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STUDIES OF EPIGENETICS AND PROPERTIES OF B CELL RECEPTOR IN WALDENSTRÖM MACROGLOBULINEMIA (WM)

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STUDIES OF EPIGENETICS AND PROPERTIES OF B CELL RECEPTOR IN WALDENSTRÖM MACROGLOBULINEMIA (WM)

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DEDICATION

I would like to dedicate my dissertation work to my family, especially to my wonderful parents Ahmad and Maryam for their continued love and support. I would like to thank my amazing sister Dr. Hoda Karbalivand for encouraging and inspiring me through my life. To my wonderful husband Dr. Jonathan Sreter for his infinite kindness and support. I hope I've made them proud.

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ABSTRACT

STUDIES OF EPIGENETICS AND PROPERTIES OF B CELL RECEPTOR IN WALDENSTRÖM MACROGLOBULINEMIA (WM)

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Waldenström macroglobulinemia (WM) is an indolent lymphoproliferative disorder with aberrant monoclonal immunoglobulin M (IgM) production that is associated with disease symptoms. Despite significant advances in our understanding of disease biology, the cell of origin remains largely unknown. Although WM therapy options have improved over the last decade, WM is still an incurable disease. Many studies have identified epigenetic dysregulation as a regulatory factor in WM malignancy. Here, we investigated the role of Mixed-lineage leukemia 1 (MLL1) histone methyltransferase in WM. We showed that MLL1 and its partner Menin are upregulated in WM patients. We also found that MLL1 knockdown and pharmacological inhibition of MLL1 complex using the menin-MLL1 inhibitor (MI-2) significantly reduced IgM levels *in-vitro* and *in-vivo*. Further we showed that MLL1 binds to multiple sites in the 5' Eμ enhancer of the IgM heavy chain (IGMH). We also found that MLL1 binding to IGH region was associated with increased histone 3 lysine 4 trimethylation (H3K4me3) enrichment at multiple MLL1 binding sites. Moreover, we described B cell receptor (BCR) repertoires of the currently available cell lines for WM (BCWM.1, MWCL-1 and RPCI-WM1). We also found abnormal transcription of immunoglobulin heavy chain (H-chain) and light chain (L-chain) transcripts with increased

L:H-chain ratio in WM cell lines compared to normal B cells and this correlated with the amount of secreted IgM by each WM cell line. Finally, we identified that MI-2 inhibitor significantly reduced light chain expression in WM cells suggesting a role for MLL1 in regulation of light chain. Taken together, these results identify MLL1 as a regulator of IgM light and heavy chains that suggest MLL1 as a new therapeutic target for WM.

CHAPTER 1

INTRODUCTION TO EPIGENETICS IN WM

Introduction

I. Epigenetics

Epigenetics refers to the heritable mechanism of gene regulation that does not account for DNA sequence modification. Cell differentiation, including the transition from a naïve B-cell to plasma cell maturation, is highly governed by a synchronized regulatory mechanism which involves epigenetics. Epigenetic events make the identity of a cell in the human body by regulating the availability of genomic DNA and transcription process[1]. In the past decade, epigenome studies identified epigenetic mechanisms that contribute to several carcinomas. This occurs through DNA methylation, histone modifications, and non-coding RNAs, all of which facilitate our understanding of biological complexity, tumor heterogeneity, pathogenesis and disease progression[2,3].

Early studies identified DNA methylation as a regulatory marker in gene expression patterns and cell differentiation[4,5]. This epigenetic mechanism involves the addition of a methyl group to the carbon 5 position of the cytosine residues making 5-methylcytosine in the CpG sequence context across the genome thus serving as an epigenetic mark for gene silencing. The distribution of CpG rich sequences is found near the transcription

start sites which are generally unmethylated centromeres, and retrotransposon elements that are mostly methylated and are also distributed in intragenic or intergenic sequences[6,7].

The eukaryotic DNA sequence is packed in an extremely meticulous structure called chromatin. Gene expression patterns are inspired by chromatin architecture. This architecture is composed of a solid dynamic structure consisting of a histone octamer protein complex (2 of each: H2A, H2B, H3 and H4) that is enveloped in 147 bp of DNA and is called the nucleosome, the fundamental chromatin subunit[8]. This naïve dedicated structure rapidly alternates in response to genetic, environmental, or metabolic aberrations which are typical events in all cancer cells, leading to uncontrolled accessibility to specific genes by regulatory factors and transcriptional machinery[9]. Accordingly, histone modifications play a fundamental role in a variety of cellular process, including transcriptional regulation, chromatin folding, DNA double-strand break response, and cell-cycle regulation[10–12]

Histone-modifying enzymes manipulate chromatin architecture through reversable covalent post-translational modifications (PTMs) that have been characterized into three main groups: writers, erasers, and readers[13]. Writers are associated with the addition of histone modification such as histone methyltransferases (HMTs), histone acetyltransferases (HATs) and kinases. Erasers are associated with the removal of histone modifications such as histone demethylases (HDMs) and histone deacetylases (HDACs). However, readers (also known as histone-binding domains) consist of a group of protein effectors that serve as detectors of specific PTMs to reinforce the recruitment of essential components of transcriptional machinery to chromatin[14]. Bromodomain was

the first identified histone effector which was linked to nuclear histone acetyltransferases for recognition of acetylated lysine[15]. Subsequent studies identified the specificity of more histone-binding domains for PTMs including methylation and acetylation readers[16]. Further developments in analyzing chromatin immunoprecipitation (ChIP)-sequencing data and mass spectrometry analysis of histone marks revolutionized our understanding of epigenetic modification communication and revealed the influential cross talk among epigenetic modifiers[17]. This information provides the rational for investigating epigenetic modifications and the interplay between epigenetic factors in the context of B-cell lymphomas.

Epigenetic mechanisms along with the chromatin landscape administrate gene expression, which affects the regulation of transcription factors, downstream signaling pathways, and eventually cell phenotype[18]. Over the years, studies revealed the N-terminal tail of histone which contains frequent lysine and arginine residues is subjected to a variety of alterations. These alterations occur through reversible covalent post transitional modifications (PTMs), with the most well studied including acetylation, methylation, ubiquitination, or phosphorylation[19]. Each of these PTMs has a different effect on nucleosome structure. Moreover, there is evidence regarding the co-occurrence of compound histone PTMs which highlights the strong crosstalk between different PTMs that make epigenetic changes a compound network of sequential events rather than simple distinct epigenome alternations[20].

Histone acetylation is known to increase gene expression by inhibiting nucleosome refolding, which results in increased DNA accessibility (ref). Unlike the straightforward function of histone acetylation, histone methylation is a multifunction marker that could

act as a transcriptional silencer or activator where histone methylation function strongly depends on the type of methylated histone residue and the methylation level of targeted residue[14,21]. On the other hand, ubiquitination and phosphorylation are reversible histone modifications that are involved in different cellular events.

Unlike other histone modifications that add chemical groups to the histones, ubiquitination results in covalently addition of a 76 amino acid protein to the histones. Histone ubiquitination, occurs in humans mainly on histone H2A (H2AK119ub1, H2AK13/15ub1) and histone H2B (H2BK120ub1) and mostly at a monoubiqutinated level while being associated with chromatin unfolding and gene activation[22]. In mammals, histone H2A and H2B monoubiquitinations are involved in DNA damage response (DDR), DNA replication and transcriptional regulation[23].

Studies have shown that histone H2A monoubiquitination maintains transcriptional silencing through the recruitment of polycomb repressive complex 2 (PRC2) complexes which results in lysine 27 methylation on histone 3 (H3K27) methylation leading to increased H3K9 trimethylation (H3K9me3) level at the gene locus, and finally chromatin folding. On the other hand, histones H3 and H4 ubiquitination can be involved in DNA replication where H3K14/18/23 that can be recognized by DNMT1 methyltransferase during DNA replication[24]**.** in addition, H3K14 ubiquitination can also regulate the activity of the Clr4 methyltransferase and its H3K9me2/3 marks for gene silencing[25].

Histone phosphorylation happens at different residues such as serine (S), tyrosine (Y), and threonine (T) within histone tails leading to gene transcription activation[26]. Histone phosphorylation adds a negative charge to the histone, leading to a more open chromatin structure[27]. Histone phosphorylation is governed by two types of enzymes, the kinases that add phosphate groups and the phosphatases that remove the phosphates[26]. Main known functions of histone phosphorylation are DDR, the chromatin remodeling during mitosis and meiosis, and gene transcriptional activation

In addition to the functions mentioned above, the role of these PTMs in transcription has been corresponded to cross-talk with other histone modifications[28]. Histone modification crosstalk can happen at different levels where one histone modification promotes a new modification on the same histone or a different histone, or it may result in the removal of another histone mark. For instance, phosphorylation of H3 on Ser10 is involved in transcription and cell division[29]. Studies have shown that phosphorylation of H3 on Ser10 on H3 tail increases H3K14 acetylation and H3K4 methylation[29]. Another example is methylation of H3K4 by the Set1 methyltransferase subunit and H3K79 by Dot1 methyltransferase need pre-ubiquitination of H2B[30]. Therefore, there is a need to better understand the sequential crosstalk among epigenetic regulations.

In addition to the direct effect of DNA methylation level and the location of DNA methylation on transcriptional suppression, the status of methylated DNA is associated with the presence of H3K4 di- and trimethylations resulting in inaccessible chromatin structure and transcription inhibition[31,32]. Therefore, studies of histone modifiers along

with their cross talk should be the focus of future epigenetic studies. Epigenetic studies and histone modification patterns have received more attention in recent years, particularly as prognostic markers in various cancers[33]. Here, we first discuss epigenetic modifications and their roles in Waldenström macroglobulinemia biology and further interplay between chromatin modifiers.

II. Waldenström macroglobulinemia (WM)

Waldenström macroglobulinemia (WM) is an indolent, low-grade lymphoplasmacytic lymphoma (LPL) which arises from a neoplasm of small B lymphocytes and plasma cells, which is characterized by secreting monoclonal immunoglobulin-M (IgM) cells and disease localization in the bone marrow[34]. WM clinical features include thrombocytopenia (low blood platelet count), hepatosplenomegaly (enlargement of liver and spleen), lymphadenopathy (swollen lymph nodes), and hyperviscosity (increased blood viscosity)[35–37]. The most common symptoms at diagnosis include fever, fatigue, night sweats, and weight loss[35]. The transcription profile of WM at the mRNA level was performed and compared with chronic lymphocytic leukemia (CLL; a mature B cell cancer) and multiple myeloma (MM; a plasma cell cancer) indicates WM shares features with both diseases[38].

III. Myeloid Differentiation primary response 88 (*MyD88*) gene mutation

Genome wide sequencing has revealed an oncogenic point mutation in the myeloid differentiation primary response 88 (*MYD88*) gene, resulting in a leucine to proline exchange (L265P). This leucine to proline point mutation is prevalent in >90% of WM cases, and in a 30% of activated B-cell diffuse large B-cell lymphomas and IgM monoclonal gammopathy of undetermined significance patients (MGUS)[39]. Interestingly, MGUS patients harboring *MYD88* L265P mutation are more likely to progress to WM (more than 40% of MGUS cases)[40,41].

V. C-X-C chemokine Receptor type 4 (*CXCR4*) gene mutation

In addition, whole genome sequencing found an activating somatic mutation in the G-protein coupled receptor, *CXCR4* gene. This chemokine receptor represents a mutation in C-terminal domain of >30% of WM patients[42]. The *CXCR4* gene mutation could be nonsense or frameshift mutations[43]. These mutations in *CXCR4* gene regulatory C terminal domain result in persistent activation of AKT serine/threonine kinase 1 (AKT1) and mitogen-activated protein kinases (MAPK1)[43]. Subsequent studies revealed that these predominant mutations can lead to disrupted mechanisms of gene expression in downstream signaling pathways[44,45].

IV. Cellular origin of WM lymphoma

The first case of Waldenström Macroglobulinemia was initially described by a Swedish physician Jan G. Waldenström in 1944. He reported a case with bone marrow infiltration of lymphocytes, lymphadenopathy, thrombocytopenia, anemia, and serum hyperviscosity[46]. The World Health Organization (WHO) defines WM as a lymphoplasmacytic lymphoma (LPL), a neoplasm of small lymphocytes, plasmacytoid lymphocytes, and plasma cells with IgM paraprotein originated mainly in bone marrow[47]. WM has an prevalence of 1500-2000 cases in in the United States[48] . WM is common in elderly people with the average age of 65-70 at the time of diagnosis with survival rate of 7-8 years[48].

Waldenström′s Macroglobulinemia originates from malfunctioning B lymphocyte differentiation to plasma cells which leads to high heterogeneity in WM B cell population. Overall, WM clonal B lymphocytes are characterized by presence of CD19, CD20, $CD22^{low}$, CD25, CD27, $CD38^{low}$, CD45, and IgM surface markers. While normal people have both kappa and lambda light chain in WM light chain expression is limited to either kappa or lambda genes in each person[49,50]. There are two distinctive subgroups of WM tumor cells that can be classified based on immunophenotypic features including, 1) $CD19^{+}$, $CD20^{+}$ and $CD138^{-}$ lymphoplasmacytic cells, and 2) $CD19^{low}$, $CD20^{+}$, $CD38^{+}$, $CD45^{low}$ and $CD138^{+}$ plasma cells[51].

Furthermore, WM can arise from Immunoglobulin-M (IgM) monoclonal gammopathy of undetermined significance (IgM MGUS) disease. Phenotypic, molecular, and genomic comparisons of WM and IgM MGUS suggests that $CD25^{+}CD22^{+low}$ activated B lymphocytes are the common origin of this disease with a potential role for *MYD88* L265P mutation during this transition[52]. WM lymphoma is an indolent lymphoma,

while transformation of this disease to highly aggressive malignancies such as diffuse large B-cell lymphoma (DLBCL) has been reported in which histological transformation (HT) of WM to DLBCL is 1-4%[53,54]. Moreover, MyD88 mutational status is found as an independent risk factor associated to shorter HT time where the transformation rate of 5 years was 16% for patients with wild type MyD88 and 2.5% for patients with mutated MyD88[55].

VI. Somatic Hypermutation (SHM) and Class Switch Recombination (CSR) in WM

In addition to these genetics changes, studying WM malignant clones revealed hypermutation in immunoglobulin heavy chain (IgH) variable , diversity, and joining (VDJ) gene rearrangement[56]. The VDJ rearrangement is found to use mostly VH3/JH4 families in WM case[57]. However, a minor portion of WM patients showed no rearrangement of Ig heavy chain VDJ segments[58]. Despite the normal somatic hypermutation in this malignancy, WM is considered to have unswitched tumor B cells, as these cells do not undergo class switch recombination (CSR). These clonotypic B cells and plasma cells are CD20+, expressing exclusively IgM and IgD isotypes[59]. CSR process associates the rearranged Ig heavy VDJ exon segment to a specific downstream constant region (C_H), resulting in isotype switching.

In addition to all these genetic abnormalities, subsequent studies have revealed dysregulation in the expression of epigenetic modifiers as significant factors in WM pathogenesis[42,60,61,62]. While genome-wide studies have identified genetic aberrations in

WM, there is a need for more investigation at genetic and epigenetic levels to provide insight into understanding of cellular and molecular events regulating the biology of this disease.

VII. DNA methylation patterns

In mammals, DNA methylation is regulated by a group of three major DNA methyltransferases (DNMTs) enzymes, DNMT1, DNMT3A, and DNMT3B. DNMT3A and DNMT3B initiate methylation pattern alternations during embryogenesis and early development and are known as *de novo* DNMTs. On the other hand, DNMT1 is leading in DNA replication to transfer DNA methylation pattern to newly replicated DNA strands[63–65]. Despite global DNA hypomethylation, abnormal promoter hypermethylation of tumor-suppressor genes in CpG islands that lead to transcriptional silencing and genomic instability is also a common phenomenon in cancer[66,67]. Studies have been shown that epigenetic alterations through DNMTs (DNMT1, DNMT3A, and DNMT3B), dysregulation, and modification are involved in the carcinogenesis and progression of a variety of tumors including solid tumors as well as hematological malignancies[68–70].

Hematological malignancies, such as myeloid and lymphoid malignancies, represent aberrant DNA methylation patterns. Genome wide methylation profiling of acute myeloid leukemia (AML) has shown a similar methylation pattern among AML patients along with a recurrent mutation in *DNMT3A* gene[71].

Anomalous DNA methylation has also been associated to WM pathogenesis. Hypermethylation and hypomethylation of oncogenes and tumor suppressor genes is

considered to be an important aspect in WM epigenome abnormalities[72]. Recently, Hao *et al*. compared gene expression profiles of plasma cells among healthy donor and Waldenström (WM-PCs), multiple myeloma and tonsil donors. This comparison of WM-PC with the control group revealed 242 differentially expressed genes. Among these 242 differentially expressed genes, 46 exhibited greater than 2-fold overexpression, and of those 46 genes, *DNMT1* was also upregulated drastically in WM-PCs[62]. As mentioned earlier, DNMT1 is a member of the DNMTs family and is required for conservation of the methylation landscape of the genome during cell replication. *DNMT1* gene alternation has been recognized in many malignant transformations, such as deletion in lymphomas, mutation in colon cancer, and over expression in breast, liver, and pancreatic cancer[68,70,73–75].

VIII. Role of Non-coding RNA

Non-coding RNAs (ncRNAs) are regulators of gene expression that are involved in many cellular functions such as differentiation, apoptosis, cell proliferation and immune response [76]. ncRNAs include 98.5% of human genomic transcripts[77]. Primarily, ncRNAs have been classified as short sequences with less than 200 nucleotides, called microRNAs (miRNAs) and long ncRNAs (lncRNAs) with more than 200 nucleotides. miRNAs have been implicated pro-tumoral function in both solid tumors and hematological malignancies, including WM[78–80].

Due to the heterogeneity of the WM tumor clones, there is no unique miRNA signature for WM. miRNA analysis identified high expression of miRNA-*363*, -*206, -494,*

-155, *-184*, *-542-3p* in WM patients[81]. Among these miRNAs, miRNA-155 has been shown to have an important role in WM biology *in-vitro* and *in-vivo* where miRNA-155 loss of function inhibited WM tumor cell growth by downregulation of MAPK/ extracellular regulated MAP kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, and NF-κB pathways[78]. In addition to this, overexpression of miR-155 in WM cell lines limits the apoptotic effect mediated by *FOXO3a* (Forkhead Box O3a) and *BIM* (BCL2L11) gene expression[82]. Studies have also shown significant overexpression of miRNA-21, -125b, -181a, -193b, -223, and -363 in WM[83]. Genomic studies identified increased levels of miR-192-5p, miR-320b, and miR-21-5p and reduced levels of let-7d as reliable biomarkers for WM progression[84]. In a recently published study, miR-4532, miR-20, and miR-1260 microRNAs (miR) and their associated genomic pathways such as Interleukin 8 (IL-8), and Signal transducer and activator of transcription 3 (STAT3) pathways were identified as important epigenetic regulators preceding IgM-MGUS progression to WM[85]. Reduced expression of miRNA-9 in WM has been linked to HDAC4 and HDAC5 down-regulation and acetyl-histone-H3 and H4 up-regulation where treatment of WM cells with the pan-HDAC inhibitor LBH589, induced miRNA-9 dependent apoptosis in WM cells[86].

Long non-coding RNAs (LncRNAs) have been shown to act as an important driver of gene expression, proliferation and survival in WM and other lymphomas[87,88]. Research has shown that the signature of lncRNAs is different between IgM-MGUS and WM. For example, overexpression of lncRNA Plasmacytoma variant translocation 1 (PVT1) inducing WM cell oncogenic proliferation has been reported in WM but not in IgM-MGUS[89]. Increased levels of lncRNA PVT1 expression is prevalent in multiple solid tumors and B cell malignancies[90]. Other overexpressed lncRNAs in WM, including

LncRNA TCB1D27 and lncRNA RP11-25K21.1 are in close proximity of oncogenes[89]. Moreover, the recruitment of lncRNAs or decoy of epigenetic modifiers is considered an important dynamic in the development of B, T and NK hematological malignancies causing malignant proliferation, survival, and invasion[87].

IX. Acetylation and deacetylation of nucleosomal histones in WM

Histone acetylation on lysine residues is one of the most studied covalent post-translational modifications (PTM) associated with the regulation of gene expression. As discussed earlier, nucleosomes are at the core of epigenic changes in genomes where HATs and HDACs constantly modify their structural and biochemical properties. Histone acetyltransferases (HAT) are associated with gene activation by loosening the chromatin structure, which allows specific gene transcription. However, histone deacetylases (HDACs) are linked to gene repression through condensation of chromatin structure, limiting the access of the transcription machinery to the gene sequence. There are three main groups of HATs including GCN5-related N-acetyltransferase (GNAT), Moz, Ybf2/Sas3, Sas2, Tip60 (MYST) and coactivators CREB binding protein (CBP)/p300 families[91]. To date, 18 human HDACs have been identified, and are grouped into four classes (classes I, II, III, and IV) based on their homology to yeast HDAC classes[92,93].

Cancer cells have a unique acetylation profile that modifies gene expression and cellular metabolisms in favor of cancer cell survival[94]. Deregulation of HATs and HDACs is linked to multiple human diseases, including hematological malignancies[92]. Histone deacetylase inhibitors (HDACi) are currently an emerging new class of drugs for

cardiovascular and neurodegenerative disorders, as well as cancer[95–97]. HDACis are predominantly generated by targeting the catalytic pocket of these proteins. SAHA (Verinostat) and FK288 (Romidepsin) were the first FDA-approved HDAC inhibitors for the treatment of T-cell lymphomas[98]. Additionally, the identification of single or combinational strategies using treatment with HDACsi is in clinical trials for treatment of multiple B-cell lymphomas such as DLBCL, MM and WM[99–101].

LBH589 (Panobinostat) is a pan-HDAC inhibitor which was initially approved by the FDA for treatment of multiple myeloma in 2015. LBH589 has also shown significant antitumor activity in WM *in-vitro*, where it induced intrinsic and extrinsic apoptotic pathways. Additionally, LBH589 increases histone H3 and H4 acetylation in a dose dependent manner and inhibits the canonical and non-canonical NF-κB pathway[102]. Further studies have shown the active therapeutic effect of panobinostat in relapsed or refractory WM patients[103]. In WM aberrant expression of proto-oncogene such as c-MYC play an important role in triggering proliferation and anti-apoptotic pathways in WM[104–106] Combined therapy of MYC oncogenes using bromodomain inhibitors, JQ1 and LBH589 synergically induced apoptosis in WM cells[105].

X. Methylation and Demethylation of Nucleosomal Histones in WM

Histone methylation is involved in multiple cellular functions such as establishment and maintenance of heterochromatin structure, regulation of gene transcription, DNA damage response , and RNA splicing[107,11]. Many studies have found the role of these histone modifiers in carcinogenesis through gene silencing or activation, resulting in cell

immortality, epithelial–mesenchymal transition (EMT), invasion, migration, and tumor formation[108].

Histone methylation is the result of enzymatic activity of lysine methyltransferases (KMTs) or arginine methyltransferases (PRMTs) and KDMs (lysine demethylases). KMTs are capable of mono-, di-, and trimethylation of lysine residues, while PKMTs can only mono- or di-methylate the arginine residue[109,110]. Lysine residue methylation and demethylation are associated with gene activation and repression, respectively. More specifically, H3K4, H3K36, and H3K79 are known lysine methylation marks of active genes, and H3K9, H3K27, and H4K20 are lysine demethylation marks associated with silenced genes[111,112].

There are two families of lysine methyltransferases, the disruptor of telomeric silencing (DOT1-like) proteins (non-set domain-containing methyltransferases) and SET domain-containing proteins. DOT1-like methyltransferases are associated with poor survival and more aggressive solid tumors[113]. DOT1L enzyme is a conserved mammalian DOT1-like protein that methylates histone H3K79 in humans. DOT1L and its H3K79 methylation mark play a role in normal SHM[114]. Inhibition of DOT1L using DOT1L inhibitor Pinometostat (EPZ-5676) have shown promising results in clinical trials for treatment of different leukemia and lymphomas[115,116].

On the other hand, most of methyltransferases are among SET domain-containing proteins which methylate histones as well as some other proteins.

The SET domain was initially identified as a conserved sequence in three *Drosophila melanogaster* proteins including Suppressor of variegation 3-9 (SUV39h1), Enhancer of zeste E(z) , and the trithorax (Trx)[117,118]. The SET domain is conserved and consists of a sequence of almost 130 amino acids[119]. To date, there are ten known class of SET domain proteins E(z), ASH (ASPM, SPD-2, Hydin), Trx/MLL, PRDM, SUV, SET and MYND domain-containing proteins (SMYD), and SETD[120]. Studies have shown that proteins in the Trx/MLL family, which methylates H3K4 lysine residues, are associated with solid tumors and hematologic malignancies[121]. The Trx/MLL family members are MLL1 (KMT2A), MLL2(KMT2B), MLL3 (KMT2C), MLL4 (KMT2D), SETD1A (KMT2F) and SETD1B (KMT2G). Many studies have recently revealed the important role of histone methylation regulation in the development of lymphoma[122]. The mutation of histone methylation modifiers such as *KMT2A*, *KMT2D*, and *KDM6A* have also been reported in cases of WM[123].

XI. Mixed Lineage Leukemia 1 (MLL1)

Mixed lineage leukemia (MLL1) family of methyltransferases has a highly evolutionary conserved sequence from yeast to humans[124,125]. There are six H3K4 methyltransferases in humans including MLL1 (KMT2A), MLL2 (KMT2B), MLL3 (KMT2C), MLL4 (KMT2D), SETD1A (KMT2F) and SETD1B (KMT2G), where each is responsible for a different level of H3K4 methylation. The major function of these H3K4 methyltransferases is chromatin architecture modification through mono-, di-, or tri-methylation of histone 3 at lysine 4 (H3K4) leading to gene transcriptional regulation[126].

The MLL1 protein complex consists of multiple domains, including Suppressor of variegation 3–9, Enhancer of Zeste, Trithorax (SET), the FY-rich (phenylalanine/ tyrosine-rich) domain in N-terminal (FYRN) and FY-rich domain C-terminal (FYRC), plant homeodomain (PHD), a bromo domain (BRD), a cysteine-rich (CXXC) , and AT hooks DNA-binding motifs in MLL1-N terminal (Figure1)[127,128].

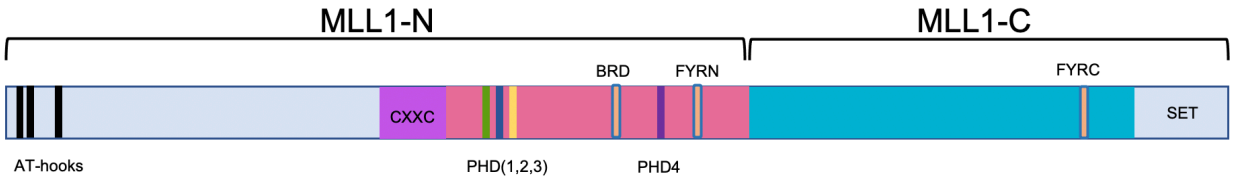


Figure 1: MLL1 functional domains. AT-hooks (Binding to AT rich DNA region), CXXC domain (Binding to unmethylated CpG rich DNA), plant homeodomain (PHD), bromo domain (BRD), FY-rich domain in N-terminal (FYRN) and FY-rich domain C-terminal (FYRC) and Suppressor of variegation 3–9, Enhancer of Zeste, Trithorax (SET). Created with BioRender.com

MLL1 is proteolytically cleaved into two fragments by a nuclear protease called taspase1. The smaller fragment MLL1-C (180 kDa) binds to multiple partners, including WD repeat containing protein 5 (WDR5), Retinoblastoma Binding Protein 5 (RbBP5), Absent-Small-Homeotic-2- Like protein (ASH2L), and Dumpy-30 (Dpy30), altogether are called the WARD complex (Figure2)[127]. The partners to which MLL1-C binds, increase the enzymatic activity of MLL1, whereas the WARD complex is required for H3K4 dimethylation and trimethylation[129,130].

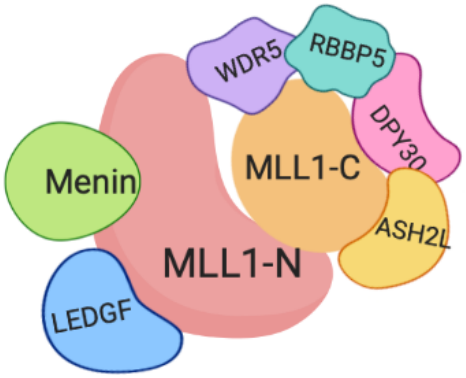


Figure 2: MLL1 protein complex. MLL1 N-terminal, MLL1 C-terminal, WD repeat containing protein 5 (WDR5), Retinoblastoma Binding Protein 5 (RbBP5), Absent-Small-Homeotic-2- Like protein (ASH2L), Lens Epithelium-Derived Growth Factor (LEDGF) and Dumpy-30 (Dpy30). Created with BioRender.com

The larger fragment of cleaved MLL1 is named the MLL1-N fragment (320 kDa), and it interacts with Menin, a protein that is required for recruitment of MLL1 to the chromatin structure of target genes[131]. Menin also binds to chromatin-binding protein Lens Epithelium-Derived Growth Factor (LEDGF), another important chromatin associated protein for MLL1 function (Figure2)[132].

XII. Menin and Carcinogenesis

Menin is a 67 kDa nuclear protein encoded by the *Multiple Endocrine Neoplasia Type I* (*MEN1*), located on chromosome 11q1[133]. Menin mutation is responsible for

MEN1 syndrome, an inherited condition leading to the development of tumorigenesis in the endocrine system[134].

Menin proteins are found to partner with multiple other proteins to activate or silence target genes, demonstrating there are many cellular and physiological functions associated to this protein function[133]. Menin directly interacts with JunD transcription factor which belongs to family of the AP1 transcription factor complex which are important in tumorigenesis and other cellular processes. Binding of menin to JunD inhibits its transcriptional activity[135]. Moreover, menin can also interact with mixed lineage leukaemia protein 1 (MLL1) with the same binding pocket as the JunD protein. This allows menin to regulate transcription of target genes. Menin continues to interact with the N-terminal of MLL1-fusion-protein in leukemia and act as a critical oncogenic cofactor in the MLL protein complex[136]. The MLL1-Menin complex is involved in tumorigenesis of different cancers, including prostate, breast, ovarian, pancreatic, and leukemia cancers[137–140]. For example in breast cancer, menin directly interacts with the estrogen receptor (ER), leading to the recruitment of MLL1 to activate the expression of estrogen target genes[140,141]. Menin has also been shown to interact with *MLL2*, *c-Myc*, and *Foxa2* (Forkhead box A2) oncogenes[142–144].

XIII. Pharmaceutical Inhibition of MLL1-menin Interaction

Due to the importance of the MLL1-menin complex in tumorigenesis, many inhibitor molecules were developed to disrupt this interaction, such as MI-463, MI-503,

MI-2, MI-2-2, BAY-155, VTP50469, and MI-1481. These were developed both to produce more potent inhibitor molecule and to evaluate druglike parameters of these small inhibitor molecules[145,146]. Among all MLL1-menin inhibitors, only KO-539 (Ziftomenib) and SNDX-5613 (Revumenib) have been successfully approved by FDA in clinical trials as orphan drugs for AML[147,148].

XIV. Role of MLL1 in Normal Development and Cancer

MLL1 also regulates the *HOX* (*Homeobox*) gene family. Members of the HOX family are characterized as transcription factors containing conserved homeodomain that are involved in cell fate during fetal development[149]. *HOX* genes are involved in both normal and malignant hematopoiesis[150].

In addition, MLL1 is essential for the self-renewal of fetal and adult hematopoietic stem cells (HSCs) and progenitors in the bone marrow, where MLL-deficient bone marrow cells showed self-renewal defects[151,152]. Moreover, MLL1 regulates pre-BCR signaling and B-cell differentiation, where MLL1 impairs pre-B-cell receptor (pre-BCR) survival and diminished B cell differentiation associated with deficiencies in the RAS/MAPK signaling pathway[153].

During gene transcription, MLL1 is associated with RNA polymerase II (Pol II) near the transcription start site, promoters, and enhancers of active genes[121,154]. Although the histone methyltransferase (HMT) activity of MLL1 is not responsible for the maintenance

of target gene expression in hematopoietic stem/progenitor cells (HSPCs), it regulates transcriptional initiation and elongation through H4K16 acetylation modification in complex with lysin acetyltransferases 8 also known as MOF[155].

The MLL-family of histone methyltransferases (MLL1–4) were previously linked to tumorigenesis through translocation, duplication, mutation, and abnormal expression [156]. For example, MLL2 is mutated in a large number of different cancers including diffuse large B-cell lymphoma, follicular lymphoma, and solid cancers such as breast, prostate, and head and neck carcinomas[157–160]. Additionally, frequent somatic mutations of MLL2 are frequently found in about 25% of WM cases[161].

MLL1 is located at chromosome 11q23 encoding the MLL protein with 3969 amino acids. MLL1 was named after being initially discovered in leukemia patients[162]. Different chromosomal translocations were detected in the MLL1 gene locus which result in production of MLL fusion oncoproteins (MLL-FPs) which do not maintain the H3K4 methylation of wild-type MLL1 target genes[163]. MLL1 fusion protein is found in acute myeloid (AML) and acute lymphoblastic (ALL) leukemias[131].

Finally, MLL1 expression is irregulated in many solid tumors[138,164,165]. MLL1 is upregulated in all stages of human colon cancer, which is correlated with increased nuclear β-catenin, the indicator of Wnt signaling pathway[164]. Wnt/ β-catenin signaling is associated with colon cancer. In addition, increased MLL1 expression is associated with poor survival of colon cancer patients where depletion of MLL1 significantly reduced colon

cancer self-renewal and xenograft tumor[164]. Mice xenograft models of cervical tumor showed that MLL1 is overexpressed in the hypoxic regions and modulates tumor growth and angiogenesis thorough regulation of hypoxia inducible factor 1 subunit alpha (HIF1α), vascular endothelial growth factor (VEGF), and CD31[166].

XV. B Cell Receptor Repertoires in Adaptive Immune System

B lymphocytes are an important of part of the adaptive immune system that were evolutionarily developed to dynamically evolve and generate an effective response against constantly changing pathogens. Immunoglobulin or antibody (Ig) molecules produced by B lymphocytes can function as secreted antibodies or member-bound Ig antigens called B-cell receptors (BCRs) that can detect and bind to a specific antigen resulting in immune response by B lymphocytes[167]. The BCR repertoire refers to the unique diversity of BCRs which corresponds to them having certain antigen specificities[168]. BCRs are composed of two identical heavy chain molecules and two identical light chain molecules bound together with disulfide bonds[169]. These heavy and light chains are encoded with Immunoglobulin heavy (*IGH*) and Immunoglobulin lambda (*IGL)* or Immunoglobulin kappa (*IGK)* genes respectively[170].

There are multiple copies of certain genes called variable (V), diversity (D), and joining (J) genes in the variable gene locus of an antibody. During B cell development in bone marrow, one copy of V, D, and J genes are joined to form a distinct BCR sequence through a process called V(D)J recombination[171]. DNA rearrangement during V(D)J

recombination provides BCR diversity at two levels: 1) Selection of V(D)J segments 2) Deletion or addition of nucleotides at V(D)J segment junctions which the later introduces greater diversity to BCR sequence[171].

XVI. Differentiation Pathways of B Lymphocytes

After they encounter antigen, B lymphocytes can differentiate into two distinct types of plasma cells, short-lived plasma cells or long-lived plasma cells. B lymphocytes differentiate into short-lived plasma cells immediately after antigen encounter in secondary lymphoid organs and produce low affinity IgM antibodies. On the other hand, long-lived plasma cells are generated by B lymphocytes that migrate to the germinal center (GC) of the spleen for affinity maturation. Affinity maturation is the process that results in production of highly specific antibodies by B lymphocytes. After affinity maturation, mature B lymphocytes can differentiate to memory B cells[172].

XVII. B Lymphocytes Affinity Maturation

Affinity maturation includes two major mechanisms called somatic hypermutation (SHM) and class switch recombination (CSR). Activation-induced cytidine deaminase (AID) enzyme has an important role in initiation of both mechanisms.

During SHM, the naïve B cell migrates to the GC for a T cell dependent antigen encounter. Then, the activated B cell will proliferate, and single nucleotide mutation will

be introduced by AID enzyme to a specific part of BCR sequence called complementary determining regions (CDRs). CDRs are a hypervariable domain of Ig molecules that generate specific antigen binding site of BCRs[173]. AID activity is focused at WRC (W = A/T, R = A/G) hot-spot motif within CDRs regions[174].

However, CSR is the process of substituting to a different Ig isotype from initial IgM producing B cells through the replacement of heavy-chain constant region by other constant genes. This DNA recombination changes the effector activity of the BCR without changing the BCR specificity[175].

XVIII. B Cell Receptor Gene Region

The immunoglobulin molecule is comprised of two identical heavy (IgH) chains located on chromosome 14 (1250 kb) in humans and two identical immunoglobulin light (IgL) chains that could be either both kappa (Ig*κ*) located on chromosome 2 or both lambda (Ig*λ*) encoded on chromosome 22 in human (Figure3)[176].

The constant region of Ig molecule dictates the Ig isotype. IgH chains have five main constant regions including *C*μ, *C*α, *C*γ, *C*δ and *C*ε which encode IgM, IgA, IgG and IgD respectively. In addition, *C*γ *gene region* of IgG generates four subtypes of IgG molecules (IgG1/2/3/4) by *Cγ1*, *Cγ2*, *Cγ3* and *Cγ4* genes. Also, IgA isotype encoded by *C*α gene, encodes two subtypes of IgA using *Cα1* and *Cα2* genes[177,178].

On the other hand, the sequence of the variable region of the antibody provides the polymorphism required for antibody selection against the antigen by VDJ recombination and SHM in IGVL and IGVH regions. The human IGH variable locus consists of ~129 V_H exons genes (88 exons belong to 7 subgroups and 44 pseudogenes), 27 D_H segments (23 functional exons) and 9 J_H segments (6 functional exons)[179,180].

Because of the complexity in sequence variation, human Ig variable genes have specific nomenclature, whereas *IGVH* or *IGVL* gene has a number for the subgroup. For example, *IGHV4*, followed by a hyphen and a number that represents the 3' to 5' location in the gene locus (IGHV4-30). Also, allelic polymorphisms are shown by a hyphen and a number such as IGHV4-30-1[181,182].

The human IG light Kappa chain located on short arm of chromosome 2 (1820 kb) includes one single *IGKC* gene, 76 *IGKV* genes in 7 subgroups (31~36 functional genes within 5 subclasses), and 5 *IGKJ* genes[183,184]. The *IGKV* genes are located in two separate variable gene clusters with a 800 kb sequence in between where the distal cluster contains 36 genes, and the proximal cluster has 40 genes[185,186].

The human IG light Lambda chain located on 22 (1050 kb) consists of 7-11 *IGLC* genes (4-5 functional constant genes), 73-74 *IGHV* genes (29-33 functional genes within 10 subclasses), and 7-11 *IGLJ* (4-5 functional genes) genes[187,188]. Due to the importance of *IGHV* genes polymorphism in BCR repertories specificity, most of the studies are focused on the IGH variable region.

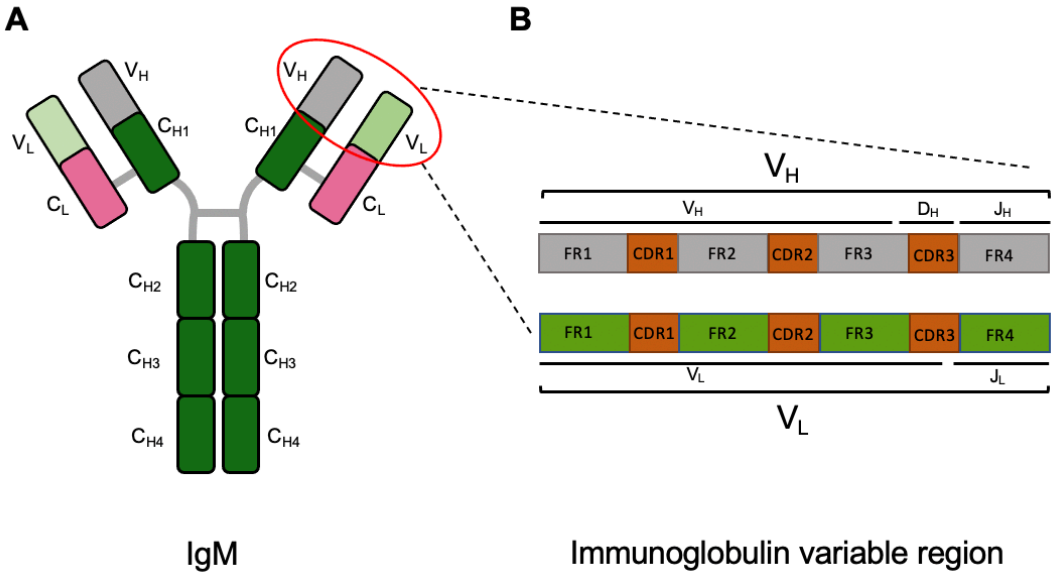


Figure 3: Schematic representation of Immunoglobulin M (IgM) structure and variable region. A) IgM is made up of two heavy chains and two light chains. C_{H1-4} (Constant Heavy chain), V_H (Variable Heavy chain), C_L (Constant Light chain), and V_H (Variable Light chain). B) Representation of Immunoglobulin variable region. Complementary determining regions (CDR1, CDR2 and CDR3) and framework regions (FR1, FR2, FR3 and FR4). Created with BioRender.com

XIX. Complementary Determining Regions (CDRs) of antibody

The variable domain of antibody includes an antibody binding site that consists of three hypervariable loops known as complementary determining regions (CDR1, CDR2 and CDR3). The CDRs are flanked by four conserved sequences called framework regions (FR1, FR2, FR3 and FR4) (Figure3). Both the sequence and length of CDRs are

highly variable. Among CDRs, CDR3 region has the most variability through the addition of nucleotides or deletion in V(D)J junction and SHM. Studies have shown that antibody selection is associated to the CDR3 region of the heavy chain where the CDR3 sequence differs between immature and mature B cells[189–191]. This represents the importance of the CDR3 sequence in antibody specificity[192]. In general, mature B cells (antigen encountered B cells) have a shorter CDR3 that is more negatively charged. Antibodies that have positively charged CDRs have been proven to have low specificity[193].

XX. Current WM treatment

Commonly used agents in the management of WM are anti-CD20 antibodies (Rituximab and Ofatumumab), Chemoimmunotherapy (mostly rituximab-based combination therapies), Bruton's tyrosine kinase (BTK) inhibitor (Ibrutinib) and Proteasome inhibitors (Bortezomib). Among these therapies, ibrutinib is the only US FDA-approved drug for WM[194].

CHAPTER 2

CHARATERIZATION OF MLL1 ROLE AS A MODULATOR OF GENE EXPRESSION IN WM

Parts of the following chapter are taken from my publication in Leukemia research[195].

Karbalivand M, Almada LL, Ansell SM, Fernandez-Zapico ME, Elsawa SF. MLL1 inhibition reduces IgM levels in Waldenström macroglobulinemia.

doi: 10.1016/j.leukres.2022.106841. Epub 2022 Apr 19. PMID: 35462170.

I. Introduction

Waldenström macroglobulinemia (WM) is an indolent, low-grade lymphoma defined by infiltration of small B lymphocytes and plasma cells in the bone marrow leading to the excessive secretion of monoclonal immunoglobulin-M (IgM)[37]. These malignant B cells and plasma cells are $CD20^{+}$ and exclusively express IgM and IgD isotypes[59]. WM has well-established recurrent genetic mutations[196]. However, despite extensive sequencing efforts, few additional druggable targets have been identified. In the absence of these targetable mutations, the Bruton's tyrosine kinase (BTK) inhibitor Ibrutinib is the only FDA approved therapy for WM patients; however, resistance to Ibrutinib is common due to disease heterozygosity and the dependance on the tumor microenvironment (TME)[197,198]. Therefore, consideration must be given to non-genetic drivers to enhance our understanding of WM pathogenesis and to inform novel and more effective treatment

strategies. The dysregulated expression of epigenetic modifiers has been identified in WM[42,199]. Among epigenetic modifiers, mutations, translocations and aberrant expression of the MLL/COMPASS family is common in lymphoma and has reported in WM[42,200–202]. However, the biological significance of these proteins has not been described to date.

KMT2A/MLL1 is a member of the COMPASS-like family of Histone 3 Lysine 4 (H3K4) *N*-methyltransferase[152]. MLL1 function is highly dependent on its interaction with Menin, a critical component of the MLL1 protein complex that is encoded by *MEN1* (Multiple Endocrine Neoplasia I) gene. Menin interacts with MLL1 to facilitate the recruitment of MLL1 to target genes[131]. Pharmacological inhibition of the Menin-MLL1 interaction provided effective treatment in various cancers including breast cancer, lymphoma and leukemias[201,203204205]. However, despite the importance of epigenetic modifiers in gene regulation and in lymphomagenesis, the role these epigenetic proteins remain unexplored in WM. Thus, investigation of the role of regulators of histone methylation is important for our understanding of WM biology.

The immunoglobulin heavy chain (IGH) region undergoes unique alternations during B cell differentiation that are coordinated by two super-enhancer elements: a 5' Eμ enhancer and a 3′ regulatory region (3′ RR). The *IGH* intronic Eμ enhancer and the mu intervening (I) promoter (Iμ) are located upstream of the Cμ constant region and are essential in the regulation of *IGH* expression[206].

In this study we identify MLL1 as a regulator of IgM expression and secretion in WM. We demonstrate that *KMT2A* and its cofactor *MEN1* are overexpressed in WM cell lines and patient samples. We show that MLL1 depletion and disruption of the menin-MLL1 complex using MI-2, a highly potent and small molecule inhibitor of the menin-MLL1 interaction[207,208] significantly reduces IgM expression and secretion in WM. Although neither MLL1 knockdown nor MLL1 inhibition had any effect on WM cell proliferation and viability. Further analysis revealed that MLL1 directly binds to the *IGH* gene enhancer region. We also found H3K4me3 deposition, a mark of MLL1 function, in the *IGH* region in WM cells. Moreover, we show that pharmacological disruption of menin-MLL1 complex significantly reduced H3K4me3 deposition at MLL1 binding sites. Finally, we show that menin-MLL1 inhibition reduces IgM secretion in tumor-bearing mice. Although, consistent with our *in-vitro* experiments, menin-MLL1 inhibition had no effect on tumor growth. Taken together, these results identify a role for MLL1 in the regulation of *IGH* expression and IgM secretion and suggest that targeting menin-MLL1 may provide threptic benefit by reducing IgM levels in WM patients.

II. Materials and Methods

Cell lines and patient samples

BCWM.1 cells were kindly provided by Dr. Steven Treon (Dana Farber Cancer Institute, Boston[209]. MWCL-1 cells were kindly provided by Dr. Stephen Ansell (Mayo Clinic, Rochester, MN)[210]. RPCI-WM1 cells were kindly provided by Dr. Asher Chanan-Khan (Mayo Clinic, Jacksonville, FL)[211]. WM cell lines were maintained in RPMI1640

supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were passaged ever 2-3 days. THP-1 cells were purchased from ATCC and cultured in RPMI1640 supplemented with 10% FBS. Peripheral blood serum samples from normal controls and WM patients were used after receiving Mayo Clinic Institutional Review Board approval. Bone marrow biopsies from WM patients were collected and fractionated to isolate **CD19+CD138+** cells from other cell types and non-cellular components. WM patients involved both untreated and post-treatment patients.

RNA isolation and RT-qPCR

Total RNA was extracted using TRIsure reagent (Bioline, London, UK), according to the manufacturer's instructions. cDNA was synthesized using Promega M-MLV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI). For Cμ expression, quantitative PCR reaction (qPCR) was performed using Applied Biosystems TaqMan 2X Universal PCR Master Mix (Applied Biosystems, cat #: 4304437) and the results were analyzed using Applied Biosystems ViiA 7 Real-time PCR Instrument (Life Technologies, Grand Island, NY). The primers used for *IGHM/Cμ* (Applied Biosystems cat #: 4331182) *GAPDH* (Applied Biosystems cat #: 4331182) were purchased from Applied Biosystems. Primers for studying *KMT2A*, *MEN1*and *TBP* expression and MLL1 binding sites in *IGH* region are listed in table 1.

Target	Forward primer (5'-3')	Reverse primer (5'-3')
KM2TA	ACATCGTCAGCCTCCTGATACA	ACACCAACTGCCTCCTAGAA
MEN1	GGGAAGACGAGGAGATCTACAA	GTGCCCTGGCTTTGCTC
TBP	GGTTTGCTGCGGTAATCATGA	CTCCTGTGCACACCATTTTCC
IGH-MLL1 BS1	ATTAGAGTCAAGATGGCTGCAT	CACCTCTTCACAACCAGAAGT
IGH-MLL1 BS2	TGAAGACAGGACTGTGGAGA	TAGGCCAGTCCTGCTGA
IGH-MLL1 BS3	CCTTGTTAATGGACTTGGAGGA	TTAAGAGAAGCAAATGCAGCAG
IGH-MLL1 BS4	GTCTGGGTCACTCCCATTTAAC	ATCATTACCCATGCCATCCC

Table1. Sequence of the oligonucleotide primers used for qPCR

Reagents and antibodies

The menin-MLL1 inhibitor, MI-2 was purchased from Selleckchem (Houston, TX). Dimethyl sulfoxide (DMSO) and trypan blue dye were purchased from Sigma-Aldrich (cat #: D8418) and Corning incorporated (cat #: 25-900-CL) respectively. Lipopolysaccharide (LPS) from *E.coli, 0111;B4* and β-actin antibody were purchased from Millipore Sigma (St. Louis, MO). MLL1 (cat #: 14197S), Menin (cat #: 6891S), IgG (cat #: 4096S) and H3K4me3 (cat#: 9727L) antibodies were purchased from Cell Signaling Technologies Inc (Danvers, MA). LPS (*E. coli* O111:B4) was purchased from MilliporeSigma (St. Louis, MO).

KMT2A knockdown using RNAi

The short hairpin RNA (shRNA) used to target MLL1 in WM cells and scrambled shRNA (shScr) were purchased from OriGene Technologies (Rockville, MD). WM cells transfection was done by electroporation using BTX ECM 630 (Holliston, MA) following the manufacturer's instruction with some modifications. In brief, 4×10^6 cells were resuspended in OPTI-MEM media and 5 mg either shScr or shMLL1 were added to individual cuvettes. After a brief period (10 minutes), cells were electroporated at 240v; 25ms and allowed to rest an additional 15 minutes. Cells were cultured for 48 hours followed by harvesting and analysis using the appropriate assay.

Immunoblotting

Cells were lysed in RIPA buffer (ThermoFisher Scientific cat #: 89901) supplemented with a protease inhibitor cocktail (ThermoFisher Scientific cat #: A32955). Total Protein concentrations were determined using BCA assay (ThermoFisher Scientific cat#: 23228) and were equally adjusted between samples in each experiment. Proteins were mixed with SDS Loading dye (1X final concentration) and denatured on a heating block at 98 °C for 5 minutes. Cell lysates were loaded onto an SDS-PAGE gel (MLL1: 5%, Menin and β-actin:10% gels) and ran for 20 minutes at 100 volts and then 60 minutes at 130 volts.

Immunoprecipitation

To identify disruption of Menin-MLL1 interaction with the MI-2 inhibitor, 5×10^6 WM cells were treated with 5 mM inhibitor for 24 hours. Cells were harvested and immunoprecipitation was performed using Pierce™ Classic Magnetic IP/Co-IP kit (ThermoFisher Scientific cat #: 88804) following the manufacturer's instruction. Immunoprecipitated proteins were detected by immunoblotting using an antibody specific for Menin as described under immunoblotting.

Cell viability and proliferation assay

Cell viability was determined using trypan blue exclusion. Cells were counted using a Luna-II™ automatic cell counter based on manufacturer's instructions. Cell proliferation was evaluated by XTT assay as previously published[212]. For this experiment, WM cells were suspended at 0.25×10^6 cells/ml and 100 ml were cultured in each well of a 96-well plate. Cells were treated with 5 mM MI-2 inhibitor or DMSO control in triplicate for 72 hrs. Subsequently, XTT labeling mixture was prepared based on manufacturer's instruction (R and D system, cat #: 4891-025-K) and was added to each well. After 3 hours of incubation at 37° C, sample absorbance was determined using a SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA, USA). Data is represented as relative proliferation of treated wells to DMSO control treated wells.

Enzyme-linked Immunosorbent assay (ELISA)

An ELISA assay was performed to measure IgM concentration as previously published [105]. Briefly, 100 ml of diluted supernatants from BCWM.1, MWCL-1 and

RPCI.WM1 cells were used to quantify IgM levels. For animal experiments, mice sera were harvested and diluted 1:50 before performing the assay. Human IgM ELISA kit was used following the manufacturer's instruction (Bethyl labs, Montgomery, TX). All ELISA data was read on a SpectraMax M2e microplate reader.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was used to study MLL1 binding to IGH region according to our previously published protocol [213]. For each ChIP 10×10^6 cells were cross-linked for 10 minutes using a 1% formaldehyde solution at room temperature. The reaction was stopped using 125 mM Glycine followed by washing of cells with cold PBS. Cells were then lysed in cell lysis buffer supplemented with protease inhibitor cocktail. Chromatin was sheared by sonication for 20 minutes (30s on/30s off) using QSonica Q800R3 sonicator (ThermoFisher). Chromatin fractions were incubated with magnetic Protein A/G beads (ThermoFisher Scientific cat#: 88803), MLL1, I H3K4me3 or IgG isotype control antibody while rotating overnight at 4^{o} C. Beads were magnetically collected and washed three times. Protein-DNA complexes were eluted using an elution buffer and then reverse cross-linked by adding proteinase K, RNase A while shaking on a heating block at 65° C for 2 hours. DNA was purified using GeneJET PCR Purification Kit (ThermoFisher Scientific cat #: K0702) and used for qPCR using primers specific for MLL1 binding site in the *IGH* locus (Table 1).

***In-vivo* xenograft experiments**

Hairless SCID Outbred (SHO) mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Mice (n=13/group) were subcutaneously implanted with 10×10^6 BCWM.1 cells. Upon 90% tumor appearance, mice were randomly assigned to two groups: 1) MI-2 treated and 2) Vehicle (2% DMSO+30% PEG 300+2% Tween 80+ddH2O) treated. Treatments were administrated every other day using intraperitoneal (i.p.) injections for 4 weeks. Tumor sizes were recorded every other day using digital calipers (Fisher Scientific, Waltham, MA) over the course of experiment. The following formula was used to calculate tumor volume as previously published[214]: length (mm) x width $(mm)^2/2$. Mice weights were recorded every other day and mice sera were harvested post euthanasia at experiment endpoint and stored at -80^o C to use for later experiments.

Statistical analysis

All statistical analysis were done using GraphPad Prism software 9.2.0 (San Diego, CA). Data were analyzed using two-tailed t test or ANOVA for 2 or more variables respectively, and are presented as the mean of at least three independent experiments (performed in triplicate) ± SEM.

RNA sequencing and differentially expressed genes analysis

Total RNA was extracted using TRIsure reagent (Bioline, London, UK), according to the manufacturer's protocol. RNA qualities were evaluated using a SpectraMax M2e

microplate reader. The DNA library construction and data analysis were conducted by LC-Sciences (Houston, TX USA). Paired-ended sequencing was conducted using Illumina's NovaSeq 6000 sequencing system. StringTie transcript assembler [215] was used to achieve expression level of mRNAs by calculating FPKM. The differentially expressed mRNAs were nominated using R package edgeR where log2 fold change >1 or log2 fold change <-1 and statistical significance of p value < 0.05 was used[216]. Gene ontology (GO) (http://www.geneontology.org) analysis was used to perform enrichment analysis. KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used (http://www.kegg.jp/) for enrichment analysis of differentially expressed genes.

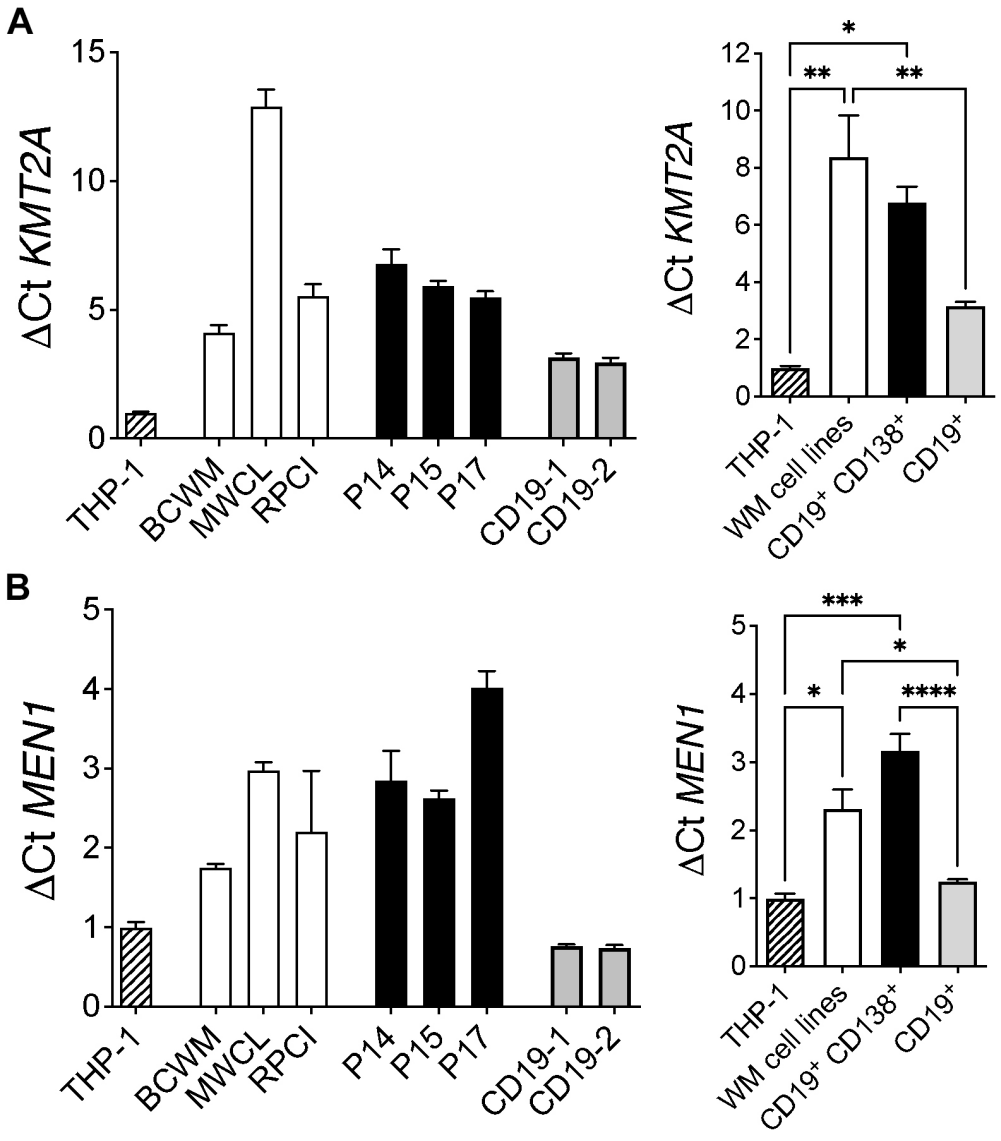


Figure 4: *KMT2A* and *Menin* are overexpressed in WM patients and cell lines. Expression of A) *KMT2A* and B) *MEN1* in WM cells lines (BCWM.1, MWCL-1 and RPCI-WM1), $CD19^+$ $CD138^+$ cells from WM patients (P14, P15 and P17), normal $CD19^+$ cells from PBMCs were compared to expression in THP-1 cells. Right panels represent the average expression of *KMT2A* and *MEN1* in WM cell lines (n=2), CD19+ CD138+ cells from WM patients (n=3) or CD19+ cells from PBMCs (n=2). Bars represent ΔCt relative to THP-1 +/- SEM.

III. Results

***KMT2A* and *Menin* are overexpressed in WM patients and cell lines**.

To examine the contribution of MLL1 in WM biology we compared the expression of *KMT2A* and its cofactor *MEN1* in three WM cells lines (BCWM.1, MWCL-1 and RPCI-WM1), $CD19^+$ $CD138^+$ cells from WM patients and normal $CD19^+$ cells from PBMCs. *KMT2A* expression in THP-1 cells was used as a control. We found that expression of in *KMT2A* (Figure 1A) and Menin (Figure 1B) was significantly higher in WM cell lines than THP-1 cells ($p<0.001$ and $p<0.05$ respectively). Similarly, *KMT2A* and Menin expression in CD19+ CD138+ cells from WM patients were significantly higher than THP-1 cells ($p<0.05$ and $p<0.001$ respectively). Compared to CD19+ B cells from PBMCs, *KMT2A* expression was significantly higher in WM cell lines ($p<0.01$) but CD19+ CD138+ expression levels did not reach statistical significance. However, Menin expression was significantly elevated in both WM cell lines and CD19+ CD138+ WM cells compared to normal B cells ($p<0.05$ and $p<0.0001$ respectively). This data suggested *KMT2A,* and Menin may play a role in WM biology.

***KMT2A* depletion and disruption of Menin-MLL1 complex reduce IgM expression and secretion in WM**.

Since the presence of high levels of a monoclonal immunoglobulin M protein (IgM) in patient serum is one of the defining features of WM[217], we investigated the role of

menin-MLL1 on IgM production in WM cells . We transfected WM cells with shKMT2A or scrambled control (shScr) for 48 hr followed by quantification of IgM levels in the cell culture supernatant by ELISA. Effective MLL1 knockdown was confirmed by immunoblotting (Figure 2A). We found that *KMT2A* knockdown significantly reduced IgM secretion in BCWM.1 ($p<0.001$) and MWCL-1 ($p<0.0001$) but not RPCI-WM1 cells (Figure 2A). Next, we tested the effect of *KMT2A* knockdown on IgM expression using primers specific for $C\mu$ region. We found a significant reduction in $C\mu$ expression in BCWM.1, MWCL-1 and RPCI-WM1 cells (Figure 2B). To investigate the possibility of targeting MLL1 as a therapeutic strategy in WM, we investigated the efficacy of the MLL1 inhibitor, MI-2 on IgM production in WM cells. WM cells were treated with 5 μM MI-2 inhibitor followed by quantification of IgM levels by ELISA. We found a significant reduction in IgM secretion by WM cells (Figure 2C). This also resulted in a significant reduction in IgM/Cμ expression by qPCR (Figure 2 D). We confirmed that MI-2 treatment reduced the interaction between Menin and MLL1 (Figure 2E). Taken together, these results show that MLL1 plays a role in WM biology by regulating Cμ expression and IgM secretion.

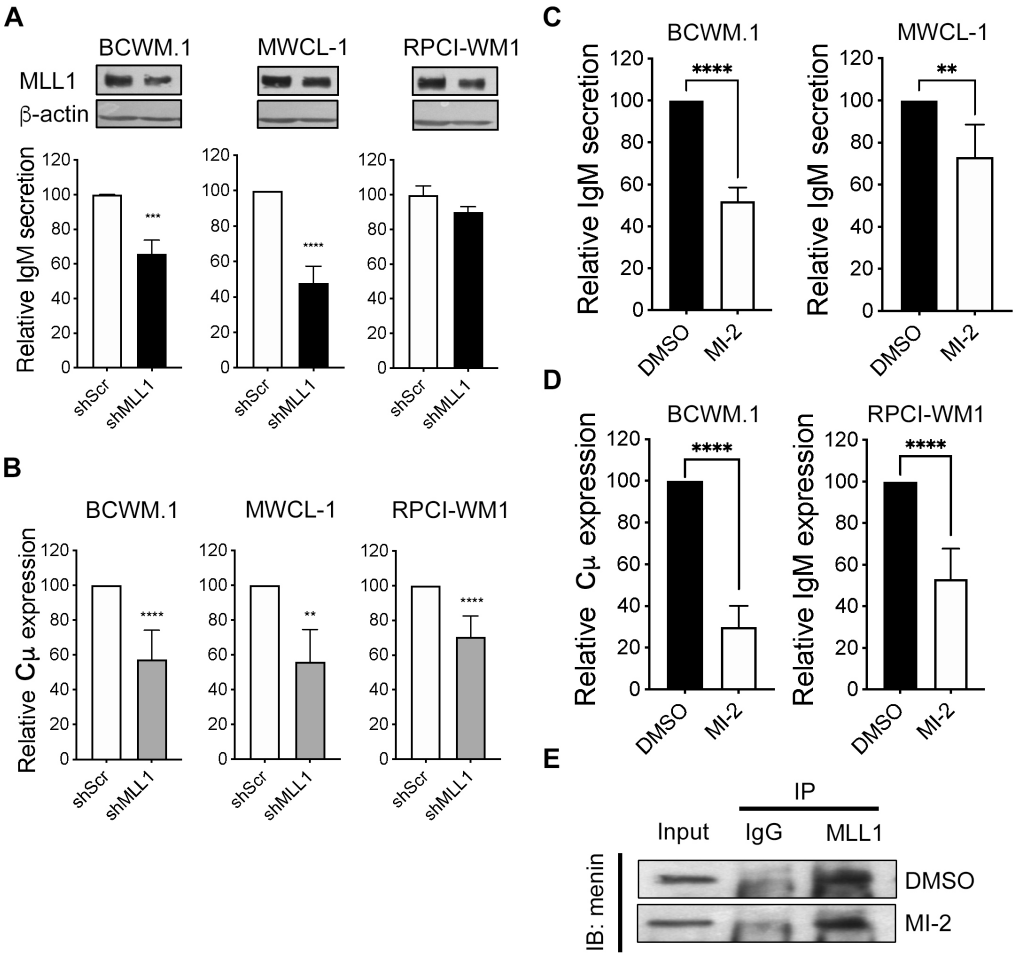


Figure 5: MLL1 depletion and disruption of menin-MLL1 complex reduce Cμ expression and secretion in WM. A) WM cells (4 x 10^6) were transfected with shMLL1 or scrambled control (shScr) for 48 hr followed by quantification of IgM levels in the culture supernatant by ELISA. An additional set of cells were transfected and lysed in RPIA buffer followed by western blot to confirm MLL1 knockdown (blots shown above each graph). B) WM cells were transfected with shMLL1 or shScr for 48 hr. Cells were harvested and RNA was purified and used to determine Cμ expression by qRT-PCR. C) WM cells (2 x 10^6) were treated with 5 mM MLL1 inhibitor MI-2 or DMSO control for 72 hr followed by

ELISA to quantify IgM levels or D) qRT-PCR to determine Cμ expression. These experiments were repeated at least 3 times with similar results and the bars represent the average of 3 independent experiments performed in triplicate +/- SEM. E) Immunoprecipitation assay was performed to assess inhibition of Menin-MLL1 interaction following treatment with 5 mM MI-2. BCWM.1 cells were lysed and MLL1 protein was immunoprecipitated using aMLL1 antibody. IgG pull-down was used as negative control. Menin protein was detected by western blot using a Menin antibody.

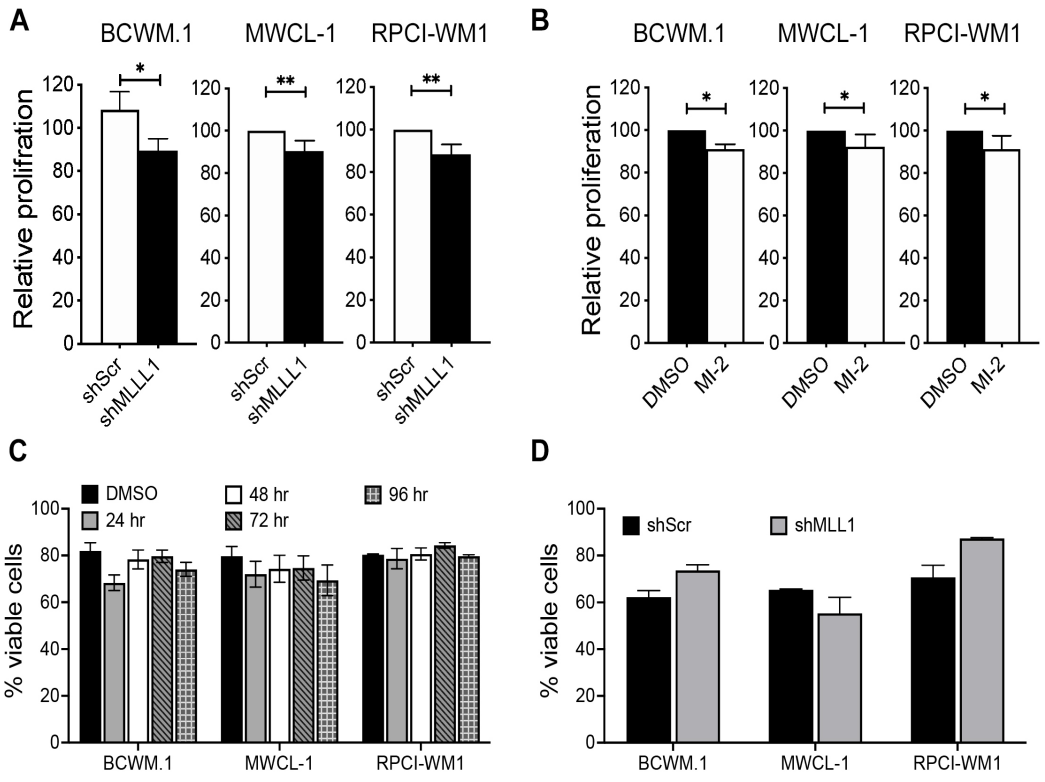


Figure 6: MLL1 inhibition does not affect WM cell viability. A) WM cells (2 x 10^6) were transfected with shMLL1 or shScr. Cells were resuspended in 0.6 ml and 100 ml were plated/well in 96-well plates. After 3 days of culture, cell proliferation was assessed using XTT assay. B) WM cells (25 x 10^3 cells/well) were plated in 96-well plates and treated with either 5 mM MI-2 or DMSO control. After 3 days, cell proliferation was assessed using XTT. C) WM cells (0.25 x 106 cells/ml) were cultured in triplicated wells in 24-well plates and treated with 5 mM MI-2 or DMSO control. Cell viability was assessed daily using trypan blue exclusion. D) WM cells (4 x 10^6) were transfected with shMLL1 or shScr for 72 hr followed by examination of cell viability by trypan blue exclusion. Bars represent the average of at least 3 independent experiments performed in triplicate +/- SEM.

***KMT2A* depletion and MLL1 inhibition does not affect WM cell viability**.

To determine if the reduced IgM levels observed in the presence of *KMT2A* knockdown or MI-2 inhibitor were a result of a reduction in WM cell growth or viability, we performed an XTT on WM cells transfected with shKMT2A (Figure 3A) or treated with MI-2 inhibitor (Figure 3B). We found a significant reduction in WM cell growth, although this reduction did not exceed 10%. However, neither *KMT2A* knockdown nor treatment with the Menin MLL1 inhibitor, MI-2, had any effect on WM cell viability (Figure 3C and D). Taken together, these data suggest that targeting KMT2A/MLL1 may reduce IgM levels directly without affecting cell viability and with modest effects on WM cell proliferation.

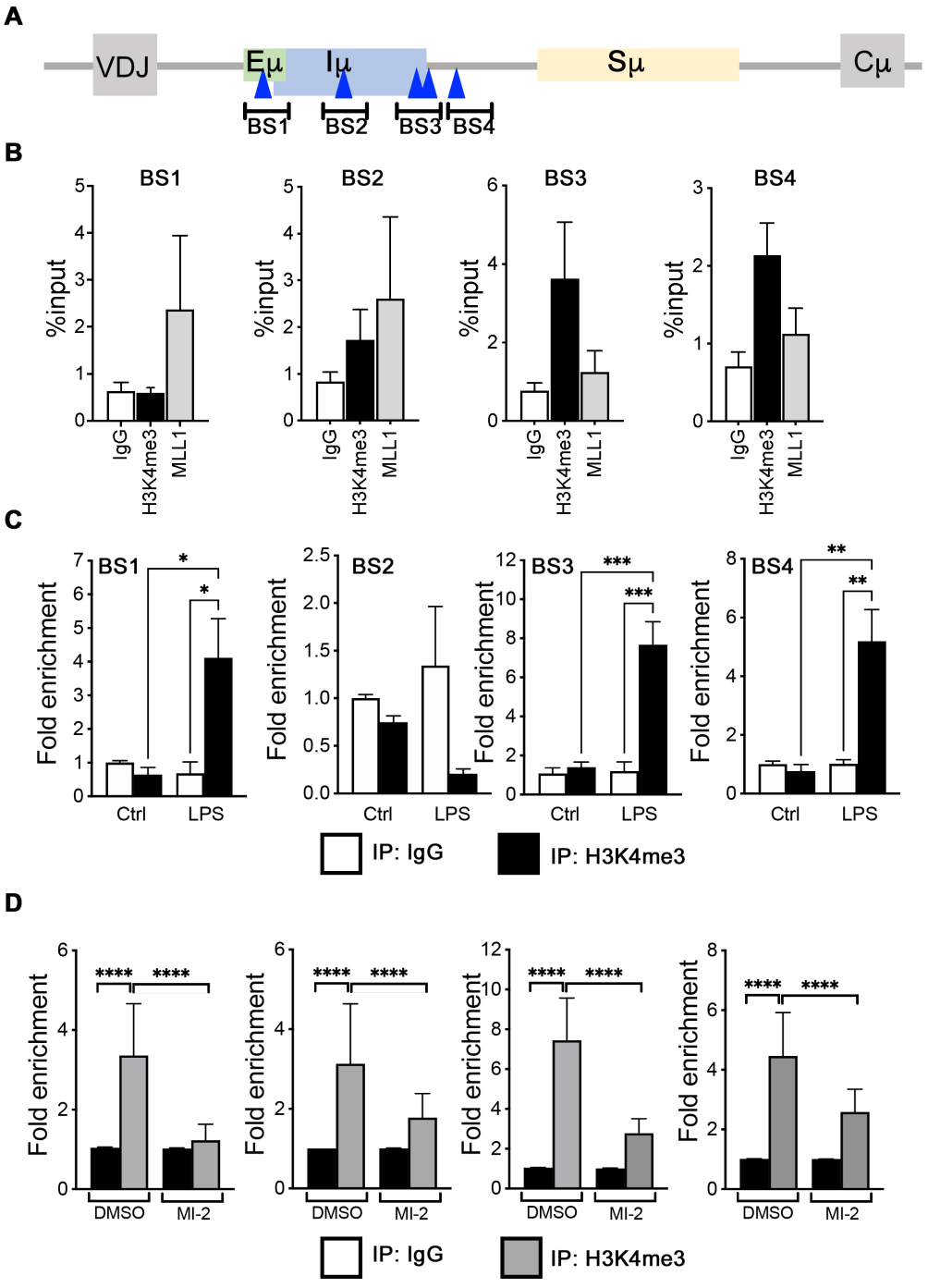


Figure 7: H3K4me3 deposition near *IGH* Eμ enhancer and Im region. A) Schematic of *IGH* region. Variable region (VDJ) and constant region (Cμ) are shown in gray. Blue

triangles represent five candidate MLL1 binding sites. The Eμ enhancer (green), Iμ region (blue) and switch (Sμ)) region are indicated. Black brackets indicate primers used for ChIP-qPCR to detect binding to MLL1 binding sites (BS1, BS2, BS3 and BS4). B) BCWM.1 cells (10 x 10^6) were fixed and lysed as indicated in materials and methods. Lysates were used in ChIP assay followed by qPCR to determine basal MLL1 or it's mark H3K4me3 deposition in the *IGH* region in WM cells. C) CD19+ cells were purified from PBMCs of healthy donors followed by stimulation with LPS for 3 hr to activate B cells. Cells were then used in ChIP-qPCR to determine H3K4me3 deposition in the IGH region. D) BCWM.1 cells were treated with 5 mM MI-2 or DMSO control and cells were used to determine H3K4me3 deposition in *IGH* region by ChIP-qPCR. These experiments were performed at least 3 times.

H3K4me3 deposition near *IGH* Eμ enhancer and Iμ region.

The human *immunoglobulin heavy chain* (*IGH*) locus consists of multiple transcription regulatory regions[218]. The intronic Eμ enhancer and Iμ region are located between the J_H segment and Cμ constant region exons[219] and strongly control *IGH* gene expression [206,220]. We identified five candidate MLL1 binding sites (BS)[221] near Eμ enhancer and Iμ region with 2 candidate BS with close proximity to one another (Figure 4A). Since tri-methylation of histone 3 on lysine residue 4 (H3K4me3) is the mark of MLL1 function and transcriptional gene activation [121,222,223], we first determined MLL1 binding and enrichment

of H3K4me3 mark in *IGH* Eμ enhancer and Iμ region in unstimulated WM cells that secrete IgM. Chromatin immunoprecipitation (ChIP) assay revealed that MLL1 binds to all five MLL1 binding sites in Eμ enhancer and Iμ region and H3K4me3 mark was enriched in these regions (Figure 4B). Similarly, stimulation of purified CD19+ cells from PBMCs with LPS to activate B cells showed increase H3K4me3 deposition at three of the 4 BS regions (Figure 4C). We treated WM cells with the MI-2 inhibitor and found that disruption of menin-MLL1 significantly reduced H3K4me3 enrichment at MLL1 BSs. These findings indicate that *IGH* gene is direct target of the MLL1 methyltransferase enzyme and suggest that MLL1-mediated reduction in IgM production occurs via MLL1/H3K4me3 binding to the *IGH* region.

MLL1 inhibition reduces IgM secretion in tumor-bearing mice.

After characterization of the role of MLL1 in *IGH* transcription *in-vitro*, we investigated the efficacy of the menin-MLL1 inhibitor *in-vivo* using SCID mice bearing WM tumors. SCID mice were subcutaneously implanted with WM cells followed by initiation of MI-2 therapy upon tumor appearance for a total of 4 weeks (Figure 5A). Mice were randomly assigned into 2 groups, Vehicle and MI-2. Mice sera was harvested at experiment end-point and human IgM levels were quantified by ELISA. We found that mice treated with MI-2 inhibitor had significantly lower levels of IgM in their sera (Figure 5B). However, consistent with our *in-vitro* results, MLL1 inhibition had no effect on tumor growth as there was no difference in tumor volume between the Vehicle and MI-2 treated groups (Figure 5C). In addition, there was no difference in body weight of MI-2 treated

and Vehicle-treated mice which suggests that MI-2 inhibitor was not toxic (Figure 5D). Taken together, these results confirm that MLL1 inhibition plays a role in the regulation of IgM but not in WM cell growth and suggests that targeting MLL1 in WM patients may be beneficial in reducing IgM levels.

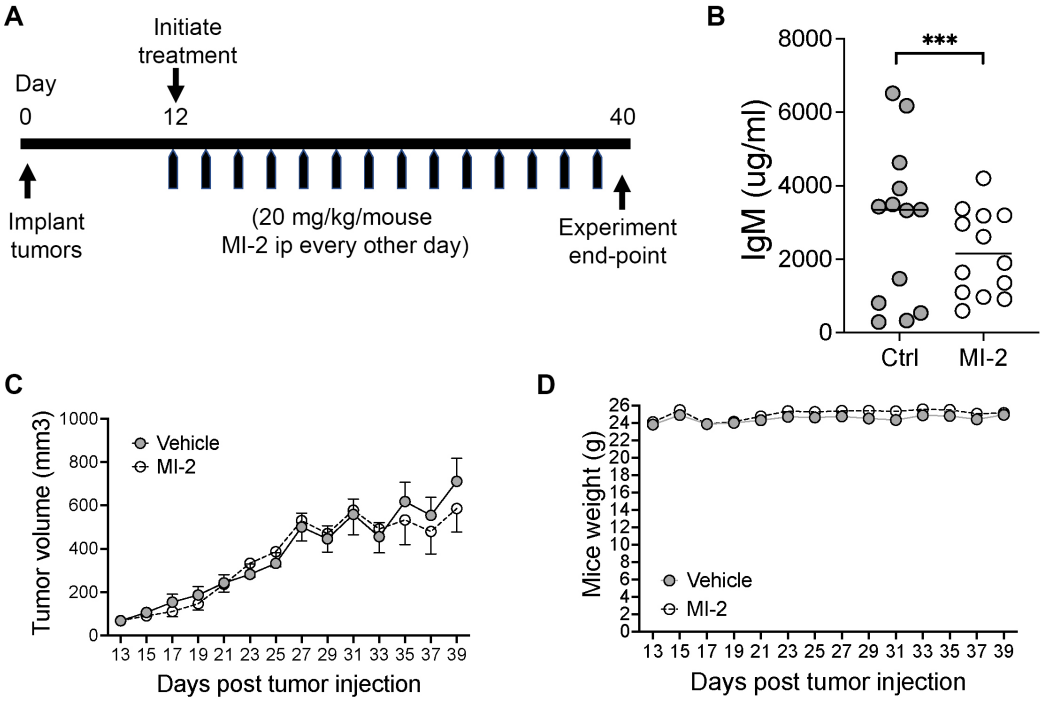


Figure 8: MLL1 inhibition reduces IgM secretion in tumor-bearing mice. A) Hairless SCID mice were implanted with 10 x 10^6 BCWM.1 cells. Groups of mice were treated with either 5 mM MI-2 or vehicle control. B) Human IgM levels were quantified in mice sera at experiment end-point using ELISA. Shown are IgM levels in individual mice and line represents Median value. C) Tumor volume in MI-2 and Vehicle treated mice. D) Mice weight was determined in MI-2 and vehicle treated mice.

Genome-wide investigation of MLL1 gene networks in WM

To study MLL1 function on gene expression profile in WM, Illumina-based RNA-sequencing analyses was performed using the three WM cell lines treated with small molecular inhibitor MI-2 that target Menin-MLL1 interaction. As the control, we treated WM cells with DMSO. RNA-seq analysis identified 99 differentially expressed genes was identified between the control group and MI-2 treated cells (Figure 6). Of those genes, 53 were significantly downregulated and 46 were upregulated in the MI-2 treated cells.

Statistical significance of differentially expressed genes in WM cells treated with MLL1 inhibitor versus fold change (FC) is presented with volcano plot (Figure 7) where the upregulated genes are shown in red color and the downregulated genes are shown in blue. In addition, gene ontology (GO) cluster analysis indicated that differentially expressed transcripts correlate with multiple GO terms including biological process, cellular component, molecular function, protein binding, cytoplasm, cytosol, nucleus, nucleoplasm, and metal ion binding (Figure 8). Moreover, based on Gene Ontology (GO) enrichment analysis MLL1-regulated genes were enriched for 20 significantly GO terms including regulation of humoral immune response, type 1 interferon receptor activity and sphingolipid transporter activity, etc (Figure 9), supporting a diverse role of MLL1 in regulating the WM transcriptional programing. Representative genes that were altered by MI-2 treatment are shown in table 2. As listed in table 2, inhibiting MLL1 led to the downregulation of oncogenic genes in WM with average log2 fold change of −2 [224–228].

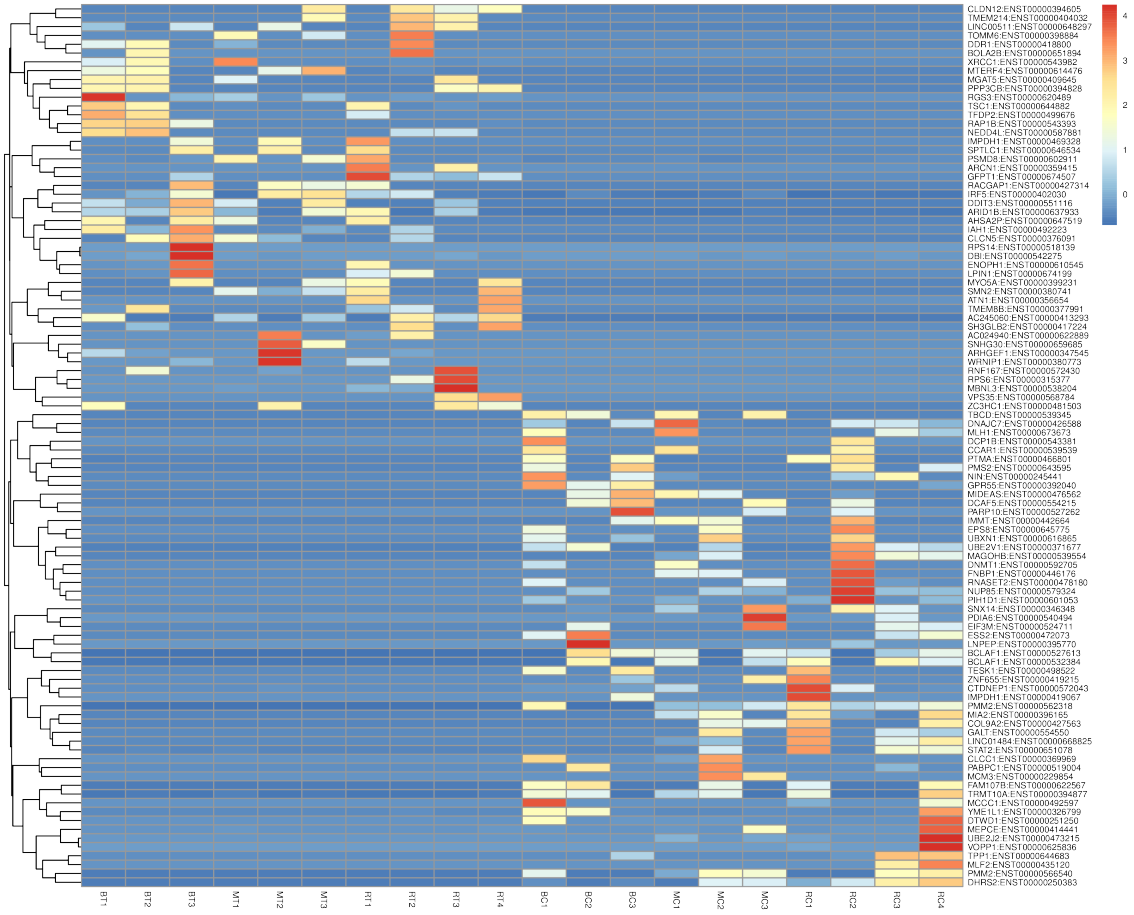


Figure 9. Heat map of differentially expressed genes in WM cells treated with MI-2 inhibitor. WM cells ($4x10^6$ cells/ml) were treated with MI-2 inhibitor for 48 hr then harvested for RNA isolation. RNA-seq analysis was performed to determine the transcriptional target of MI2 inhibitor in WM cells. We identified a total of 99 differentially expressed genes between the control group and MI2 treated cells, of which 53 were significantly downregulated and 46 were upregulated.

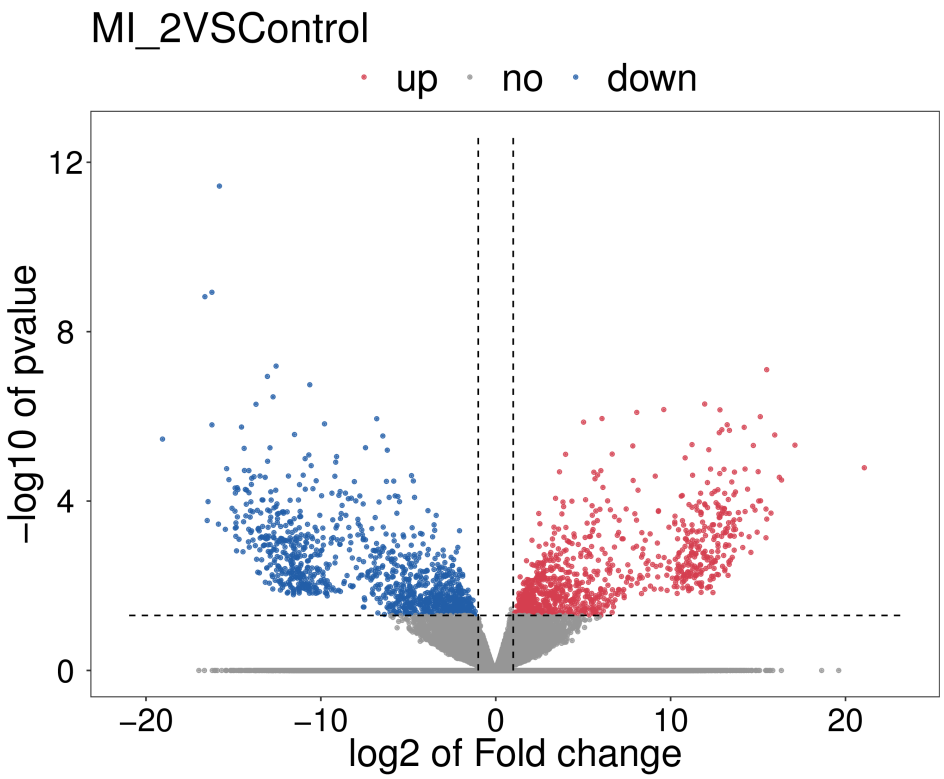


Figure 10. Volcano plot of differentially expressed genes in WM cells treated with MLL1 inhibitor. Red dots represent significantly upregulated genes; blue dots are, significantly downregulated genes where the most upregulated genes are towards the right, the most downregulated genes are towards the left. The plot shows statistical significance (P value) versus log fold change.

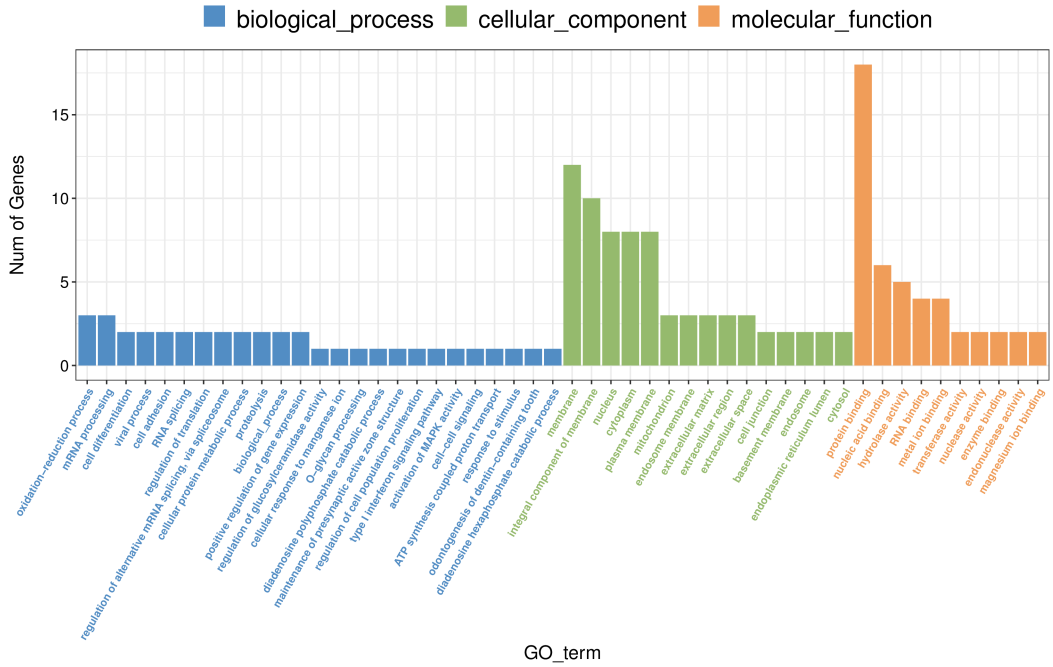


Figure 11. Gene ontology (GO) cluster analysis of differentially expressed genes in WM cells treated with MLL1 inhibitor. Investigation of biological process using gene ontology (GO) cluster analysis indicated that this differentially expressed mRNAs were functionally assigned to multiple GO terms including biological process, cellular component, molecular function, protein binding, cytoplasm, cytosol, nucleus, nucleoplasm, and metal ion binding.

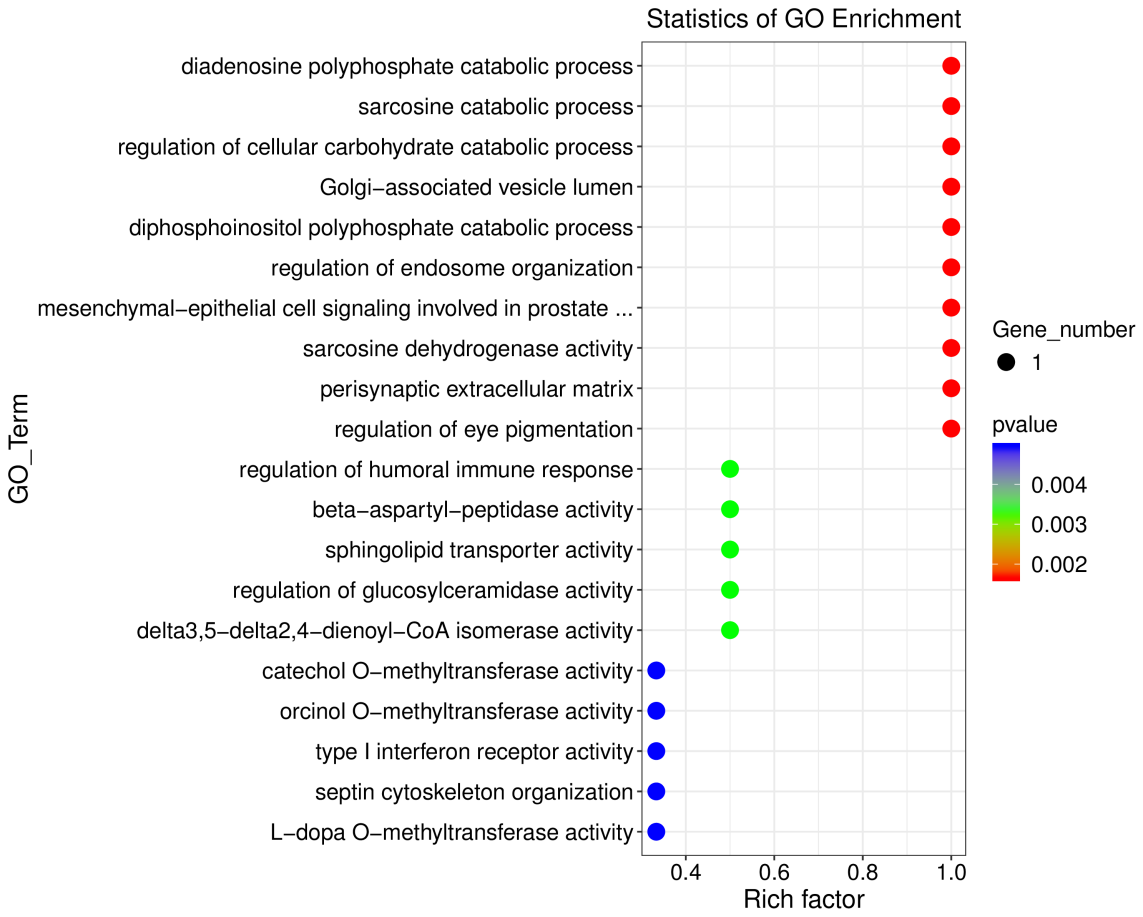


Figure 12. Gene Ontology (GO) enrichment analysis of differentially expressed genes in WM cells treated with MLL1 inhibitor: Circle size represents the number of genes, and the color represents the p value. Based on GO enrichment analysis 20 significantly enriched GO terms were identified including regulation of humoral immune response, type 1 interferon receptor activity and sphingolipid transporter activity, etc.

Table 2. Top modulated genes (based on Log2 Fold-Change in gene expression level) by MLL1 inhibititon. Positive FC value representing up-regulated genes and negative FC value representing down-regulated genes. P-value ≤0.5.

Gene symbol	Gene ID	Log2(FC)	*P*-value	Description
FBXO39	ENSG00000177294	-3.66	0.01	F-box protein 39: Oncogene
TNC	ENSG00000041982	-3.46	0.00	Tenascin C : Oncogene
LAIR1	ENSG00000167613	-2.35	0.00	Leukocyte associated immunoglobulin like receptor 1 (CD305) : Immune chekpoint
GPR68	ENSG00000119714	-1.75	0.00	G protein-coupled receptor 68: Oncogene
NDUFA4L2	ENSG00000185633	-1.29	0.03	NDUFA4 mitochondrial complex associated like 2: Oncogene
TNFAIP8	ENSG00000145779	-1.05	0.02	TNF alpha induced protein 8 : Oncogene
QKI	ENSG00000112531	1.06	0.01	QKI, KH domain containing RNA binding :Tumor suppressor
SNORD22	ENSG00000277194	3.27	0.01	Small nucleolar RNA (scoRNA)::Tumor suppressor

IV. Discussion

Despite current therapeutic options, WM remains incurable. Genetic and epigenetic abnormalities and a supportive tumor microenvironment result in a low response to chemotherapies and in Ibrutinib resistance in WM[229]. Therefore, there is a need to develop new therapies targeting novel pathways that are critical in WM biology. We have previously shown that epigenetic inhibitors have successful therapeutic effect in WM[105]. To further understand epigenetic modifications in WM, we investigated the role of the MLL1 methyltransferase in WM biology.

The menin-MLL1 complex plays an oncogenic role in hematological malignancies[230231] which made targeting this protein complex a potential therapeutic

option in lymphoma. Here, we demonstrate that the MLL1 complex plays a role in WM lymphomagenesis by regulating IgM production. We also show that the small molecule inhibitor of the menin-MLL1 interaction (MI-2) reduces IgM secretion in WM without *in-vivo* toxicity; therefore, may have therapeutic benefit for WM patients.

The MLL family of proteins play a critical role in regulating gene transcription through histone modification and genomic DNA accessibility to transcription machinery in normal and malignant B cells[232]. In the current study, we found that expression of *KM2TA* and its partner *MEN1* are higher in WM cell lines and CD19+ CD138+ cells from WM patients compared to THP-1 cells (which express very low levels of both genes) (Figure1A and B). Consistent with these findings *KM2TA* and *MEN1* expression were significantly higher in WM cell lines compared to CD19+ B cells from PBMCs of healthy donors (Figure1A and B). This suggested that MLL1 and Menin may play a role in WM biology. Previous studies also have reported somatic mutations in several histone modifying enzymes in WM including *KM2TD* (MLL2), *SETD2* and *KDM6A*[23342]. In addition, mutation of histone remodelers such as *ARID1A* and *ARID1B* were found in WM patients [42]. These studies indicate modulation of epigenetic pathways; however, a biological understanding of their role in WM is poorly understood.

WM is defined as malignant IgM-secreting lymphoplasmacytic lymphoma where uncontrolled production of monoclonal IgM in the serum is associated with hyperviscosity syndrome along with other health complications[234]. Here, we show that MLL1 knock down significantly reduced IgM secretion in WM cell lines via a reduction in Cμ expression

(Figure 2A & 2B). Similarly, pharmacological inhibition of menin-MLL1 interaction using the MI-2 inhibitor significantly reduces IgM expression and secretion (Figure 2C& 2D). Our results demonstrate that the reduced IgM is not due to a decrease in WM cell growth in response to MI-2 treatment or MLL1 knockdown (Figure 3A and B). Although we detected a reduction in WM cell viability, this reduction was relatively minor suggesting the role of MLL1 in the modulation of IgM is independent of cell growth and survival (Figure 3). It is well understood that epigenetic regulation plays an important role in B cell maturation[235]. MLL1, as the core of menin-MLL1 complex, with H3K4me3 mark of transcriptional activation is involved in the regulation of adult hematopoietic stem cells (HSCs) and progenitors[236].

The regulation of immunoglobulin transcription is a unique mechanism that is highly dependent on DNA recombination events and dynamic epigenetic changes[237]. Studies have shown that H3K4me3 modification is required for *IGH* rearrangement in B cells [238239]. WM cells are characterized as mature B cells where the transition to mature B cell happens upon encountering antigen in the periphery[240]. These activated B cells are more prone to malignant alteration, since epigenetic dysregulation of gene regulatory enhancer elements has been reported in B cell malignancies where these cells represent district chromatin configuration and histone modifications [241]. Here, we characterized the role of MLL1 in *IGH* regulation and found that MLL1 binds to Eμ enhancer and Iμ regions in WM cells (Figure4A and B). In addition, we found enrichment of H3K4me3 at multiple MLL1 binding sites in these regions (Figure4B).

Consistent with our findings, genome-wild studies have shown high MLL1 enrichment at promoter and enhancers of immune cells[163,223]. Additionally, MLL1 has been shown to preferentially bind gene enhancers (79% of target regions) than promoters and TSSs in leukemia cells where histone mono-, di-, and tri-methylation marks were significantly enriched near MLL1 binding sites[163]. Our studies revealed a higher enrichment of H3K4me3 in MLL1 binding sites in *IGH* gene regulatory regions in LPS-stimulated CD19+ cells from PBMCs of healthy individuals (Figure4C). We also found that treatment of WM cells with the MI-2 inhibitor significantly reduced enrichment of the H3K4me3 mark in the *IGH* region (Figure4D) supporting a role for the menin-MLL1 complex in the regulation of *IGH*/IgM. Other studies suggest that the MLL family of methyltransferases affect gene expression not only by altering histone modifications and accessibility of promoter and enhancer regions, but also through bringing several other proteins in close vicinity of these gene regulatory regions[242]. However, there is limited information regarding MLL1 interaction with other proteins such as transcription factors and epigenetic modifiers in lymphoma.

Taken together, our results highlight a role for the menin-MLL1 complex in the regulation of *IGH* regulation. Our *in-vitro* studies are validated *in-vivo* where we show the efficacy of the menin-MLL1 inhibitor MI-2 and subsequent reduction in IgM secretion in WM tumor-bearing mice (Figure5B). These results are consistent with other studies showing the efficacy of the MI-2 inhibitor *in-vivo*[243,244]. As expected, we found no significant reduction in tumor volume upon MI-2 treatment which is consistent with our *in-vitro* results (Figure5C). This finding indicates similar drug mechanism in both *in-vitro* and

in-vivo studies where the MI-2 inhibitor had no effect on WM cell proliferation and survival. Finally, administration of the MI-2 inhibitor shows no *in-vivo* toxicity (Figure5D). Together, these findings suggest the pharmacological efficacy of the MI-2 inhibitor in reducing IgM levels in WM. Moreover, genome-wide studies of the effect of MLL1 inhibition in WM using the MI-2 inhibitor showed that among MLL1 target genes, the immune checkpoint inhibitor Leukocyte Associated Immunoglobulin like Receptor 1 (LAIR1: CD305) gene is significantly downregulated (Table 2). Downregulation of CD305 is reported in WM cases[249]

Immune inhibitory receptors are receptors that communicate inhibitory signals to immune cells moderating the balance between self-tolerance and eliminating the foreign antigen. Most studied inhibitory immune receptors including LAIR1, programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte protein 4 (CTLA-4) rely on immunoreceptor tyrosine-based inhibitory motifs (ITIMs) for signaling[245].

LAIR1 is an important transmembrane glycoprotein that functions as an inhibitory immune receptor, and is expressed on almost all immune cells such as B cells, T cells, NK cells, dendritic cells and monocytes[246,247].

The clinical significance of LAIR1 (CD305) expression has been reported in chronic lymphocytic leukemia[248]. CD305 is considered a valuable marker for diagnosis of light-chain restricted B-cells lymphomas[249]. In addition, down regulation of tumor

suppresser genes such as RNA-binding protein Quaking (QKI) and IncRNAs (SNORD22) id detected using MLL1 inhibition[250,251].

These results suggest that Menin-MLL1 inhibitor promotes WM cell differential expression by targeting the expression of both oncogenes and tumor suppressor genes transcription and genes regulating the humoral immune response.

CHAPTER 3

CHARACTERIZATION OF B CELL RECEPTOR (BCR) SEQUENCES IN WALDENSTRÖM MACROGLOBULINEMIA CELL LINES

I. Introduction

Waldenström macroglobulinemia (WM) is a B-cell lymphoma characterized by the overproduction of a monoclonal immunoglobulin-M (IgM)[34]. Currently, there are three cell lines derived from WM patients that are used to study WM: BCWM.1, MWCL-1, and RPCI.WM1 [209–211]. However, the B cell receptor sequence of WM cell lines has not been described. WM B cells do not undergo class switch recombination (CSR) to produce other Ig isotypes[252].

Ig molecules are composed of two identical heavy (H) and 2 identical light (L) chains, which include a variable region (V_H, V_L) and one or more constant regions (C_H, C_L)[253].

The H-chain V-region consists of recombined $V_H(D_H)J_H$ gene segments and the L-chain includes V_LJ_L segments[254]. V(D)J gene recombination occurs sequentially where H-chain genes recombine first followed by L-chains[255]. Each variable region contains

three hypervariable complementary determining regions (CDR1, CDR2, and CDR3) flanked by four conserved framework regions FR1, FR2, FR3 and FR4 (Figure 1A)[256]. The International Immunogenetics Information System (IMGT) has a unique definition for CDR and FR[257]. In the V_H region, CDR3 has the highest sequence variability suggesting an important role in antigen specificity[258]. A study of CDR3 length in WM patients indicated antigenic selection in 77% of cases[259]. Following antigen exposure, B cells undergo somatic hypermutation (SHM) and CSR resulting in increased affinity and a change in antibody isotype. During SHM, a high rate of point mutations or (rarely) insertions/deletions are introduced in the V-region.

Here, we describe the B cell receptor repertories of WM cell lines and identify VDJ gene segment usage. We found high levels of L-chain expression in WM cell lines with a L:H-chain ratio of 20-100 compared to 2 in normal B cells and this correlated with secreted IgM levels[260].

We have previously shown in chapter 2 that MLL1 methyltransferase inhibition reduced Ig heavy chain expression and secretion in WM. We therefore decided to evaluate the effect of MLL1 inhibition on light chain regulation. Taken together, this study provides information on BCR sequences of WM cell lines and provides a platform to study the regulation of immunoglobin expression.

II. Methods

Cells:

BCWM.1 cells were kindly provided by Dr. Steven Treon (Dana Farber Cancer Institute, Boston [209]. MWCL-1 cells were kindly provided by Dr. Stephen Ansell (Mayo Clinic, Rochester, MN) [210]. RPCI-WM1 cells were kindly provided by Dr. Asher Chanan-Khan (Mayo Clinic, Jacksonville, FL) [211]. WM cell lines were maintained in RPMI1640 supplemented with 10% FBS and 1% antibiotic-antimycotic.

RNA extraction:

Total RNA was extracted using PureLink RNA Mini kit according to the manufacturer's recommendations (ThermoFisher Scientific cat #: 12183018A). RNA was quantified with Qubit High Sensitivity RNA Assay kit (ThermoFisher Scientific cat# Q32852). WM cell lines were authenticated using STR profiling (Labcorp DNA Identification lab, Burlington, NC) prior to RNA extraction.

B cell receptor sequencing:

Bulk B cell receptor sequencing was performed by Medgenome (Foster City, CA). To generate sequencing libraries, Takara's SMARTer Human BCR IgG IgM H/K/L Profiling Kit . For this experiment 1 ug total RNA was used as input. Separate libraries were made for heavy chain (IgG/IgM) and light chain (IgK/IgL). Final libraries were pooled

and sequenced (paired end, 300 bp) on Illumina MiSeq to a minimum depth of 100k reads per sample.

Bioinformatic analysis:

VDJ sequences were provided by Medgenome. CDR3 DNA sequences were aligned using SnapGene 6.0.4 software. Additional CDR3 analysis was performed using IMGT/V-Quest and junction analysis v3.5.28. CDR3 amino acid sequences were aligned and graphed using PRRN algorithm and Weblogo 3.7.4. Sequence of the variable region was aligned to the closest germline sequence using IMGT.

Enzyme-linked Immunosorbent assay (ELISA):

To quantify IgM secretion, a human IgM ELISA kit from Bethyl labs (Montgomery, TX) was used following manufacturer's recommendations as previously published [23].

Light:Heavy chain ratio calculation:

BCR sequencing was performed using duplicate RNA samples for each cell line. The clone count for each sample was used to generate L:H-chain ratio and an average of the 2 samples was used to generate graphs.

Gene copy number analysis

Total RNA was extracted using TRIsure reagent (Bioline, London, UK), according to the manufacturer's protocol. Then, cDNA was synthesized using Promega M-MLV reverse transcriptase kit following the manufacturer's protocol (Promega, Madison, WI).

mRNA specific primers were designed for heavy and light chains where forward primers were in variable region and the reverse primers were in associated constant region in each cell line. After PCR amplification of heavy and light chain sequence, PCR products where ran on the 1% low melt agar gel and the DNA was purified from the gel using a GeneJET Gel Extraction kit (Thermo Scientific) and DNA quality was quantified by SpectraMax M2e microplate reader. For generating the qPCR standard curve, 1 μg of DNA was used to make 15 serial dilutions. The results were analyzed using Applied Biosystems ViiA 7 Real-time PCR Instrument (Life Technologies).

Finally, qPCR mRNA specific primers for the Cμ, Kappa and Lambda genes were designed. Data analysis was performed using Applied Biosystems ViiA 7 Real-time PCR Instrument (Life Technologies, Grand Island, NY). The primers used for this experiment are listed:

Cμ, 5′-GAACACGTGGTGTGCAAAG-3′ (forward) and 5′-CAGTCGCTCTCTTTGATGGT-3′ (reverse); Lambda IGV-C1/C2 region, 5′-GCTGAGGATGAGGCTGATTATTA-3′ (forward) and 5′-CTGTAGCTTCTGTGGGACTTC-3′ (reverse); Kappa IGV-C region, 5′-GGGACAGAATTCACTCTCACAA-3′ (forward) and 5′-AGGCGTAGACTTTGTGTTTCT-3′ (reverse). IgM, 5′-GGAGTGCGTCTCAACCATTAG-3′ (forward) and 5′-GTGGACTTGGTGAGGAAGATG-3′ (reverse); Lambda C1/C2 region, 5′-

GTGACAGTGGCCTGGAAG-3′ (forward) and 5′- CTGGCCGCGTACTTGTT-3′ (reverse); Kappa C region, 5′- ATCTGGAACTGCCTCTGTTG-3′ (forward) and 5′- GTTATCCACCTTCCACTGTACTT-3′ (reverse).

III. Results

V(D)J usage

Bulk BCR sequencing identified V(D)J gene usage in WM cell lines. in BCWM.1 cells, *IGHV3-23*, *IGHD6-19* and *IGHJ3* are used in the H-chain (Figure 13). In addition, *IGL2-8* and *IGLJ2* are used in the L-chain. The length of the CDR3 region in *IGH* and IGL were calculated based on the IMGT numbering system[261], which were 13 and 9 amino acids respectively (*IGH* AAs: CAKYAGWLDAPFDIW and IGL AAs: CSSYAGTTVVF).

MWCL-1: These cells use VH3-15 as previously reported (97.12% sequence identity)[211] and we identified *IGHD1-1* and *IGHJ4* gene segments usage. *IGKV1-17* and *IGKJ1* are used in the L-chain. CDR3 length of *IGH* was 16 AAs (CTTILTGTWHGASIGHYW) and *IGL* was 8 AAs (CLQHNSMWTF).

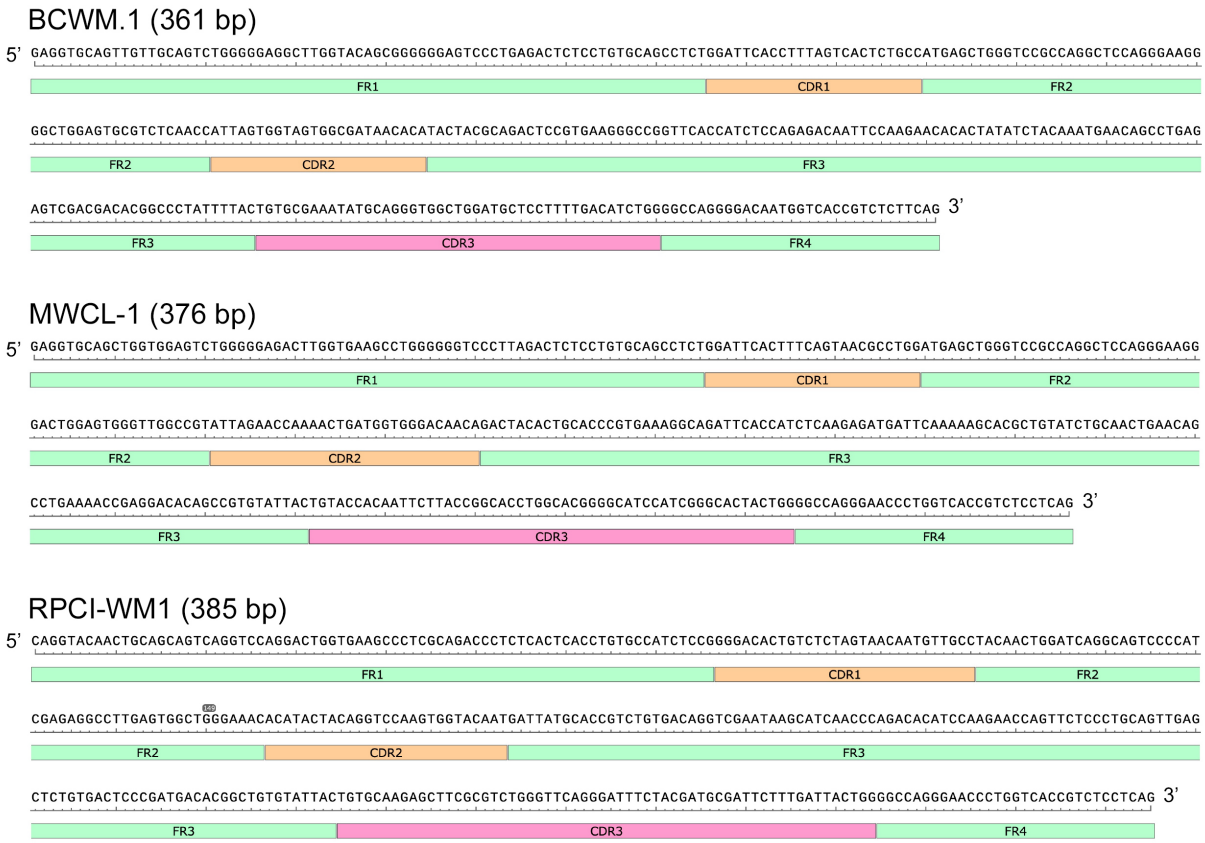
RPCI-WM1: Unlike BCWM.1 and MWCL-1 cells which use the VH3 family in their H-chain, the *IGHV6-1* gene segment is used in RPCI-WM1 cells[262]. *IGHD3-10* and *IGHJ4* segments are used. For the L-chain, *IGKV3-20* and I*GKJ4* gene segments were identified and the CDR3 length was 18 AAs (CARASRLGSGISTMRFFDYW) for *IGH* and 10 AAs (CLQHNSMWTF) for *IGL*.

CDR3 analysis

The length of the H-chain CDR3 is variable based on V_H germline usage[263]. RPCI-WM1 cells have the longest CDR3 sequence followed by MWCL-1 then BCWM.1 cells. We aligned the H-chain CDR3 DNA sequences using SnapGene and identified overlapping and different CDR3 sequences in WM cell lines (Figure14A). Using IMGT junctional analysis, the CDR3 amino acid sequence of WM cell lines is numbered based on the IMGT numbering system and AA physiochemical classes are shown according to the IMGT color coding system (Figure14C and D)[264]. The CDR3 amino acid sequence includes a Cys-XXXX-Trp/Phe domain representing a conserved cysteine at amino acid 104 (second-Cys) and a conserved tryptophan/phenylalanine at amino acid 118 (Figure14B)[257]. Using WebLogo software to compare amino acid distribution, we show the CDR3 amino acid similarities and differences between WM cell lines (Figure14C). CDR3 was mostly occupied by neutral amino acids in MWCL and RPCI cell lines. Amino acid residues 104-105 (CA) in BCWM-CDR3 were conserved in RPCI-WM1 cells, and 117–118 (YW) were conserved between MWCL-1 and RPCI-WM1 cells.

A

B



C

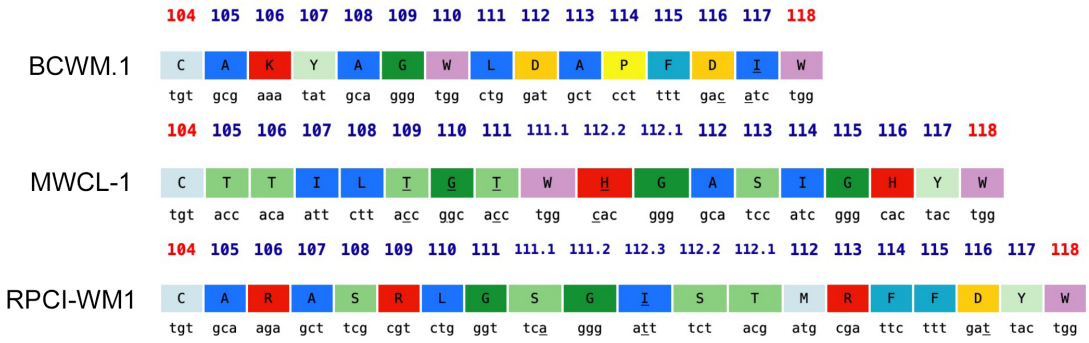
Cell line	Reads	V usage	D usage	J usage	CDR3-IMGT length	CDR3 AA sequence
BCWM.1	8130	IGHV3-23	IGHD6-19	IGHJ3	13	CAKYAGWLDAPFDIW
	168434	IGLV2-8		IGLJ2	9	CSSYAGTTVVF
MWCL-1	3333	IGHV3-15	IGHD1-1	IGHJ4	16	CTTILTGTWHGASIGHYW
	80710	IGKV1-17		IGKJ1	8	CLQHNSMWTF
RPCI-WM1	1259	IGHV6-1	IGHD3-10	IGHJ4	18	CARASRLGSGISTMRFFDYW
	143634	IGKV3-20		IGKJ4	10	CQHYSGSPPITF

Figure13: IGHV, IGK/L V segment usage and sequence of CDR3 region of WM cell lines. A) Schematic of IGH region representing the location of complementarity

determining regions (CDRs) and framework regions (FR) and the relative location of CDR1 and CDR2 sequences in the V gene segment and CDR3 sequence in the VDJ joining region. B) Entire IGHV sequence in WM cell lines including the four conserved framework regions (FR1, FR2, FR3, and FR4) and three complementary determining regions (CDR1, CDR2, and CDR3). C) *IGH* and *IGL* chain gene segments usage in WM cell lines. CDR3 length, mutational status and aminoacidic sequence are obtained from Using IMGT/V-Quest version 3.5.28 from IMGT website.

A

B



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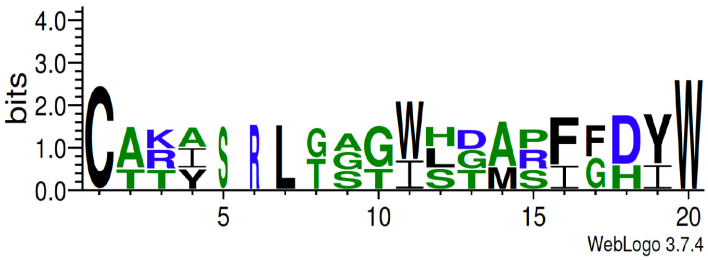
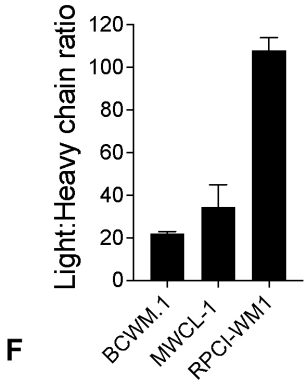


D

Cell line	V-region	FR1	CDR1	FR2	CDR2	FR3
BCWM.1	22	5	4	3	3	7
Silent	6	1	1	0	1	3
Non silent	16	4	3	3	2	4
MWCL-1	8	2	0	1	2	3
Silent	2	1	0	1	2	3
Non silent	6	1	0	0	0	0
RPCI-WM1	20	1	5	4	1	9
Silent	5	1	2	0	1	1
Non silent	15	0	3	4	0	8

E



F

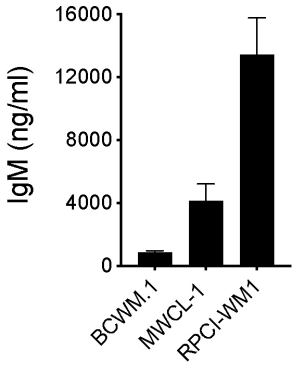


Figure14: Analysis of CDR3 in WM cell lines. A) Sequence alignment of CDR3 DNA sequence in the H-chain region of WM cell lines were aligned using SnapGene 6.0.4. The identical nucleotides are highlighted. Gaps are used for alignment comparison to indicate

insertion or deletion. B) Amino acid sequence of CDR3 region of WM cell lines included a Cys-XXXX-Trp/Phe domain. Codons are numbered based on IMGT numbering system and amino acid physiochemical classes are shown according IMGT color coding system. Mutated nucleotides and amino acids are underlined. Figures were generated using IMGT-junction analysis. C) Consensus amino acid sequence of CDR3 residues of H-chain in WM cell lines. Sequences were aligned using PRRN algorithm then Weblogo 3.7.4 was used to display the plots. D) Mutational analysis of *IGH* region in WM cell lines. E) Abnormal L-chain to H-chain (L:H) ratio in WM cell lines was calculated based on clone count in BCR-sequencing results. F) WM cells (0.5×10^6 cells/ml) were cultured in triplicate wells of a 24-well plate and cultured for 2 days. Supernatants were harvested and used to quantify IgM levels by ELISA.

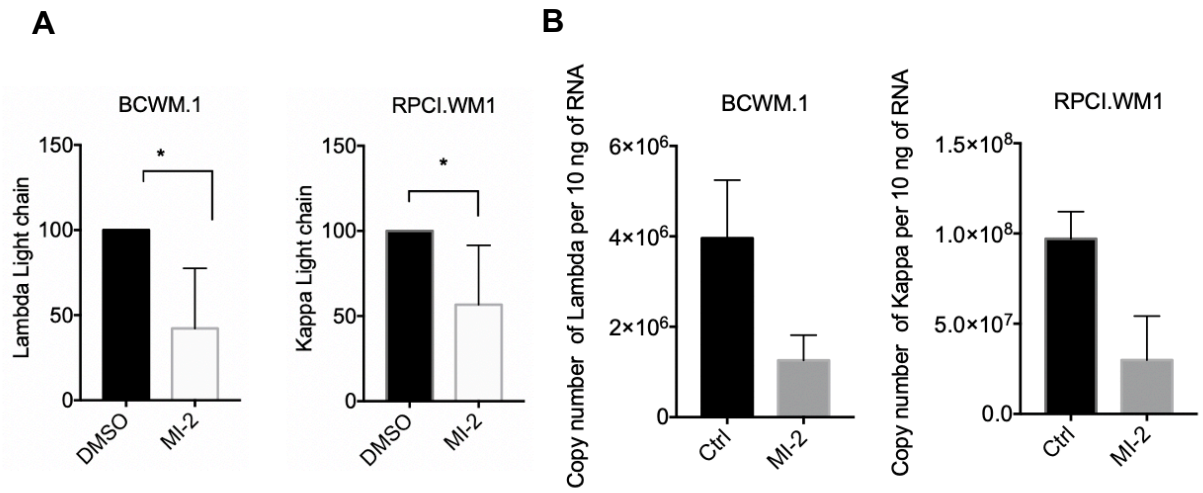


Figure15: Disruption of menin-MLL1 complex reduce light chain expression in WM. A) WM cells (2 x 10^6) were treated with 5 μM MI-2 inhibitor or DMSO control for 48 hr followed by qRT-PCR and B) copy number analysis to determine Ig kappa and Lambda light chain expression. All the experiments were performed at least 3 times.

V region mutation analysis

Using IMGT alignment, BCWM.1 cells has multiple mutations in AID hotspots motifs (AGCT and AGCA) in CDR1, CDR2, FR1 and FR2 regions. MWCL-1 cells had mutations within the CDR and FR regions, but CDR1 sequence was unmutated. RPCI-WM1 cells had only one silent mutation in FR1 and CDR2 (Figure 14D).

Additional attributes

While L:H chain ratio is 0.96-2.30 in normal human B cells[260], this ratio was 10 to 100 times more in WM cell lines (Figure 14E). This ratio correlated with IgM secretion

where BCWM.1 secreted the least IgM and RPCI-WM1 secreted the most IgM (Figure 14F).

MLL1 modulates immunoglobulin light chain in WM cells

Because we observed an abnormally high ratio of L:H chain in our BCR sequence analysis, and this correlated with secreted IgM by each cell line (Figure 14F), we investigated the effect of MLL1 inhibition on IGL expression. Interestingly, WM patients have abnormal immunoglobulin light chain expression, which correlates with a poor prognosis[265]. We treated WM cells with the MLL1 inhibitor MI-2 and examined the expression of kappa light chain (for MWCL-1 and RPCI-WM1 cells) and lambda light chain (for BCWM.1 cells). We found a significant reduction in light chain expression by qPCR (Figure 15A). To determine if MLL1 inhibition affected the ratio of L:H chain, we used qPCR copy number analysis to compare light chain copy number with heavy chain copy number. Our results indicate that MLL1 inhibition did not affect the ratio of L:H chain in WM (Figure 15B). Additionally, using copy number analysis, we were unable to replicate the L:H chain ratio of the BCR sequencing.

IV. Discussion

There is massive variation in germline DNA at *IGH* and *IGL* variable regions allowing the generation of B cell receptor (BCR) repertoires. We analyzed BCR attributes

of the currently available WM cell lines. In all three cell lines, a dominant functional *IGH* and *IGK/L* (Figure 1B) and infrequent other rearranged sequences (reads<30) were detected. We also identified the V_H, D_H and J_H gene segment usage for each cell line (Figure1C). While V_H gene segment usage for WM cells lines has been reported, no reports exist for D_HJ_H gene segments or V_LJ_L gene segment usage in these cells.

A comparison of H-chain rearrangement in patient-derived WM B cells to normal B cells revealed that the D_H and J_H gene segment usage had similar occurrence to normal B lymphocytes, but V_H1 and V_H3 were less abundant and highly prevalent in WM cases respectively[266]. Therefore, the BCWM.1 and MWCL-1 cells are consistent with a dominant V_H3 family in WM patients, while the RPCI-WM1 cells utilize the less abundant V_H1 segment. The most common V(D)J segments in WM were V_H3-23 family, D_H1 and D_H6 families (54%), and $J_H4/6$ families (59%) which represent overexpression of some gene segments in WM suggesting possible shared antigen reactivity. In addition, among WM patients, *IGHV3-30* was associated with a longer H-chain CDR3[267]. In addition, the long CDR3 sequence identified in WM cell lines is consistent with previous studies that reported a majority of WM patients have long H-chain CDR3 along with biased IGHV usage[267]. Based on CDR3 sequence alignment, a conserved region in CDR3 does not appear to exist in WM cell lines.

Despite the lack of CSR in primary WM cells, SHM was detected in >90% of WM cases[259]. Mutations of the V region during SHM (induced by activation-induced cytidine deaminase (AID) enzyme in specific AID hotspots) plays an important role in affinity

maturation[268]. Using IMGT alignment to the closest germline sequence, we identified SHM in all cell lines (Figure 2D). Previous studies have shown that despite the detection of hypermutated *IGH* in WM patients, the CDR and FR mutational patterns indicate the lack of antigen driven selection in a group of patients[57], suggesting that SHM in WM is not necessarily induced by antigenic selection.

We found aberrant transcription of H-chain and L-chain resulting in an abnormally high L:H chain ratio. This ratio correlated with IgM secretion level in WM cell lines (Figure 2F). This finding is in agreement with abnormal IgM L:H chain ratios in patient-derived WM B cells where this ratio correlated with total IgM levels in patient sera[269].

Finally, we identified that treatment of WM cells with MI-2 inhibitor significantly reduced light chain expression in WM cells (Figure 3) suggesting a role for MLL1 in regulation of light chain. Consistent with this data, we previously showed that MLL1 modulates Ig heavy chain expression in WM[195] which supports a role for MLL1 in immunoglobulin transcriptional regulation.

This work describes the BCR repertoire in WM cell lines and provides a foundation to study immunoglobulin regulation in WM.

CHAPTER 4

CONCLUSIONS AND SUMMARY

The term “epigenetics” describes the heritable modification of genome beyond DNA sequence variability that needs to be maintained during cellular processes of specific cell type. Disruption of epigenetic modifications including DNA methylation, histone modifications, and non-coding RNAs result in mis regulation of gene expression leading to severe cell pathological changes and cancer[270]. Differentiation of B cells is one of the important cellular processes that is tidily regulated by genetics and epigenetic factors. For instance epigenetic mechanisms play a critical role during B cell maturation in germinal center of spleen where many B cell lymphomas arise[271]. WM is considered a bone marrow infiltrating lymphoplasmacytic lymphoma that cells secret high level of an immunoglobulin M (IgM) monoclonal antibody[249]. Despite clinical progresses in treatment of lymphomas using monoclonal antibodies, proteasome inhibitors, and BTK-inhibitors, WM remains an incurable disease[194].

One of the main WM clinical manifestations is serum hyperviscosity syndrome due to accumulation of excessive IgM in serum. Based current guideline initiation of WM therapy is recommended only for patient with symptomatic hyperviscosity[272] . Therefore, it is critical to understand how immunoglobulin production is coordinated in WM. Next-generation sequencing identified exclusive mutations, deletion and gene rearrangements in WM which result in modified epigenetic signature in this disease. One of the important

epigenetic modifications is histone methylation which regulates the active and inactive status of gene region for transcriptional machinery.

MLL1 methyltransferases enzyme is the main regulator of *HOX* gene family that is mainly involve in cell fate. Moreover, MLL1 is required for self-renewal of hematopoietic stem cells (HSCs) and progenitors in the bone marrow[151]. MLL-family of members are associated to tumorigenesis including lymphomas[157,158]. For example, MLL2 is frequently mutated in WM patients[161]. Additionally, other epigenetic regulatory molecules such as DNMT1, miRNA-155 and miRNA-21, *SETD2* and *KDM6A* are modulated in WM [8362].

Despite the genetic similarly of B cell malignancy, they have unique epigenetic landscape. Therefore, it is critical to investigate epigenetic modifications to identify possible new target for development of new therapies in WM. Here, we hypothesized that MLL1 is regulating important gene expression in WM. To examination this hypothesis, we first evaluated expression of MLL1 and its partner Menin in WM cell lines and patient samples. We found that the expression of these genes is higher in WM cell lines and CD19+ CD138+ cells from WM patients compared to THP-1 cells suggesting a role for MLL1 and Menin in WM biology. We showed that MLL1 knock down using RNAi, resulted in significant reduction in Cμ expression. Additionally, inhibition of menin-MLL1 interaction using the MI-2 inhibitor significantly reduces IgM expression and secretion. Furthermore, the importance of H3K4me3, mark of transcriptional activation is described for *IGH* rearrangement in B cells[238,239].

We identified enrichment of H3K4me3 in multiple MLL1 binding sites in *IGH* gene regulatory regions in CD19+ cells from PBMCs of healthy individuals after treatment with LPS. We also found that MLL1 is involved in the regulation of immunoglobulin gene activation by directing H3K4me3 modification of $C\mu$ gene regulatory region. We found that MLL1 binds to Eμ enhancer and Iμ regions in WM cells and enriched H3K4me3 level at multiple MLL1 binding sites in $C\mu$ gene regulatory regions. Consistent with our data, treatment of WM cells with the MLL1 inhibitor significantly reduced H3K4me3 level in the *IGH* region. The role of MLL1 in regulating IgM was further confirmed by *in-vivo* experiment in which we show the efficiency of MI-2 inhibitor leading to significant reduction in IgM secretion in WM tumor-bearing mice. These results and previous genome-wild studies supporting a supervisory role for MLL1 regarding IgM transcriptional regulation [163,223,242]. In addition to MLL1 regulatory role in IgM transcription, our RNA-seq data suggested that MLL1 may play a role in regulation of immune response of b cells by targeting transcription of tumor suppressor genes and immune checkpoint inhibitors such as LAIR1.

Additionally, to better understand IgM regulation in WM, we examined BCR of three available WM cell lines using next generation sequencing. We recognized the V_HDJ_H gene segment usage and V_LJ_L genes for each cell line. V_H3 family was identified in BCWM.1 and MWCL-1 cell line which is the most popular family in WM BCR repertoires[266].

In addition, alignment analysis identified the long CDR3 sequence in WM cell lines which is consistent with CDR3 length reported for most WM patients[267]. Also, IMGT alignment analysis identified SHM in all cell lines. Interestingly, RNA-seq results showed aberrant transcription of H-chain and L-chain in WM cells which subsequently results in high L:H chain ratio in these cells. These abnormal high L:H chain ratio was previously shown in patient-derived WM B cells[269].

In addition, we found that WM cells treatment with MI-2 inhibitor significantly reduced light chain expression in WM cells that is in agreement with our previous data showing MLL1 regulates IgM transcription[195]. These data strongly suggest a role for MLL1 in regulation of IgM light and heavy chain. Finally, this study suggests MLL1 as a new therapeutic target for WM and other lymphoplasmacytic lymphomas.

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