrosis among K562 and HL-60 cells when treated with 0.5μM of the drug. The observation that EOL-1 cells exhibit the greatest apoptotic and necrotic response after drug treatment is in agreement with the proliferation results from Figure 9.

Our mass spec data indicated an enriched interaction among MLL-PTD and STAGA complex members, which includes the acetyltransferase KAT2A, in comparison to MLL-WT. Therefore, we next tested a KAT2A inhibitor, MB-3, to assess its ability to impede MLL-PTD leukemic cell proliferation. MB-3 prevents the binding of the substrate acetyl-CoA with KAT2A. The literature shows that disruption of KAT2A's acetyltransferase activity reduces leukemic burden in MLL-fusion cancers (Tzelepis et al., 2016). However, it has yet to be tested in MLL-PTD. Inhibition of KAT2A with MB-3 suggests that EOL-1 cells have a time and dose-dependent response resulting in the lowest IC50 value. This difference is significant when compared to HL-60, K562 and MV4-11 cells only 48h post-treatment (Figure 11). However, at 59µM after 6 days, this IC50 value for EOL-1 cells is higher than the most sensitive MLL-fusion mutations published in the literature (~30µM) (Tzelepis et al., 2016). Despite this, because the MLL-PTD cell line is significantly more sensitive than those expressing MLL-WT, it does validate the importance of KAT2A for PTD mediated leukemogenesis. Consistent with the literature, we observe that the MLL-AF4 cell line MV4-11 is not sensitive to MB-3 (Tzelepis et al., 2016; Figure 11). This suggests that perhaps only a subset of MLL-rearranged leukemias are truly suitable for KAT2A inhibition.

Finally, we tested a drug currently in clinical trial to treat MLL-PTD leukemia in order to compare it to MEN1 and KAT2A inhibition (Stein et al., 2015). DOT1L is the only known H3K79 methyltransferase. Small molecule inhibitors were originally developed to treat MLL-fusion leukemias upon discovery that the C-terminal fusion partner often retained its native ability to bind DOT1L (Bernt et al., 2011). The MLL^N terminus would

then aberrantly recruit DOT1L to gene loci resulting in misregulation and leukemogenesis (Bernt et al., 2011; Okada et al., 2005). Treatment using a DOT1L inhibitor on MLL-PTD cells demonstrated efficacy including decreased leukemic burden, and improved survival in murine xenograft models (Kuhn et al., 2015).

Consistent with the literature, our results show MLL-PTD positive EOL-1 cells have reduced proliferation after treatment with the DOT1L inhibitor (Figure 12). Our IC50 value of 0.58μM closely matches the published value of 0.68μM (Kuhn et al., 2015). In addition, there is very little difference between the IC50 values of EOL-1 cells and MV4-11, which harbour the MLL-AF4 mutant. Finally, we observe that EOL-1 cells respond faster to menin inhibition than they do to DOT1L inhibition (Figure 13).

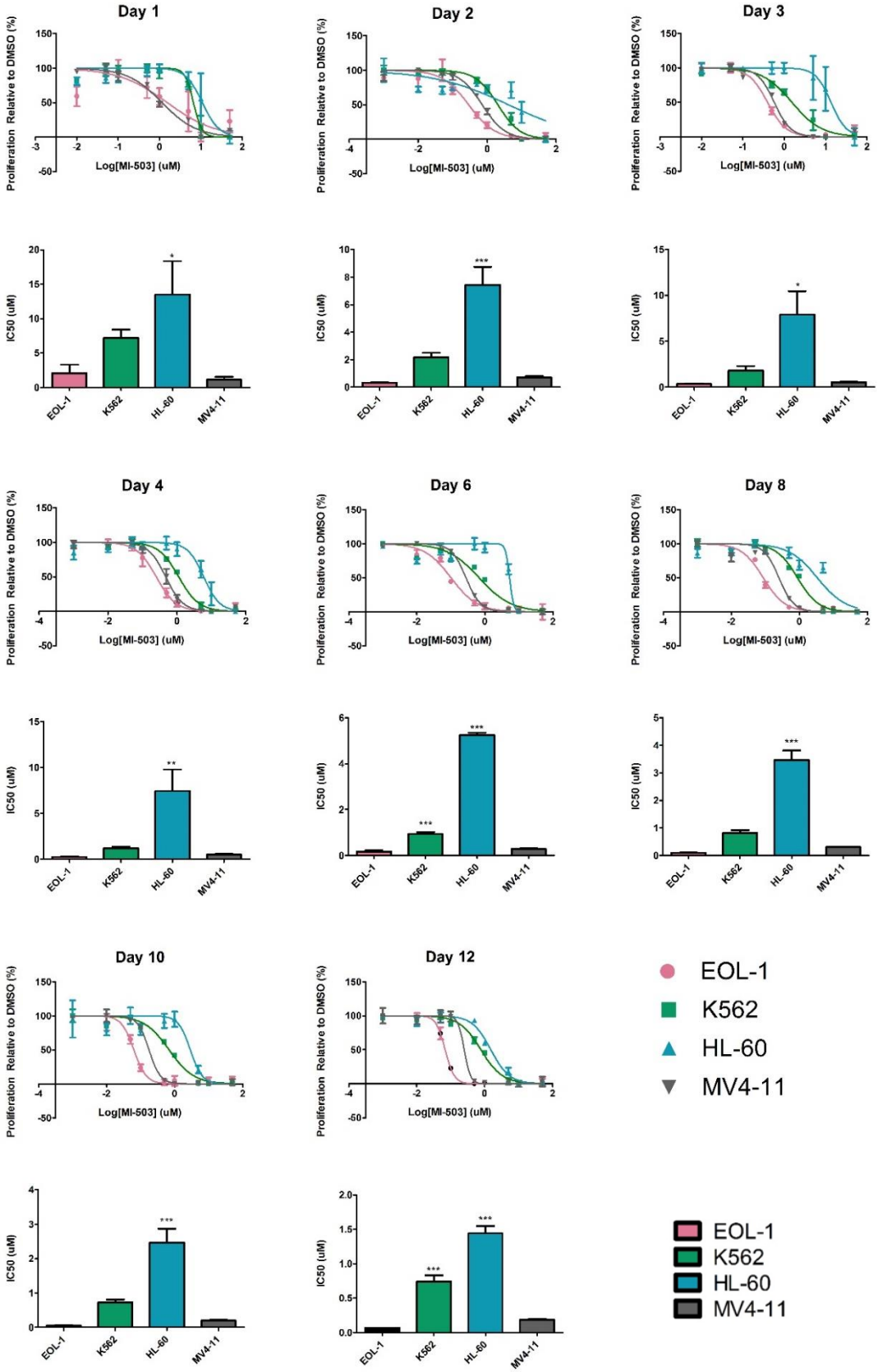
Day 1
Day 2
Day 3
Day 4
Day 6
Day 8
Day 10
Day 12
Proliferation Relative to DMSO (%)
Log[MI-503] (uM)
IC50 (uM)
EOL-1
K562
HL-60
MV4-11

Figure 9. Menin inhibition selectively kills MLL-rearranged leukemias. MTS proliferation assay performed on human leukemic cell lines EOL-1 (MLL-PTD), K562 (MLL-WT), HL-60 (MLL-WT) and MV4-11 (MLL-AF4). The effect of menin inhibitor MI-503 on the proliferation of drug treated cells was analyzed every 24-48h for 12 days and compared to a 0.2% DMSO control. The IC50 values from the four cell lines are shown below each proliferation graph. Error bars represent the SEM of N=4 replicates. Asterisks indicate significance relative to EOL-1 based on one-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

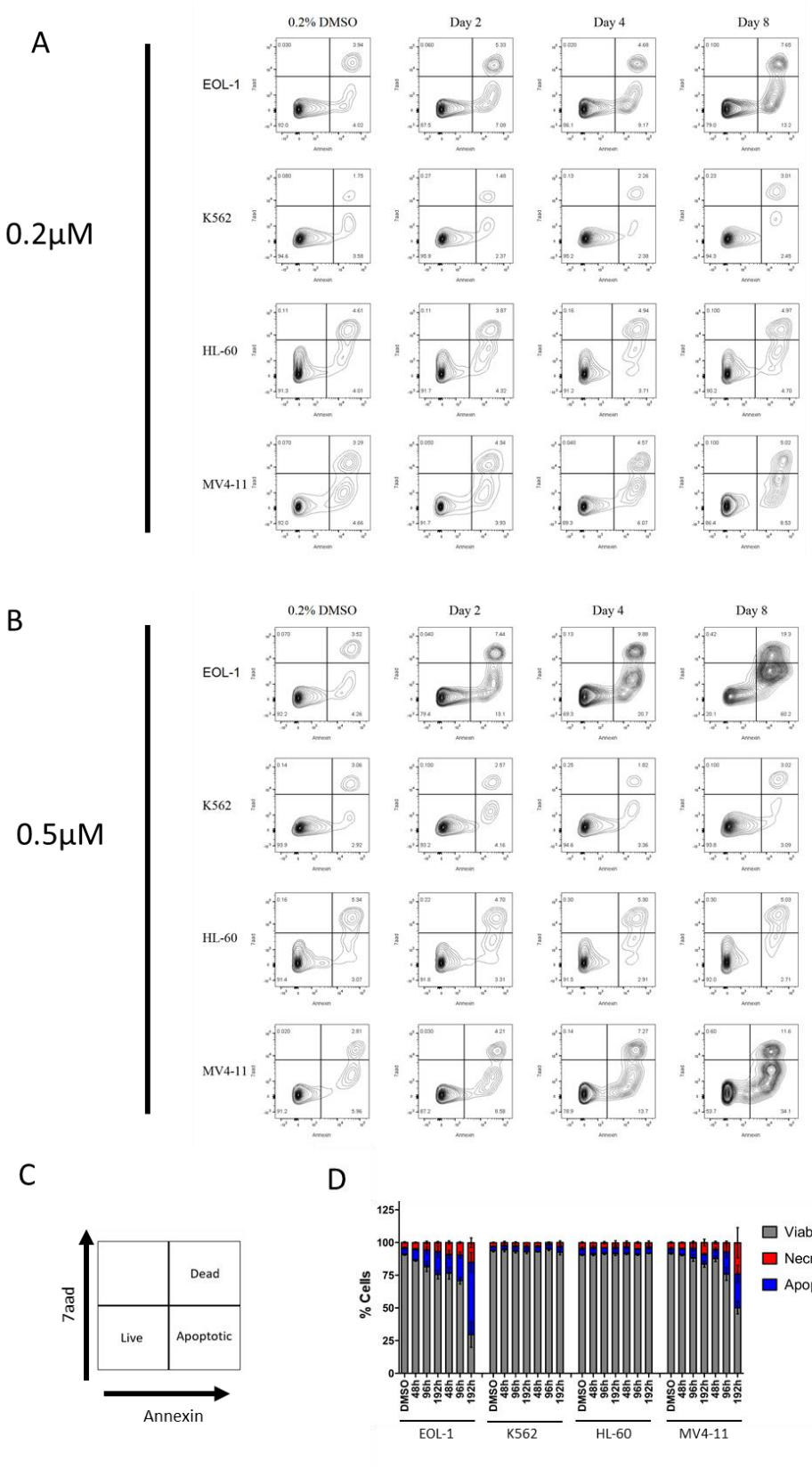
A
0.2µM
0.2% DMSO
Day 2
Day 4
Day 8
EOL-1
K562
HL-60
MV4-11
B
0.5µM
C
7aad
Annexin
Dead
Live
Apoptotic
D
% Cells
Viable
Necrotic
Apoptotic
DMSO
48h
96h
192h
EOL-1
K562
HL-60
MV4-11

Figure 10. Menin inhibition induces apoptosis in MLL-rearranged leukemic cell lines. Leukemic cell lines EOL-1 (MLL-PTD), K562 (MLL-WT), HL-60 (MLL-WT) and MV4-11 (MLL-AF4) treated with 0.2µM (A) or 0.5µM (B) of the menin inhibitor MI-503 for 8 days and compared to a 0.2% DMSO control. Cells were analyzed using flow cytometry for markers 7aad and annexin (C). The percent of viable, necrotic and apoptotic cells for each treatment condition are indicated (D). Error bars represent the SEM of N=3 replicates. Panels A and B show one representative experiment.

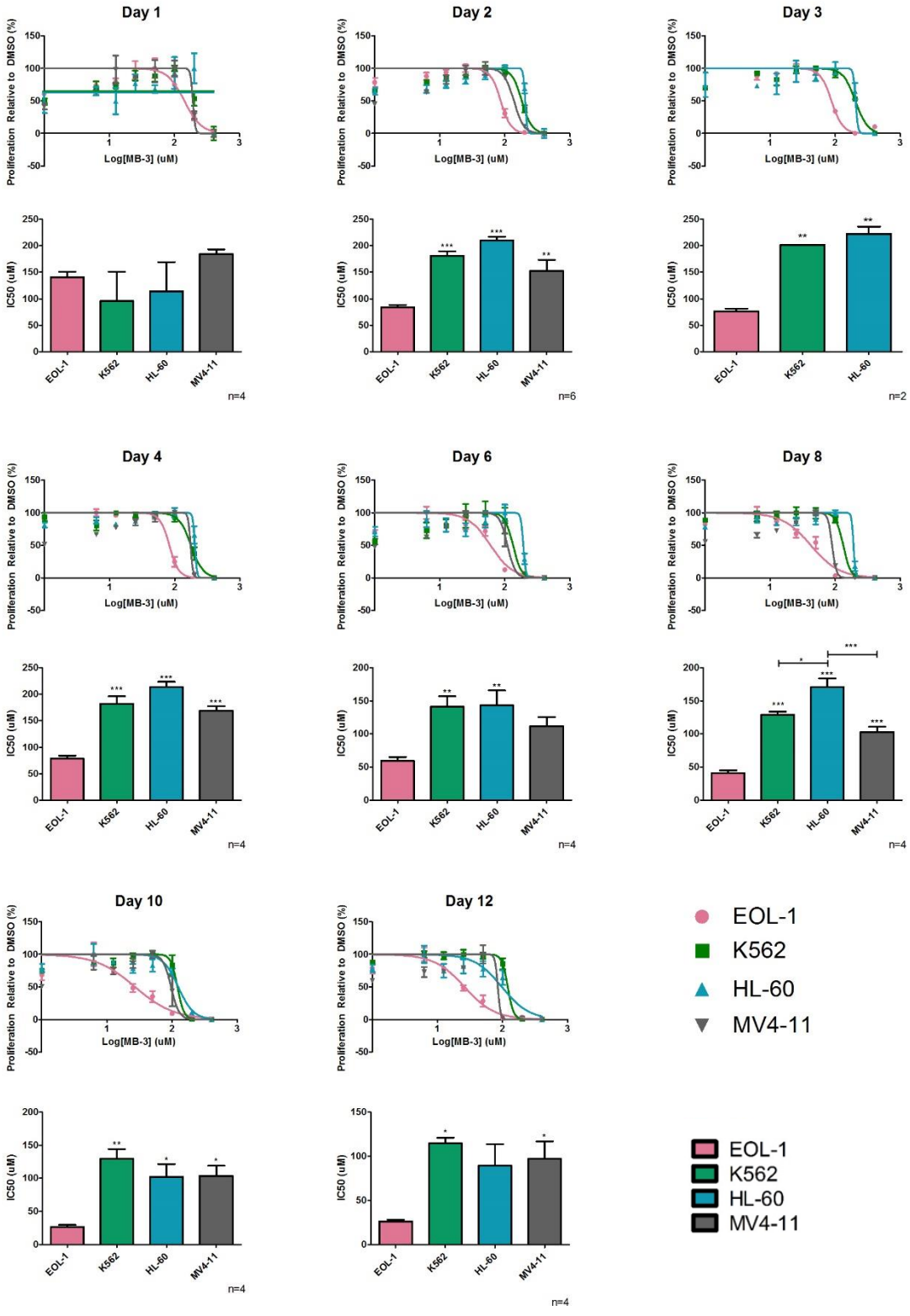
Day 1
Day 2
Day 3
Day 4
Day 6
Day 8
Day 10
Day 12
Proliferation Relative to DMSO (%)
Log[MB-3] (uM)
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
n=4
n=6
n=2

Figure 11. MLL-PTD is sensitive to KAT2A inhibition. MTS proliferation assay performed on human leukemic cell lines EOL-1 (MLL-PTD), K562 (MLL-WT), HL-60 (MLL-WT) and MV4-11 (MLL-AF4). The proliferation of cells treated with the KAT2A inhibitor MB-3 were analyzed every 24-48h for 12 days and compared to a 0.4% DMSO control. The IC50 values from the four cell lines are shown below each proliferation graph. Error bars represent the SEM of N=2 or 4 as indicated. Unless otherwise marked, asterisks indicate significance relative to EOL-1 based on one-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

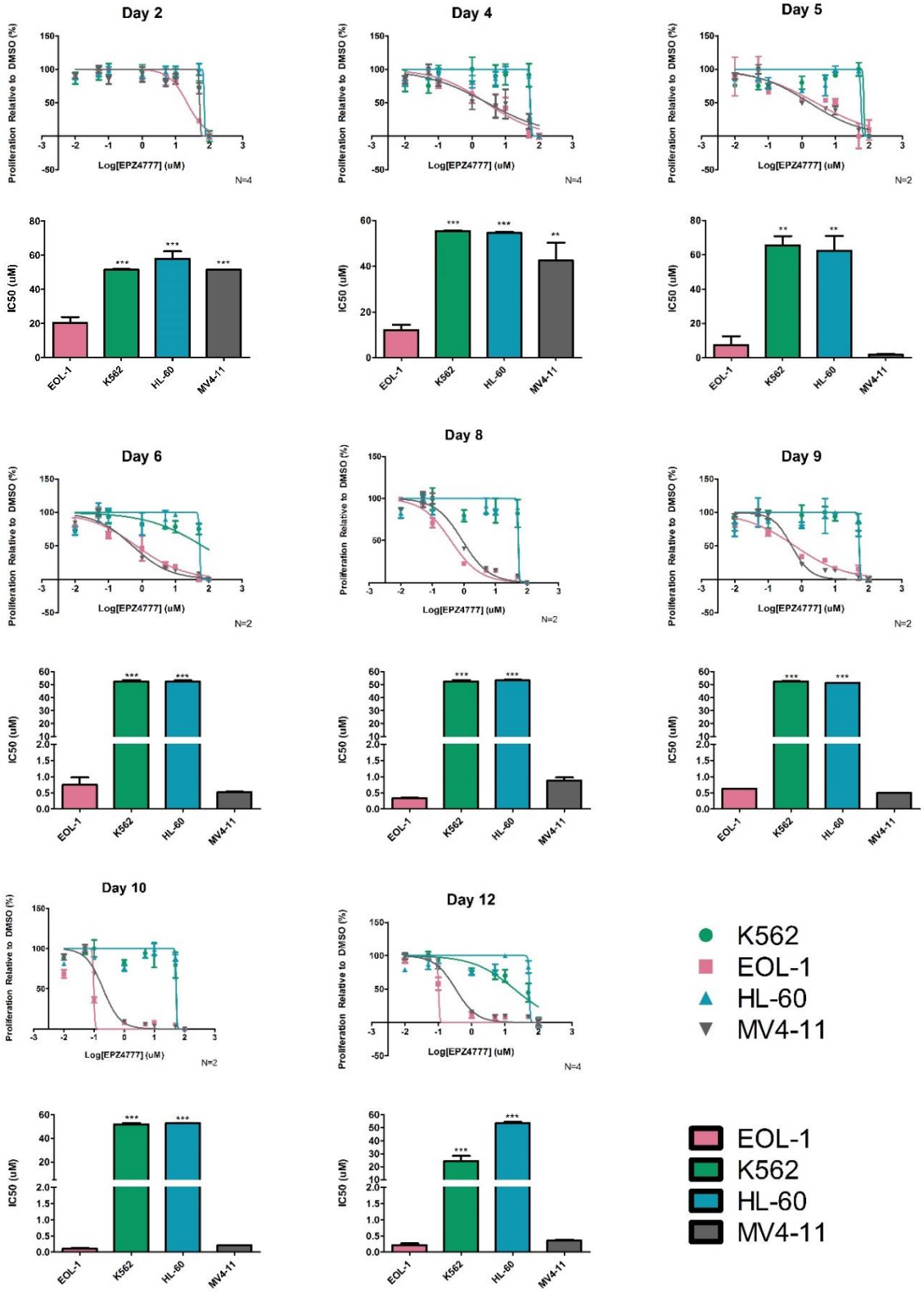
Day 2
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=4
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 4
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=4
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 5
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=2
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 6
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=2
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 8
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=2
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 9
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=2
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 10
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=2
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
Day 12
Proliferation Relative to DMSO (%)
Log[EPZ4777] (uM)
N=4
IC50 (uM)
EOL-1
K562
HL-60
MV4-11
K562
EOL-1
HL-60
MV4-11
EOL-1
K562
HL-60
MV4-11

Figure 12. DOT1L inhibition selectively kills MLL-rearranged leukemias. MTS proliferation assay performed on human leukemic cell lines EOL-1 (MLL-PTD), K562 (MLL-WT), HL-60 (MLL-WT) and MV4-11 (MLL-AF4). The proliferation of DOT1L inhibitor EPZ4777 treated cells was analyzed every 24-48h for 12 days and compared to a 0.2% DMSO control. The IC50 values from the four cell lines are shown below each proliferation graph. Error bars represent the SEM of N=2 or 4 as indicated. Asterisks indicate significance relative to EOL-1 based on one-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

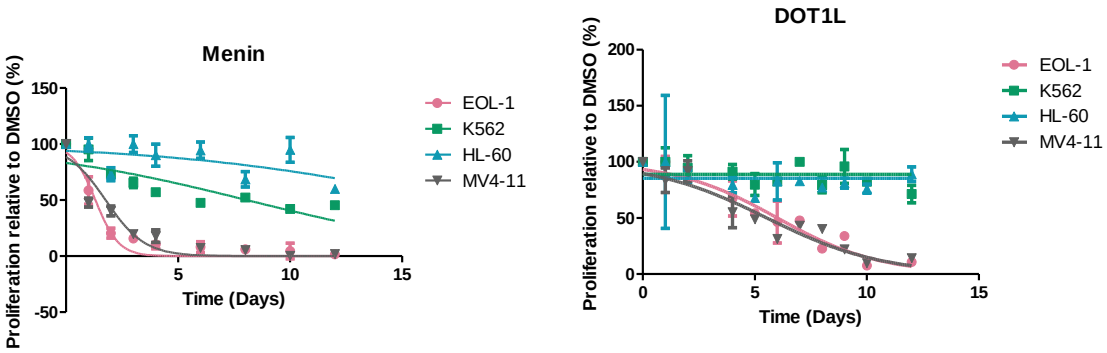

Figure 13. Menin inhibition kills MLL-rearranged leukemic cells faster than DOT1L inhibition. MTS proliferation assay performed on human leukemic cell lines EOL-1 (MLL-PTD), K562 (MLL-WT), HL-60 (MLL-WT) and MV4-11 (MLL-AF4). The proliferation of cells treated with 1µM of menin inhibitor MI-503 or DOT1L inhibitor EPZ4777 was analyzed every 24-48h for 12 days and compared to a 0.2% DMSO control. Error bars represent the SEM of N=2.

4. Discussion

4.1 Discussion and Significance of Results

The MLL-PTD mutation is found in ~5% of acute myeloid leukemia and confers a poor prognosis. This thesis endeavoured to provide additional details on the molecular mechanism for leukemogenesis of MLL-PTD in order to facilitate improved therapeutic interventions for patients. Our focus has been on MLL-PTD's proteomic interactions and we have, for the first time, identified the interacting partners of the leukemogenic histone methyltransferase MLL-PTD. We chose to focus on two co-factors of MLL-PTD, MEN1 and KAT2A, as both were more abundant in the elution of the PTD IPs compared to the WT, both are known oncogenes and both have drugs which target their activities.

Through nuclear and cytoplasmic fractionation of protein extracts from cells expressing MLL-WT and MLL-PTD, we demonstrate that MLL-PTD retains the WT's ability to enter the nucleus. Our ability to detect the HA-tagged C-terminus subunit in the nuclear fraction implies proper shuttling by the N-terminus subunit (Yokoyama et al., 2011). In addition, our ability to detect the C-terminus fragment at the appropriate size (180kDa) suggests no defect in MLL-PTD's ability to be cleaved in the cytoplasm into two subunits by taspase1. Uncleaved MLL causes homeotic transformations and misregulated cell cycle progression (Hsieh et al., 2003; Takeda et al., 2006). Our results suggest that the defects in *HOX* gene expression associated with MLL-PTD leukemia are not a result of uncleaved protein.

Expression of the *MLL-PTD* mRNA in transfected 293T cells is approximately equivalent to expression in the *MLL-PTD* mutant cell line EOL-1. This result suggests that we are not overexpressing *MLL-PTD* in our system compared with a patient-derived leukemic cell line. As overexpression can introduce irregularities such as improper protein folding and post-translational modification, this result reinforces confidence in the data obtained from our model system.

Through western blot and mass spec analysis, we showed, for the first time, that MLL-PTD retains its ability to interact with the WRAD complex. This interaction is lost in MLL-fusion leukemogenic mutants (Vedadi et al., 2017). The WRAD complex is composed of the proteins WDR5, RBBP5, ASH2L and DPY30. MLL adopts a conformation with weak methyltransferase activity when alone and requires interaction with these proteins to increase its catalytic activity. We detect ASH2L, WDR5 and RBBP5 in mass spec results from both MLL-WT and MLL-PTD samples suggesting their association with both proteins. We were unable to detect DPY30 in our immunoprecipitation experiments, however, it is the least critical protein of the WRAD complex and is the only member to not directly bind to MLL-WT (Ernst and Vakoc, 2012; Li et al., 2016; Rao and Dou, 2015). Mass spec results indicate a small decrease in MLL-PTD's interaction with RBBP5 and WDR5 compared to MLL-WT, however, by western blot we observed no difference in their ability to associate with the PTD protein compared to the WT. MLL-PTD has been shown to increase H3K4 methylation at the promoter of *Hox* genes despite the repression of the WT allele (Dorrance et al., 2008).

This is consistent with our finding that MLL-PTD retains the ability to interact with the WRAD complex, and therefore, likely maintain it's methyltransferase activity.

Multiple endocrine neoplasia type 1 (Menin or MEN1) is a 67kDa protein that acts primarily as a tumour suppressor (Chandrasekharappa et al., 1997; Lemmens et al., 1997). Mutations in this protein cause a disease by the same name which is characterized by the development of tumours in the endocrine glands: commonly in the parathyroid, pancreas and pituitary (Schussheim et al., 2001). Menin binds to proteins involved in transcriptional regulation and chromatin modification, including MLL1 and MLL2 (Huang et al., 2012). Menin helps MLL associate with its downstream target genes including the *HOXA9* promoter (Chen et al., 2006). In fact, murine menin knockout models result in greater reduction of H3K4 methylation at *Hox* loci than MLL1 knockouts (Wang et al., 2009). However, menin does not contain any DNA binding domains and therefore cannot recruit MLL to gene loci alone. Menin tethers MLL to lens epithelium derived growth factor (LEDGF) (Yokoyama and Cleary, 2008). LEDGF contains a PWWP domain which is believed to interact with H3K36me3, as well as non-specifically bind DNA, and therefore this protein contains the domains required for recruitment of MLL to promotor regions by menin (Eidahl et al., 2013). When there is genetic disruption of the MEN1-MLL interaction in cells harbouring MLL-fusion mutations, there is a decrease in *HOXA9* overexpression (Yokoyama et al., 2005). MLL-rearranged leukemias require menin expression for their leukemic initiation and maintenance. However, menin is also not a necessary cofactor for MLL during normal hematopoiesis (Li et al., 2013). Taken together, menin inhibition was identified as a

promising potential therapy for leukemia. Small molecular inhibitors of the MEN1-MLL interaction have been shown to be effective in causing differentiation and apoptosis of MLL-fusion leukemic cells (Borkin et al., 2015). Furthermore, murine xenograft models show improved survival after treatment with MEN1 inhibitors. Because of these preclinical findings, MLL-menin inhibitors are currently being prepared to enter phase 1 clinical trials (Wu et al., 2017). Despite these findings in MLL-translocation mutations, it is unknown if menin is also critical for MLL-PTD leukemogenesis.

Our mass spec data indicates that menin has increased association with MLL-PTD compared to MLL-WT. There are two menin binding motifs located within the first 43 amino acids of MLL. These are positioned 5' to the partial tandem duplication. Therefore, this result cannot be justified on the basis of additional binding domains. As a result of our finding of increased interaction between MLL-PTD and menin, we tested increasing concentrations of the small molecule MLL-menin inhibitor MI-503 for its ability to prevent cell growth in four cell lines with different mutational profiles for MLL. We found that MI-503 can kill EOL-1 (MLL-PTD) and MV4-11 (MLL-AF4) cells in a dose-dependent manner while K562 cells are slightly sensitive to the drug and HL-60 are the most resistant. MV4-11 cells were included as a positive control and our IC50 value on day 8, 0.305μM, is similar to the published value of 0.25μM (Borkin et al., 2015). The results from apoptosis analysis mirror those from the MTS proliferation assay. EOL-1 and MV4-11 cells show significant, dose-dependent increases in cell death after treatment with the inhibitor. This data suggests that menin-MLL inhibition suppresses

AML cell proliferation through apoptotic cell death. This finding provides the rationale for further preclinical testing of the menin MLL inhibitor for MLL-PTD leukemia.

In addition to menin, we chose to focus on one of the novel interacting partners discovered in our mass spec data. KAT2A was more abundant in the elution of the PTD IP compared to the WT. It is a highly conserved acetyltransferase which is capable of global and locus-specific histone modification. In addition, it is able to acetylate non-histone proteins including several transcription factors and proteins involved in cell survival and cell cycle regulation (Bondy-Chorney et al., 2018; Fournier et al., 2016). KAT2A knockout mice show similarities to MLL knockouts as they have axial skeletal defects and a shift in the expression boundary of *Hox* genes (Lin et al., 2008). KAT2A is the catalytic subunit of the STAGA complex and all ten members of this complex were at least 2x more abundant in the mass spec results from the MLL-PTD IP compared to MLL-WT. The multiprotein STAGA complex has not been completely characterized, however, it is thought to act as a coactivator through histone acetylation and by recruiting mediator complexes (Liu et al., 2008; Martinez et al., 2001). We have limited validation of our mass spec result by western blot and further work is needed to determine if it has a direct interaction with MLL (Appendix D).

KAT2A and three STAGA complex members (TAF5L, TAF6L and TADA2B) were shown in a CRISPR dropout screen to be essential to three of the five tested AML cell lines. Genetic and small molecule disruption of KAT2A caused impaired cell proliferation in cell lines with MLL-AF9 mutations but not in HL-60 or MV4-11 cells

(MLL-AF4) (Tzelepis et al., 2016). We confirmed that HL-60 and MV4-11 cell lines are not sensitive to MB-3 and our IC50 values at day 4 (181.1µM and 229.2µM) match those of the literature at day 5 (~200µM). In contrast, we found that MLL-PTD containing EOL-1 cells are sensitive to MB-3 inhibition in a dose- and time-dependent manner. MB-3 prevents KAT2A, when bound to the substrate acetyl-CoA, from associating with its histone targets (Biel et al., 2004). This suggests that KAT2A is important in MLL-PTD leukemogenesis but not necessarily in all MLL-rearranged leukemia or those harbouring different mutations. In a murine model, inhibition with MB-3 caused impaired AML cell expansion, and the genetic disruption of KAT2A caused impaired cell proliferation and prolonged mouse survival (Tzelepis et al., 2016).

As summarized in the introduction, there are few published articles about the molecular mechanisms through which MLL-PTD induces and/or maintains leukemia. The limited information available about the role of MLL-PTD in hematopoiesis and leukemia is derived mainly from a murine knock-in published by Dorrance et al. (Dorrance et al., 2006). Their key finding revealed that although $MLL^{PTD/WT}$ mice did not develop leukemia, the animals experienced abnormal hematopoiesis and axial skeletal defects. This paper found an increase in H3 and H4 acetylation and H3K4 methylation in addition to a decrease in H3K9 methylation at the *Hoxa7* and *Hoxa9* loci of bone marrow cells and splenocytes. Our proteomic work provides a potential mechanism for this result (Figure 14). Our data, which reveals the increased association of MEN1 with the PTD mutant compared to the WT, could cause increased binding of MLL at the *HOXA9* locus. This could, therefore, cause the increase in H3K4 methylation seen in the $MLL^{PTD/WT}$

mice. Furthermore, the binding of MLL to CpG nucleotides in the *HOXA9* promoter protects these clusters from repressive DNA methylation (Erfurth et al., 2008). KAT2A is known to acetylate targets on histone H3 (K9, 14, 18, 23, 27, 36) as well as H4 (K8) (Huynh and Casaccia, 2013). Genetic and small molecule inhibition of KAT2A caused a decrease in histone acetylation at the promoter of *HOXA9* and *HOXA10* (Tzelepis et al., 2016). The novel interaction of KAT2A with MLL-PTD could cause the increased histone acetylation seen in the *HOXA9* promoter region of PTD knock-in mice. Furthermore, we could speculate that the decrease in repressive H3K9 methylation in these animals could be a result of the increased competition of activating acetylation at this same lysine residue by KAT2A (Gates et al., 2017). Further work is needed to confirm these hypotheses.

As these epigenetic modifications are associated with gene expression, our observed changes in proteomic interactions between MLL-PTD and MLL-WT could account for the increase in *HOXA9* expression in MLL-PTD leukemia. Indeed, when MLL-rearranged cell lines are treated with MI-503 or MB-3, there is a significant decrease in *HOXA9* gene expression (Borkin et al., 2015; Tzelepis et al., 2016). Clinically, MLL-PTD patients have significantly elevated levels of *HOXA9* (Gao et al., 2016). As *HOXA9* overexpression prevents the differentiation of myeloid progenitor cells, this elevated expression would be consistent with the finding that MLL-PTD patients have early hematopoietic phenotypes and significantly lower absolute leukocyte counts as well as higher numbers of immature CD34+ cells at the time of diagnosis (Steudel et al., 2003).

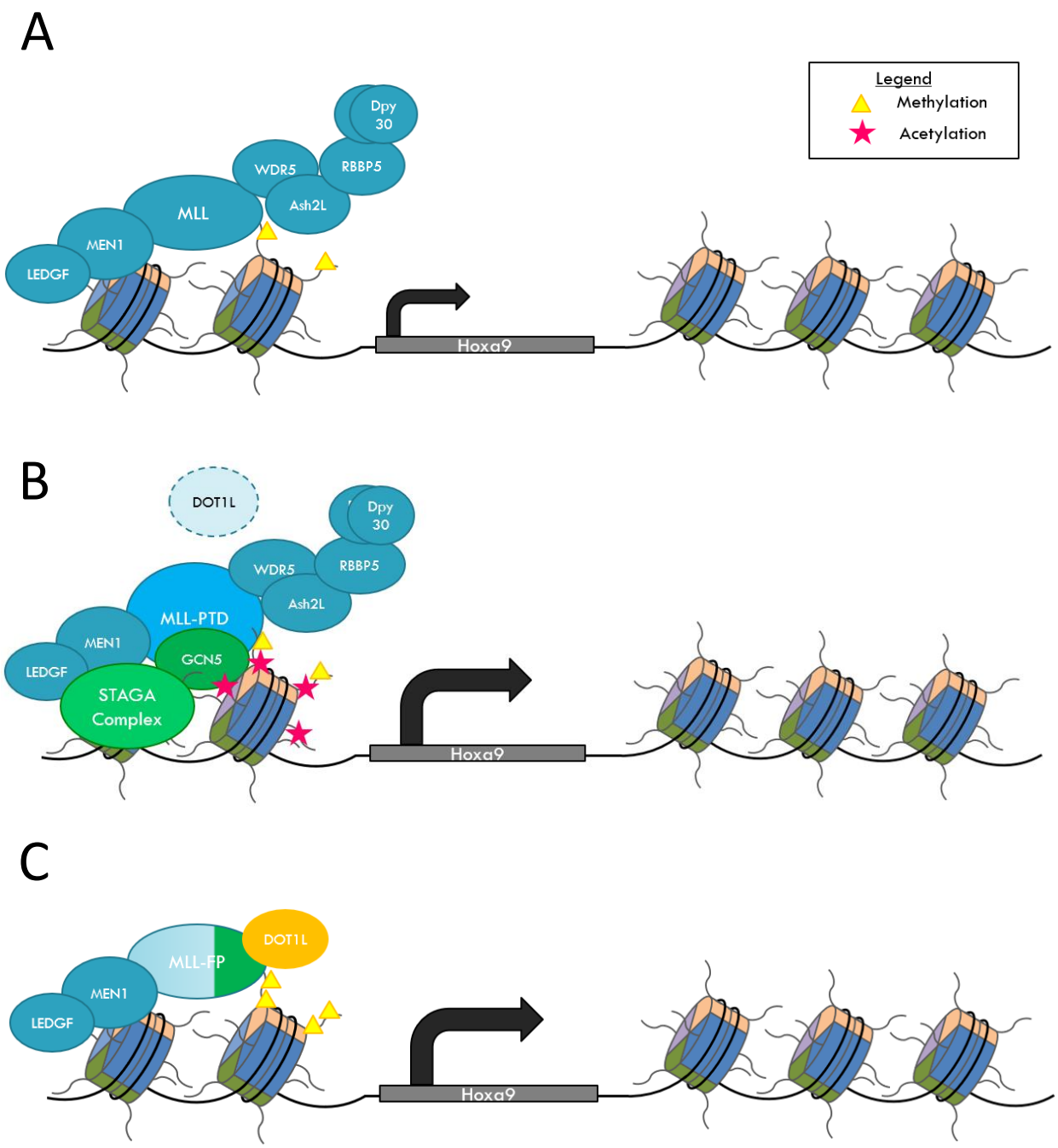

Figure 14. Working hypothesis for MLL-PTD's role in leukemogenesis. MLL-WT (A) is recruited to the *HOXA9* promoter by menin (MEN1) and other transcription factors. MLL, because of its association with the WRAD complex, can methylate histone 3. MLL-PTD (B) retains this association while MLL-fusion proteins (MLL-FP) lose the WRAD interaction (C). MLL-PTD recruits GCN5 (KAT2A) which can acetylate histones 3 and 4. MLL fusion proteins can recruit the H3K79 methyltransferase DOT1L. It is currently unknown the role DOT1L plays in MLL-PTD mediated leukemogenesis. Both MLL-PTD and MLL-FP cause overexpression of *HOXA9*.

There are currently two therapies which are undergoing clinical trials that have shown *in vitro* and *in vivo* pre-clinical efficacy on MLL-PTD leukemias. The first exploits MLL-PTD leukemias' increased global DNA methylation (Whitman et al., 2008). This silencing mark is mediated by DNA methyltransferases (DNMT). *In vitro*, the use of DNA hypomethylating agent 5'-aza-2'-deoxycytidine (5AD also called decitabine), in combination with the histone deacetylase inhibitor AR42, causes a decrease in MLL-PTD to MLL-WT ratio and this results in the death of MLL-PTD blasts (Whitman et al., 2005). *In vivo*, MLL-PTD and FLT3-ITD ($MLL^{PTD/WT}$:$Flt3^{ITD/WT}$) double knock-in mice have a 1.7-fold increase in global DNA methylation and have elevated DNMT1, 3a and 3b mRNA. Mice treated with this drug combination show increased survival and an induction of differentiation and apoptosis of blasts (Bernot et al., 2013).

The second therapy, which has just shown positive results in a phase 1 clinical trial, is the DOT1L inhibitor Pinometostat (ClinicalTrials.gov identifier: NCT01684150). DOT1L is the only known histone 3 lysine 79 methyltransferase which is an activating epigenetic mark in eukaryotes (Jones et al., 2008). Several of the common MLL fusion partners, such as the proteins AF4, AF9, AF10 and ENL, bind DOT1L. They retain this ability when fused to MLL causing aberrant recruitment of DOT1L to the loci under the regulation of MLL (Bitoun et al., 2007; Mohan et al., 2010; Mueller et al., 2007; Okada et al., 2005). These MLL-rearranged leukemias are dependent on this H3K79 methyltransferase for leukemogenic initiation and maintenance in murine xenograft models. Treatment with the DOT1L inhibitor EPZ004777 causes myeloid differentiation, a reduction of global H3K79 and impaired proliferation of MLL-fusion samples both *in*

vitro and *in vivo* (Bitoun et al., 2007; Mohan et al., 2010; Mueller et al., 2007; Okada et al., 2005). Furthermore, DOT1L inhibition has the ability to reduce cell numbers and induce differentiation both *in vitro* and *in vivo* in cell lines carrying MLL-PTD (Kuhn et al., 2015). Despite these results, the molecular mechanism through which DOT1L inhibition results in a reduction of MLL-PTD leukemic burden remains unknown, as it has not been shown to directly interact with DOT1L. Our mass spec data confirms this result as there was no DOT1L or its known interacting partners, such as β-catenin and SKP1, detected (Mohan et al., 2010).

In summary, inhibitors against DOT1L and MEN1 kill MLL-PTD leukemic cells with approximately the same efficiency as MV4-11 cells which carry the MLL-AF4 mutation. In addition, the IC50 values for MLL-PTD with the various inhibitors appears to match those in the published literature for MLL-fusion leukemias. When compared to DOT1L inhibition, menin inhibition works more rapidly at the same concentration. Taken together, this suggests that menin inhibition is a strong candidate for future therapeutic intervention for MLL-PTD patients.

Finally, there continues to be some controversy surrounding the similarities between MLL-PTD and MLL-fusion leukemogenic mechanisms. Structurally, MLL-PTD retains all the WT domains including the catalytic SET domain while the fusion mutants lack the latter. Microarray data shows that gene expression patterns from MLL-PTD primary cells are distinct from those harbouring MLL-fusion mutations (Ross et al., 2004). In addition, one hallmark of MLL-PTD leukemias is the suppression of *MLL-WT* expression

(Whitman et al., 2008). In contrast, MLL-fusion leukemias require expression of MLL for leukemogenesis (Thiel et al., 2010). Despite these differences, recent work suggests that MLL-PTD does in fact share similarities with fusion protein leukemogenesis. Both show elevated expression of *Hoxa9* and sensitivity to DOT1L inhibition (Dorrance et al., 2006; Gao et al., 2016; Kuhn et al., 2015). We have added to this debate by providing initial evidence that these two types of MLL mutations share sensitivity to menin and KAT2A inhibition. Further work is needed to elucidate differences between the fusion and PTD mutations however, they appear to share some commonalities in their leukemogenic mechanisms.

4.2 Limitations and Future Work

MLL-PTD is a very challenging protein to study, in large part because of it's 600kDa size and rapid degradation. Therefore, there are several limitations in our work since we had to make compromises from our original hematopoietic model systems. The first limitation is that our mass spec data was performed in the human embryonic kidney cell line HEK293T. We could, therefore, potentially be missing protein interactions which are specific to the hematopoietic environment. Additionally, protein expression levels could vary between 293T cells and hematopoietic cells which might influence the observed protein interactions. We attempted to overcome this by showing the impact of inhibitors of the protein interactions in hematopoietic cell lines, thereby, validating this work in a clinically relevant environment. To expand on this research, it would remain important to either perform MLL-PTD IPs in cell lines which naturally express this protein or to continue to extensively validate the HEK293T IP results in the

hematopoietic environment. Multiple attempts to visualize the MLL-PTD protein by western blot in EOL-1 cells, including enriching for MLL by IP, were unsuccessful. MLL-PTD has only been detected by western in one publication, in a patient sample, therefore demonstrating the technically challenging nature of this work (Whitman et al., 2005).

An additional limitation is that we are ectopically expressing MLL and MLL-PTD in the HEK293T cells. However, we are not knocking down the endogenous MLL-WT already expressed by the cells. Therefore, it is possible that some of the overexpressed HA tagged MLL^C subunit is interacting with the endogenous MLL-WT N-terminus and not the FLAG tagged MLL-PTD N-terminus subunit. We hoped to overcome this limitation by directly comparing samples between MLL-WT and MLL-PTD for the HA IP. Therefore, any differences seen between the two transfected 293T cells should be because of the exogenous protein and the endogenous MLL will be consistent between the two samples. We could further overcome this limitation by performing IPs using both FLAG and HA antibodies in succession. This is not a limitation for the FLAG IPs as the MLL^C subunit is identical between MLL-WT and MLL-PTD. However, this might be the reason why we see less HA tagged MLL^C pulled down in the FLAG IPs compared to the HA IPs.

A limitation of our proliferation experiments is that they were performed in only one cell line with an MLL-PTD mutation. Additional work in other MLL-PTD expressing cell lines and patient samples will be important to increase the strength of our conclusions. A

further limitation of our drug experiments is that all this work was performed *ex vivo*. The next step would test the effectiveness of these compounds in a murine xenograft model. The bone marrow of immunocompromised mice would be injected with EOL-1 cells or patient samples, recently received from a collaborator in Spain, and animals would be treated with the drugs. In parallel, we would inject with samples containing MLL-WT or MLL-fusion mutants, such as MLL-AF4. This would provide further pre-clinical evaluation of the inhibitors for MLL-PTD leukemias. In addition, it was recently shown that treating MLL-fusion cells with both DOT1L and MEN1 inhibitors showed synergistic effects for leukemic cell differentiation and apoptosis both *in vitro* and *in vivo* (Dafflon et al., 2017). Testing these two inhibitors in combination on MLL-PTD cells should be done in future work.

Finally, important follow-up work should focus on genomic analysis by performing ChIP-seq. This will determine the differential binding of MLL-WT and MLL-PTD to gene loci and would be particularly insightful because the duplication in the PTD protein affects only the two DNA binding domains. It is therefore conceivable that the mutant protein could be binding with different stoichiometry, for example, longer binding at known loci, or bind to different loci altogether compared to the WT.

5. Conclusion

This master's thesis has made progress towards elucidating the leukemogenic mechanism of MLL-PTD. Mass spectrometry analysis following FLAG and HA IPs indicates several potential cofactor candidates for MLL-PTD mediated leukemogenesis. We chose to focus on two proteins that were found to be more abundant in the elution of the PTD IPs compared to the wild-type: the known MLL-WT interacting protein MEN1 and the novel partner KAT2A. Inhibitors targeting these interactions selectively kill MLL-rearranged leukemias including a cell line expressing MLL-PTD. Finally, we have shown evidence which suggests that MLL-PTD retains its ability to undergo proper cleavage in the cytoplasm and interact with WRAD complex members.

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7. Appendices

Appendix A – MLL-PTD Clinical Statistics

Table 3. Summary of MLL-PTD incidence in various cohorts. M5 represents the FAB subtype. CN-, s- and t-AML represent cytogenetically normal, secondary and therapy related AML respectively.

Cohort	%	Incidence PTD/Total	Reference
15-39 years old	2.00%	2/103	(Kuwatsuka et al., 2018)
Karyotype Aberrant	4.03%	10/248	(Dohner et al., 2002)
	3.10%	13/426	(Steudel et al., 2003).
De Novo	7.17%	62/865	(Shih et al., 2006)
	9.86%	7/71	(Shiah et al., 2002)
	5.80%	110/1881	(Bacher et al., 2005)
	6.38%	63/988	(Shih et al., 2006)
	6.05%	176/2911	(Bacher et al., 2007)
	9.00%	13/144	(Ishikawa et al., 2009)
	6.00%	28/470	(Tang et al., 2009)
	8.30%	76/921	(Alpermann et al., 2013)
	7.09%	310/4373	(Rose et al., 2017)
	10.50%	19/181	(Ozeki et al., 2004)
	3.16%	5/158	(Libura et al., 2003)
	9.68%	9/93	(Muñoz et al., 2003)
	11.11%	9/81	(Shiah et al., 2002)
M5a	1.70%	1/58	(Haferlach et al., 2002)
M5b	4.50%	3/66	(Haferlach et al., 2002)
MDS	2.70%	10/368	(Bacher et al., 2007)
Normal Karyotype	7.70%	19/247	(Dohner et al., 2002)
	7.50%	35/466	(Steudel et al., 2003)
	8.50%	145/1706	(Weisser et al., 2005)
	10.10%	24/238	(Whitman et al., 2007)
	8.05%	84/1044	(Bacher et al., 2007)
	7.34%	47/640	(Schlenk et al., 2008)
	9.30%	5/54	(Ishikawa et al., 2009)
	12.90%	21/163	(Weber et al., 2014)
	6.63%	13/196	(Paschka et al., 2008)
	8.82%	15/170	(Langer et al., 2008)
	9.52%	8/84	(Marcucci et al., 2005)
Other AML not M5	6.10%	46/753	(Haferlach et al., 2002)
Pediatric	20.00%	2/10	(Shiah et al., 2002)
	2.50%	7/276	(Balgobind et al., 2010)

	13.40%	21/157	(Sano et al., 2012)
	4.04%	4/99	(Liu et al., 2014)
Pediatric CN-AML	5.56%	3/55	(Liu et al., 2014)
Pediatric De Novo	0.81%	1/123	(Shiah et al., 2002)
	13.29%	21/158	(Shimada et al., 2008)
	2.00%	6/237	(Balgobind et al., 2011)
Relapsed AML	8.25%	25/303	(Bacher et al., 2007)
s-AML	7.41%	28/378	(Bacher et al., 2007)
t-AML	3.29%	7/213	(Bacher et al., 2007)
Trisomy 11	25.00%	4/16	(Steudel et al., 2003)
	37.50%	3/8	(Schnittger et al., 2000)
Unselected	5.90%	29/495	(Dohner et al., 2002)
	5.00%	48/956	(Steudel et al., 2003)
	5.60%	218/3901	(Weisser et al., 2005)
	4.00%	10/250	(Olesen et al., 2005)
	6.50%	236/3629	(Bacher et al., 2007)
	5.16%	65/1259	(Dicker et al., 2010)
	4.40%	11/252	(Haferlach et al., 2012)
	4.80%	19/396	(Patel et al., 2012)
	6.00%	57/952	(Grossmann et al., 2012)
	6.00%	18/325	(Chou et al., 2014)
	4.26%	11/258	(Gao et al., 2016)
	3.40%	13/387	(Schnittger et al., 2000)

Table 4. Summary of statistics for percentage of MLL-PTD patients by FAB subtype.

FAB Subtype	Description	Weisser 2005	Steudel 2002	Olesen 2005	Shih 2006	Whitman 2007	Shimada 2008
M0 (%)	Undifferentiated acute myeloblastic leukemia	3.45	2.10	0.00	4.84	5.00	4.76
M1 (%)	Acute myeloblastic leukemia with minimal maturation	20.69	22.90	20.00	27.42	24.00	33.33
M2 (%)	Acute myeloblastic leukemia with maturation	38.62	39.60	40.00	40.32	48.00	23.81
M3 (%)	Acute promyelocytic leukemia	n/a	0.00	0.00	0.00	0.00	0.00
M4 (%)	Acute myelomonocytic leukemia	17.24	12.50	10.00	19.35	10.00	19.05
M5 (%)	Acute monocytic leukemia	3.45	14.60	10.00	4.84	0.00	14.29
M6 (%)	Acute erythroid leukemia	6.21	6.30	0.00	3.23	10.00	0.00
M7 (%)	Acute megakaryoblastic leukemia	n/a	0.00	0.00	0.00	0.00	4.76

Appendix B

Table 5. List of primers

Primer	Sequence	For/Rev
FLAG	5'-TGGACTACAAAGACCATGACGGTG -3'	Forward
	5'-TCTGCAGAATTCCACCACACTGGA-3'	Reverse
3'UTR	5'-GCTTTCCCATGCTTCTTTCGGGTT-3'	Forward
	5'-AGTGCTTACAGGCTCAGGGAACTT-3'	Reverse
PTD	5'-CTTCCAGGAAGTCAAGCAAGCAGGT-3'	Forward
	5'-GTGGGCATGTCATCAGGAAACACA-3'	Reverse

Appendix C

Table 6. List of antibodies

Antibody	Use	Company	Host	Clonality	Catalogue #
FLAG	IP, Western	Sigma-Aldrich	Mouse	Monoclonal	F3165
HA	IP	Abcam	Rabbit	Polyclonal	ab9110
HA	Western	Sigma-Aldrich	Rat	Monoclonal	11867423001
MLL^{C}	Western	Millipore	Mouse	Monoclonal	05-765
ASH2L	Western	Homemade	Rabbit	Polyclonal	-
RBBP5	Western	Benthyl	Rabbit	Polyclonal	A300-109A
MEN1	Western	Abcam	Rabbit	Polyclonal	ab2605
KAT2A	Western	SantaCruz	Mouse	Monoclonal	sc-365321
TFIIH	Western	SantaCruz	Rabbit	Polyclonal	Sc-293

Appendix D

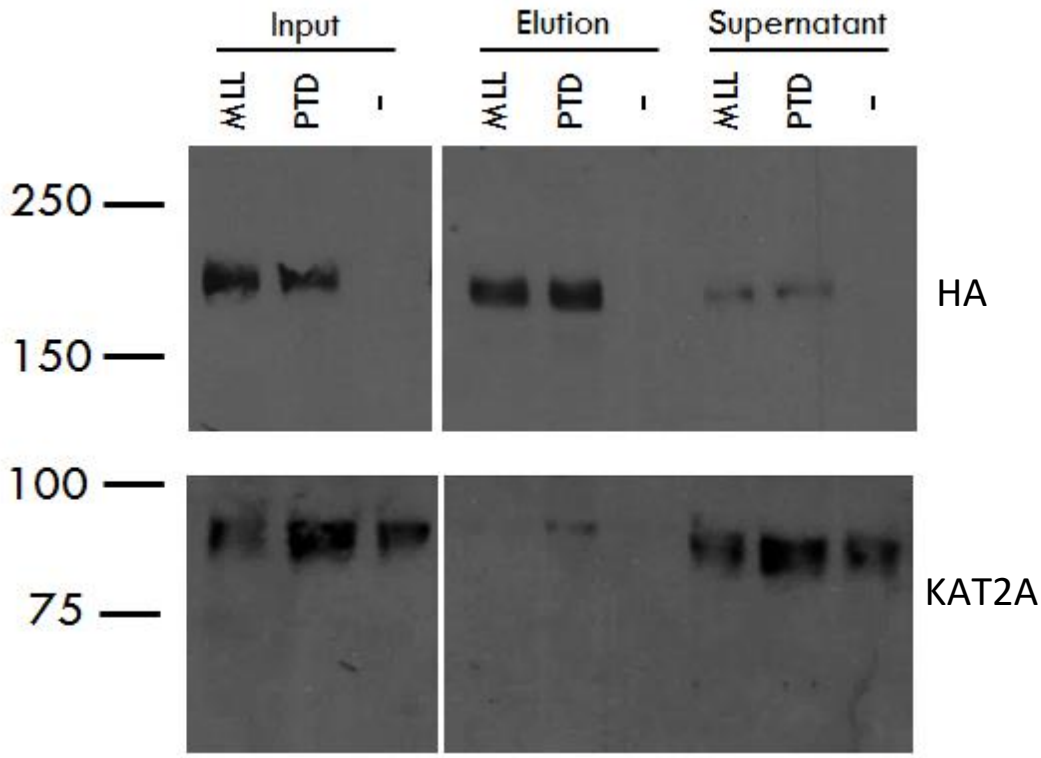
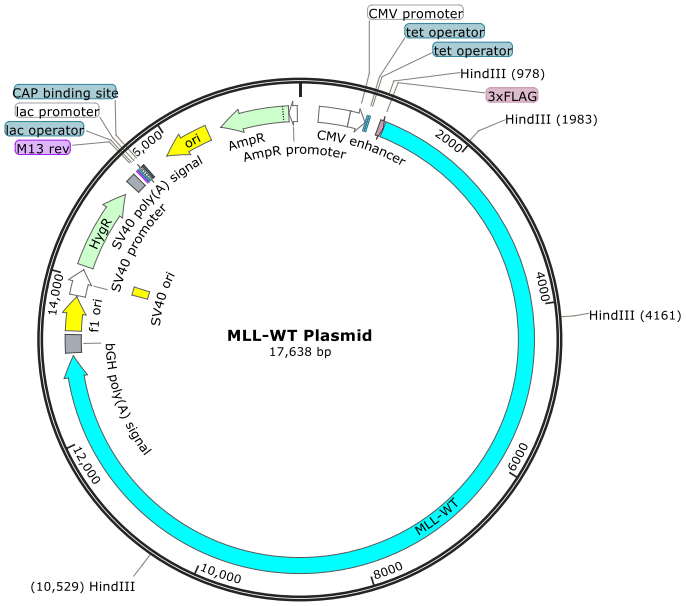


Figure 15. MLL-PTD interacts with KAT2A. Immunoprecipitation of nuclear extracts from 293T cells transfected with plasmids expressing FLAG and HA tagged MLL-WT or MLL-PTD. HA abundance in the elutions is equilibrated between MLL-WT and MLL-PTD. The novel MLL-PTD interacting partner KAT2A is co-immunoprecipitated. '-' represents the untransfected negative control. N=1

Appendix E

A



B

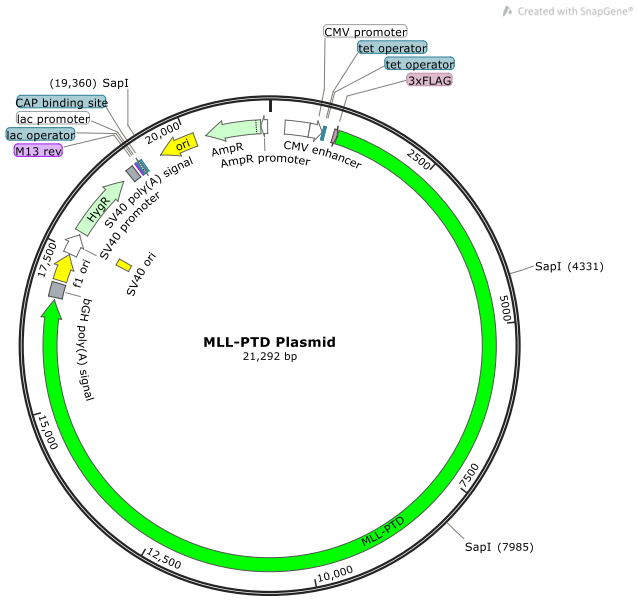


Figure 16. Plasmid maps for 3x-FLAG-MLL-WT-HA (A) and 3x-FLAG-MLL-PTD-HA (B) cloned into a pcDNA5/TO (Invitrogen) backbone. Plasmid maps generated using SnapGene software.