The role of Menin in transcriptional regulation of MLL-AF4 and NPM1-mutated leukaemia

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Declaration

I declare that the work presented in this thesis is my own, unless otherwise stated in the Methods, such as when collaborators contributed to experiments or analysis, or when public and previously published data were accessed. This thesis has not been submitted, either partially or in full, for another qualification of this University, or for a qualification at any other institution.

Contributions by collaborators and public data:

Chapters 3 and 5: ChIPmentation data for patient-derived infant acute lymphoblastic leukaemia (iALL) cells was generated by Alastair Smith in the Milne lab and has been previously published (Crump et al., 2023). Publicly available ATAC-seq data for OCI-AML3 cells was accessed from NCBI GEO: GSM5114291 (Schaefer et al., 2022). Catherine Charhour contributed code for generating Figure 3.6.

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Abstract

Inhibition of the protein-protein interaction between Menin and Mixed Lineage Leukaemia 1 (MLL, also known as KMT2A) is emerging as a promising targeted treatment for specific high risk acute leukaemias. Menin is essential in leukaemias with rearrangements of the *MLL1* gene or mutations in *NPM1* — two subtypes with distinct genetic backgrounds. However, the function of Menin in these leukaemias, and why inhibiting its interaction with MLL is effective in these subtypes and not others, remains elusive.

Menin is a scaffold protein which can interact with wild-type MLL, MLL fusion proteins, and with mutant NPM1 (known as NPM1c) to facilitate binding at their gene targets. Rearrangements of *MLL1* give rise to oncogenic fusion proteins, most commonly MLL-AF4, which form a multi-protein chromatin complex with Menin, and activate transcription of haematopoietic stem cell genes. On the other hand, mutations in *NPM1* cause the encoded chaperone protein nucleophosmin to mislocalise from the nucleolus to the cytoplasm, or to directly bind and activate self-renewal genes in cooperation with Menin and wild-type MLL. Defining how Menin regulates transcription in these two leukemia subtypes, while participating in distinct chromatin complexes, is key to understanding how Menin inhibitors can best benefit leukaemia patients.

In this thesis, I tested the transcriptional response of MLL-AF4 and NPM1c leukemia models to Menin inhibition. Despite a general loss of Menin binding at active regulatory regions in both, and a redistribution of MLL and NPM1c chromatin binding, the transcriptional responses differed. MLL-AF4 cells exhibited rapid, broad transcriptional dysregulation upon Menin inhibition, while NPM1c cells did not have a strong immediate transcriptional response and instead required long-term

exposure to alter gene expression. Identifying leukaemia subtype-specific protein-protein interactions by Menin immunoprecipitation followed by mass spectrometry revealed a greater enrichment for components of the transcriptional machinery in MLL-AF4 cells as compared to NPM1c cells. Finally, I demonstrated that Menin specifically regulates enhancer activity and maintains enhancer-promoter contacts in MLL-AF4 cells but not NPM1c cells. Together, these findings point to distinct Menin activity as a more general transcriptional co-activator and regulator of enhancer-promoter proximity in partnership with MLL-AF4 in acute lymphoblastic leukaemia, unique to its regulatory roles in NPM1c acute myeloid leukaemia.

Keywords— Transcription regulation - Leukaemia - Menin inhibition - Enhancers - NPM1-mutated AML - MLL-rearranged ALL

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List of Abbreviations

***MLL*r** ..... *MLL*-rearranged

ALL ..... Acute lymphoblastic leukaemia

AML ..... Acute myeloid leukaemia

ATAC-seq .... Assay for transposase-accessible chromatin with sequencing

ChIP-seq ..... Chromatin Immunoprecipitation followed by sequencing

DSIF ..... DRB Sensitivity Inducing Factor

EB ..... Embryoid body

GMP ..... Granulocyte-monocyte progenitors

H3K27ac ..... Histone H3 lysine 27 acetylation

H3K4me1 .... Histone H3 lysine 4 mono-methylation

H3K4me3 .... Histone H3 lysine 4 tri-methylation

H3K79me .... Histone H3 lysine 79 methylation

HSC ..... Haematopoietic stem cell

IMiDs ..... Immunomodulatory drugs

IP ..... Immunoprecipitation

KEE ..... H3**K**79me2/3 **E**nhancer **E**lements

LSK ..... $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$

MEF ..... Mouse embryonic fibroblasts

MENi ..... Menin inhibitor (VTP-50469)

mESC ..... Mouse embryonic stem cell

MLL-FP ..... MLL fusion protein

MLL-N ..... The N-terminus of MLL1

MS ..... Mass Spectrometry

NELF ..... Negative Elongation Factor

NLS ..... Nuclear localisation signal

NPM1c ..... Mutant NPM1 protein

***NUP89*r** *NUP98*-rearranged

PDX ..... Patient derived xenograft

PROTAC .... Proteolysis Targeting Chimera

RNA Pol II .. RNA Polymerase II

SEC ..... Super elongation complex

T-ALL ..... T-cell acute lymphoblastic leukaemia

TFIID ..... Transcription Factor II D

TSS ..... Transcription start site

TT-seq ..... Transient Transcriptome RNA-sequencing

UBTF-TD ... *UBTF* tandem duplications; the protein product of UBTF with a tandem duplication

1 | Introduction

1.1 Chromatin and gene regulation in leukaemia

Chromatin is the dynamic assembly of DNA and proteins in eukaryotic cells. Activating or repressive chromatin states dictate whether DNA is compacted or accessible for transcription (Strahl & Allis, 2000). This process must be strictly regulated in order to allow for the precise expression of specific genes at the appropriate developmental state or physiological condition. In haematological malignancies, the regulation of transcription is perturbed as a result of mutations in chromatin modifying proteins or transcription factors, which causes inappropriate transcription of haematopoietic stem cell genes (Fang, Rao, Crispino, & Ntziachristos, 2020; Richardson & Huntly, 2021). It is therefore key to understand how chromatin-associated protein complexes are stabilised on their target regulatory elements on DNA, how they regulate transcription, and which regulatory processes can be targeted in cancer without disrupting normal cell function (Van Mierlo & Vermeulen, 2021).

The basic subunit of chromatin is the nucleosome, in which 147 bp of DNA are wrapped around eight histone proteins (Luger, Mäder, Richmond, Sargent, & Richmond, 1997), which can be covalently modified (Lorch & Kornberg, 2017). DNA and histone tail modifications are associated with either transcription activation or repression (Strahl & Allis, 2000). For transcription to initiate at gene promoters, DNA must be made accessible to RNA Polymerase II (RNA Pol II) (Fuda, Ardehali, & Lis, 2009). Actively expressed gene promoters are therefore found in regions of accessible chromatin, with unmethylated CpGs in the promoter region, and are marked by histone modifications associated with

active promoters (Cramer, 2019). These modifications include histone H3 lysine-27 acetylation (H3K27ac), deposited by acetyltransferases EP300/CBP, and histone H3 lysine 4 tri-methylation (H3K4me3), deposited by SET/MLL family proteins (Millán-Zambrano, Burton, Bannister, & Schneider, 2022).

Transcription factors bind promoters or enhancers in a sequence specific fashion. Enhancers are cis-regulatory elements that can be more than 1 Mb away from their target promoter (Proudhon et al., 2016; Lettice et al., 2003; Cramer, 2019). Enhancers regulate transcription by coming into proximity with target promoters via DNA looping (J. H. Yang & Hansen, 2024). Highly active, clustered enhancers bound by co-activators are classified as "super-enhancers" (Sabari et al., 2018; Whyte et al., 2013). A class of transcription factors referred to as pioneer factors can regulate DNA accessibility by recruitment of chromatin remodelling complexes and histone acetyltransferases (Iwafuchi-Doi & Zaret, 2016). Through transactivation domains, transcription factors can recruit additional complexes to initiate transcription (Y. W. Fong, Cattoglio, Yamaguchi, & Tjian, 2012). RNA Pol II assembles at promoters along with a complex of general transcription factors, which together form the pre-initiation complex (Cramer, 2019; Greber & Nogales, 2019; H. Wang, Schilbach, Ninov, Urlaub, & Cramer, 2022). This assembly facilitates the recognition of promoters by RNA Pol II. Pre-initiation complex assembly is further facilitated by the Mediator complex, which links RNA Pol II to initiation and transcription factors (Richter, Nayak, Iwasa, & Taatjes, 2022). Following transcription initiation, promoter-proximal pausing of RNA Pol II must be overcome to allow for transcription elongation. This is regulated by the P-TEFb complex, which phosphorylates RNA Pol II, along with pausing factors DSIF and NELF, triggering active elongation (Y. Li, Liu, Chen, & Chen, 2018).

Regions of active transcription and a high density of DNA-bound transcription activators can form transcriptional "hubs" (Cisse et al., 2013). This relies on multivalent interactions between disordered protein domains, which can in turn trigger phase separation, and is thought to serve to concentrate transcription factors (Cho et al., 2018; Chong et al., 2018). Although the significance of this condensate formation is uncertain, it is generally associated with high levels of transcription and highly active super-enhancers (Sabari et al., 2018; Whyte et al., 2013).

Chromatin modifiers and transcription factors are frequently altered in cancer, triggering inappropriate activation of oncogenes or repression of tumour suppressor genes (Dawson & Kouzarides, 2012). As a result, chromatin regulators have been attractive drug targets. Inhibitors of DNA methyltransferases, histone deacetylases, and the histone methyltransferase EZH2 are in clinical use, and more targets are under investigation (Cheng et al., 2019; Mabe, Perry, Malone, & Stegmaier, 2024). However, an understudied element in chromatin complexes are adaptor or scaffold proteins, which regulate complex assembly and tether complexes on chromatin in a context-specific manner (Demmerle, Hao, & Cai, 2023; Z. Ye, Soshnev, & Soto-Feliciano, 2023).

Menin, the product of the *MEN1* gene, is a scaffold protein with diverse functions in gene regulation (Matkar, Thiel, & Hua, 2013), including binding at active promoters as part of the H3K4me3 histone methyltransferase MLL1 (also known as KMT2A) and MLL2 (also known as KMT2B) complexes (Yokoyama et al., 2004; Hughes et al., 2004). In leukaemia, Menin can take on oncogenic roles by stabilising transcriptional activators, including MLL-fusion proteins, at key oncogenes. Recently, a number of drugs have been developed to inhibit the interaction between Menin and MLL, which is conserved in MLL-fusion proteins, as a potential treatment for a subset of leukaemias which

depend on this for gene expression (Grembecka et al., 2012; Krivtsov et al., 2019; Borkin et al., 2015). However, Menin inhibitors have proved effective in some additional leukaemias without MLL-fusion proteins, but not in others. It remains uncertain why inhibiting the Menin-MLL interaction is effective in some genetically distinct leukaemias and not others, in part because its role in transcription is unclear. Understanding the requirement for Menin is key to defining which leukaemia patients are most likely to benefit from Menin inhibition, as well as how leukaemia cells might evade this therapeutic approach. To address this gap, this thesis explores the function of Menin in transcription regulation in different models of leukaemia.

1.2 The biology of Menin

1.2.1 Discovery of *MEN1* as the causal gene for Multiple Endocrine Neoplasia I

In 1997, the *MEN1* gene, located on chromosome 11q13 (Larsson, Skogseid, Öberg, Nakamura, & Nordenskjöld, 1988), was identified as the causal gene in the familial cancer syndrome Multiple Endocrine Neoplasia Type I (Chandrasekharappa et al., 1997). Patients with this syndrome have a 94% chance of developing a tumour by age 50 (Trump et al., 1996), commonly affecting the endocrine tissues of the parathyroid, pancreas, duodenum or anterior pituitary. Patients inherit a loss-of-function variant in one copy of *MEN1* and the second copy is subsequently lost, displaying the classic inheritance and turmourigenesis pattern of a tumour suppressor gene.

The *MEN1* locus contains 10 exons encoding a 610-amino-acid protein. Mutations associated with endocrine neoplasia are not concentrated in any specific

domain but rather spread throughout the *MEN1* coding region; an analysis of clinical data in 2008 reviewed 1,336 reported mutations, most of which generate truncated Menin variants (Lemos & Thakker, 2008). A visualisation of mutations detected in several more recent cancer genomics studies (Cao et al., 2013; Jiao et al., 2011; Scarpa et al., 2017) is presented in Figure 1.1, including known driver mutations annotated in OncoKB and variants of unknown significance (Chakravarty et al., 2017; de Bruijn et al., 2023; Cerami et al., 2012).

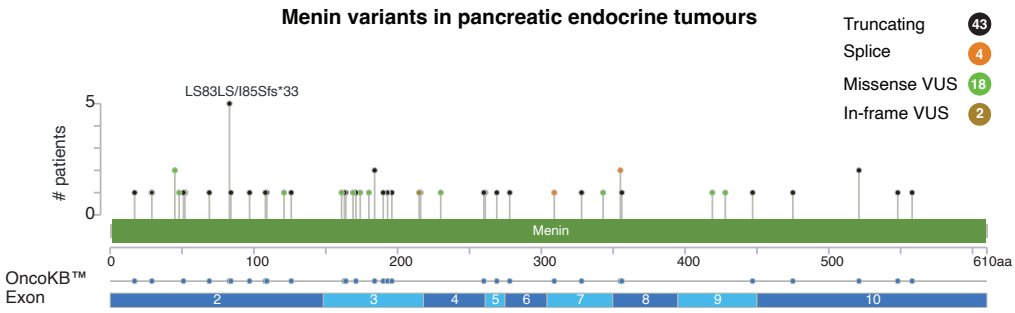


Figure 1.1: Lollipop mutation plot produced in cBioPortal showing variants in *MEN1* identified in recent genomic profiling studies of pancreatic neuroendocrine tumours. OncoKB annotation refers to known driver mutations. The data was accessed through cBioPortal.org

In mouse models, loss of Menin was found to cause embryonic lethality between E11.5-13.5 and is associated with defects in multiple organ systems (Bertolino et al., 2003). For example, Menin-null fetal mice exhibit increased apoptosis in the developing pancreas (Fontanière et al., 2008) and liver (Bertolino et al., 2003); in the fetal liver, both epithelial and haematopoietic tissue is affected. Crucially, the observation that mouse embryonic stem cells (mESCs) lacking Menin are capable of proliferation but fail to form embryoid bodies (EBs) indicates that Menin is critical for normal differentiation (Bertolino et al., 2003).

1.2.2 Menin's role in transcription regulation

The sequence of *MEN1* does not display homology to other known proteins or recognised motifs of specific domains (Guru et al., 1998). As a result, lacking these indications as to the function of the Menin protein, research groups have focused on elucidating Menin's protein-protein interactions. Menin was found to localise to the nucleus (Guru et al., 1998) and interact with DNA independently of sequence, which it achieves through positively charged residues within two nuclear localisation sequences (NLS) in its C-terminus (La et al., 2004). Menin was also shown to interact with several transcription factors, which implicated Menin in transcriptional regulation. For example, the interactions between Menin and JunD (Agarwal et al., 2003) and Nf-κb (Heppner et al., 2001) was found to repress gene targets, whereas Menin was required to activate Smad3 targets (Kaji, Canaff, Lebrun, Goltzman, & Hendy, 2001). Together, these observations suggested that Menin could function as both as a repressor and activator of gene expression, depending on its interaction partner.

The role of Menin in regulating chromatin emerged when Menin was found to associate with components of the histone methyltransferase MLL1 and MLL2 complexes and directly interact with RNA Polymerase II (Yokoyama et al., 2004; Hughes et al., 2004). Quantitative mass spectrometry analysis in HeLa cells later confirmed that Menin is a unique component of the MLL1 and MLL2 complexes, which catalyse trimethylation on lysine 4 of histone H3 (H3K4me3) via the SET domains of MLL1 or MLL2 (Nuland et al., 2013). The methyltransferase activity of the MLL1/2 SET domain is enhanced by interactions with conserved members of SET histone methyltransferase complexes — WRD5, RbPB5, ASHL2, and DPY30 — via the C-terminal

fragment of MLL1/2 (Dou et al., 2006).

H3K4me3 is associated with active promoters, but the majority of this histone modification is deposited by the SET1A/SET1B complexes (Wu et al., 2008; P. Wang et al., 2009). While MLL1/2 complexes are also enriched at many active promoters, loss of MLL1/2 affects H3K4me3 at only about 5% of promoters in mouse embryonic fibroblasts (MEFs) (P. Wang et al., 2009). Among these promoters, MLL1/2 complexes were found to be especially important for the maintenance of gene expression at the *HOX* cluster (Milne et al., 2002; P. Wang et al., 2009), which encodes a set of transcription factors that regulate development (Hubert & Wellik, 2023) and haematopoiesis (Argiropoulos & Humphries, 2007). Increased expression of the *HOX* cluster is a hallmark of leukaemias with translocations of *MLL1* (Armstrong et al., 2002; Drabkin et al., 2002), discussed in detail in Section 1.3. In complex with wild-type MLL, Menin was found to be essential for the expression of *HOX* cluster genes (Yokoyama et al., 2004; Wu et al., 2008), which are key to normal haematopoiesis (Novotny, Compton, Liu, Collins, & Chandrasekharappa, 2009; Maillard et al., 2009). In endocrine tissues on the other hand, Menin-MLL binding regulates expresion of cyclin dependent kinase (CDK) inhibitors p18Ink4c and p27Kip1 (Milne, Hughes, et al., 2005), which restricts the growth of pancreatic islet cells and thereby suppresses the endocrine tumour formation observed in *MEN1*-associated neoplasia (Karnik et al., 2005). In summary, by recruiting MLL to promoters, Menin can exert tumour suppressor or oncogenic effects depending on the cell type and the specific genes regulated in that context.

1.2.3 Menin and MLL regulate normal haematopoiesis

Mouse models of Mll and Menin loss in the embryo, and in haematopoietic stem cells (HSCs) specifically, revealed that together these proteins regulate HSC self-renewal and differentiation. Similar to Menin knock-out mice, Mll knock-out mice survive to E10.5-14.5, depending on the model, and exhibit defects in haematopoiesis and altered HOX gene expression (Yagi et al., 1998; B. D. Yu, Hess, Horning, Brown, & Korsmeyer, 1995; Hess, Yu, Li, Hanson, & Korsmeyer, 1997). While $Mll^{-/-}$ mESCs are capable of forming embryoid bodies, c-Kit+/CD41+ haematopoietic cells from these EBs fail to form colonies, indicating that Mll loss blocks haematopoietic differentiation at the stage of definitive haematopoiesis (Ernst, Mabon, Davidson, Zon, & Korsmeyer, 2004).

The severity of the haematopoietic defects induced by Mll loss can vary depending on the affected cells. For example, an inducible germline *Mll* knock-out induced bone marrow failure and reduced *Hox* gene expression in bone marrow cells in adult mice (Jude et al., 2007). However, a different mouse model, in which inducible *Mll* knock-out was constricted to haematopoietic cells using Vav Cre, instead showed normal adult bone marrow homeostasis but an inability of $Mll^{-/-}$ bone marrow cells to reconstitute haematopoiesis in irradiated mice (McMahon et al., 2007). Both models point to a deficiency of adult $Mll^{-/-}$ HSCs to self-renew. Similar to the less penetrant Vav Cre $Mll^{-/-}$ model reported by McMahon *et al.* (2007), in which Mll is specifically deleted in haematopoietic cells (McMahon et al., 2007), models of Menin loss do not show severely impaired steady state haematopoiesis (Maillard et al., 2009). Instead, $Men1^{-/-}$ cells transplanted into irradiated mice were outcompeted by wild-type cells, and did not give rise to progenitor cells, pointing to a

differentiation defect under these conditions. Decreased *Hoxa9* and *Meis1* expression in LSK cells ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}$) lacking Menin confirmed the requirement for the Menin protein at these Mll1 target promoters.

Menin loss leads to decreased pro-B, pre-B, and immature B cells in mouse models, indicating that B cell development is specifically reliant on Menin (Maillard et al., 2009). While Mll has been shown to be required for the development of both lymphoid and myeloid progenitor cells (Ernst, Fisher, et al., 2004; Jude et al., 2007), differentiated lymphoid and myeloid cells do not require Mll, suggesting that Mll is primarily important in multipotent progenitors (Jude et al., 2007). In HSCs, Mll regulates genes involved in controlling HSC proliferation beyond the *Hox* cluster, including *Pbx1*, *Prdm16*, and *Mecom*, by directly binding to their promoters, yet only a subset of its target genes depend on Menin (Artinger et al., 2013). The distinct requirements for Menin and Mll for gene expression in HSCs raised the question of whether the two proteins function strictly as a complex in haematopoiesis.

To address this, Li *et al.* (2013) compared B cell development in mice with conditional knock-outs of *Menin* and *Mll* using Rag Cre, which restricted Menin and Mll loss to the lymphoid lineage (B. E. Li, Gan, Meyerson, Rabbitts, & Ernst, 2013). Both Menin and Mll-deficient animals exhibited a reduction of B cells following the pre-B stage of differentiation, together with reduced *Meis1* and *Hoxa9* expression at the pro-B stage. However, gene expression analysis in the pro-B stage revealed distinct gene sets under Menin versus Mll control, with only nine genes found to be down-regulated in both Menin- and Mll-deficient pro-B cells. To distinguish whether the arrest of B cell differentiation was caused by the genes co-regulated by Menin and Mll, versus Menin- or Mll-specific transcriptional programs, Li *et al.* used a peptide to disrupt the interaction between Menin and Mll. While this effectively

down-regulated shared target genes, it was insufficient to recapitulate the differentiation defects observed in double knock-out B cells. This pointed to distinct requirements for Menin, Mll and the Menin-Mll interaction in normal B cell development. The observation that inhibiting the Menin-Mll interaction did not disrupt B cell development indicated that it could be selectively targeted in *MLL*-rearranged (*MLL*r) leukaemias.

1.3 Menin as a clinical target in *MLL*-rearranged leukaemia

Translocations of the N-terminal end of the *MLL* gene with over 70 known fusion partners give rise to oncogenic fusion proteins (MLL-FPs) that drive acute myeloid and B-cell leukaemias with a poor prognosis (Meyer et al., 2023). MLL translocations account for approximately 10% of acute leukaemia overall, but 80% of infant acute lymphoblastic leukaemia (ALL) (Marschalek, 2015). While the five-year survival rates of childhood ALL now reach 90%, infant ALL patients have had, until more recent clinical trials with immunotherapies, an event-free survival rate of less than 50% (Rice & Roy, 2020) — posing an unmet clinical need.

In 2023, van der Sluis *et al.* reported that adding Blinatumomab, a bispecific antibody which brings CD19+ cells into contact with CD3+ cytotoxic T cells, to the chemotherapy regiment for infant ALL improved two-year disease free survival rates to 81.6% compared to the 49.4% rate calculated based on historical data (van der Sluis et al., 2023). While these results are promising, the efficacy of Blinatumomab depends on sustained expression of CD19 on immature B-lineage leukaemic blasts. Both down-regulation of CD19

and lineage switching from ALL to AML, with the resulting loss of CD19 expression, have been reported in patients treated with Blinatumomab or Chimeric Antigen Receptor T cells (Ruella & Maus, 2016; Wölfl et al., 2018; Haddox et al., 2017; Balducci et al., 2017). Lineage switching is very rare, detectable in approximately 0.6% of pediatric ALL cases, however it is mainly associated with MLL-rearrangements and infant disease (Rossi et al., 2012) — suggesting that this may be a limiting factor to Blinatumomab efficacy in some cases. As a result, a range of treatment modalities with specificity to different MLL*r* leukaemia vulnerabilities are still needed.

1.3.1 Mechanisms of leukaemogensis of MLL fusion proteins

Fusions of the MLL1 N-terminus (MLL-N) to transcription elongation factors, including AF4, AF9, and ENL, give rise to oncoproteins that are sufficient to initiate and drive leukaemia (Meyer et al., 2023). As a result, MLL-rearranged (*MLL*r) leukaemias harbour few cooperating mutations (A. K. Andersson et al., 2015; Dobbins et al., 2013; Cancer Genome Atlas Research Network et al., 2013). The SET domain of MLL1, which has H3K4 methyltransferase activity, is lost in all translocations, whereas the Menin-binding domains are always preserved (Krivtsov & Armstrong, 2007; Ayton & Cleary, 2001). MLL fusion proteins (MLL-FP) assemble a large protein complex of transcription activators, including Menin, at gene promoters, which activates genes through both epigenetic and transcription elongation mechanisms (Milne et al., 2010; Slany, 2020; Takahashi & Yokoyama, 2020).

In *MLL*r leukaemogenesis, Menin is critical for linking MLL-FPs to the co-activator of transcription LEDGF (Ge, Si, & Roeder, 1998a) at the promoters

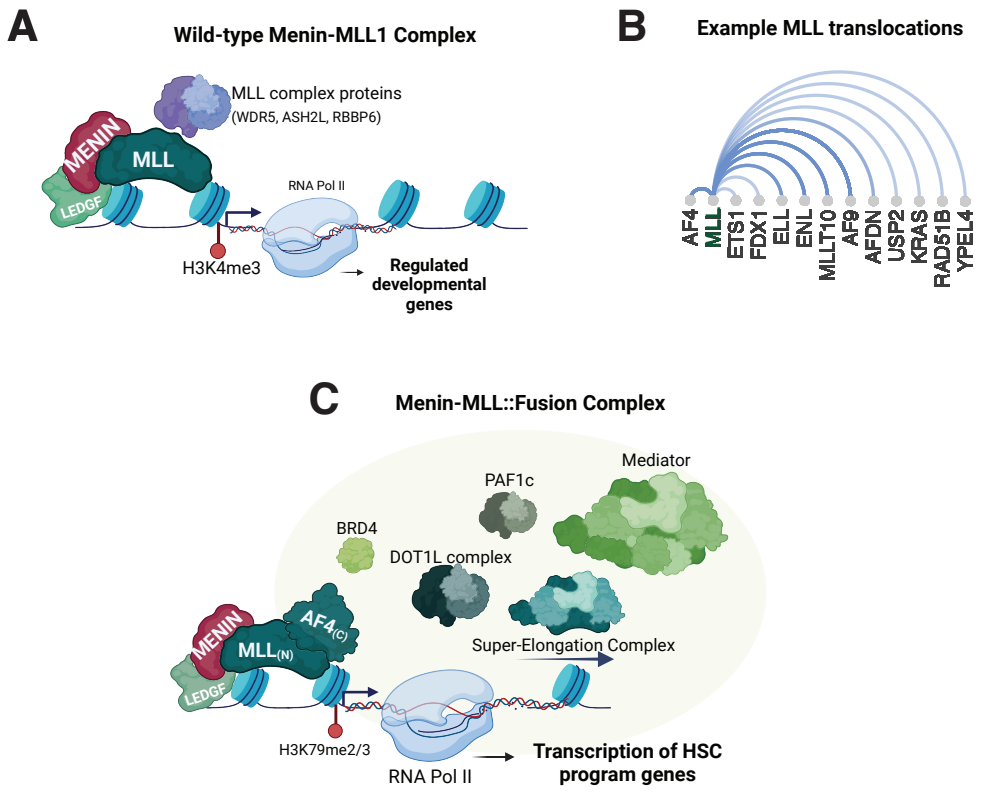


Figure 1.2: Representation of MLL-AF4 oncogenic activity (Freire et al., 2024; Yokoyama, 2017). **A.** The wild-type MLL1 complex deposits H3K4me3 at promoters. **B.** Translocations detected in genomics studies of acute leukaemias available in cBioPortal. **C.** MLL-AF4 assembles transcription co-activators and elongation factors to inappropriately sustain the expression of genes involved in haematopoiesis.

of target genes (Yokoyama & Cleary, 2008). Complexes associated with transcription elongation, including PAF1c (Muntean et al., 2010), DOT1L (Okada et al., 2005; Bernt et al., 2011) and the Super Elongation Complex (SEC) (Lin et al., 2010; Yokoyama, Lin, Naresh, Kitabayashi, & Cleary, 2010), associate with MLL-FPs and RNA Pol II to drive aberrant transcription at targets including *HOXA9* and *MEIS1* (Q. F. Wang et al., 2011). MLL-FPs induce a distinct transcriptional programme that differentiates *MLL*r leukaemias from

other acute leukaemias. This transcriptional profile is composed of genes associated with both haematopoietic progenitor cells, early B cell development, as well as myeloid specification (Armstrong et al., 2002).

1.3.2 Therapeutic targeting of *MLL*r-associated transcription

Since MLL-FPs do not themselves contain any obvious domains for inhibition, therapeutic targeting of *MLL*r leukaemia has focused on limiting the activity of other proteins in the MLL-FP complex in order to disrupt expression of oncogenes regulated by MLL-FPs. For example, Menin inhibition is achieved by disrupting the interaction between Menin and MLL fusion proteins (Grembecka et al., 2012; Krivtsov et al., 2019), discussed in detail in Section 1.6. It is important to consider Menin inhibition in the context of previous endeavours to disrupt the transcriptional activation of MLL-FP target genes. For example, BRD4, DOT1L, and ENL are chromatin proteins which interact with MLL-FP complexes and can be effectively inhibited with small molecules, leading to down-regulation of MLL-FP target genes.

1.3.2.1 BRD4

BRD4 is a member of the bromodomain and extra-terminal domain (BET) family of chromatin readers, containing two bromodomains that recognise and bind to acetylated lysines on histones — marks associated with active transcription. BRD4 promotes transcription globally by regulating the assembly of elongation factors, including SPT15, NELF components, and MED1 (a component of Mediator), thereby thereby promoting the transition of paused RNA Pol II to transcription elongation (Winter et al., 2017). In addition, BRD4 can interact with P-TEFb/SEC and with PAF1c (Dawson et al., 2011), which

are both involved in MLL-FP transcriptional activation (Milne et al., 2010; Muntean et al., 2010). This made the inhibition of the BRD4 bromodomain a promising therapeutic target in *MLL*r leukaemias, given their dependence on transcription elongation (Yokoyama et al., 2010; Lin et al., 2010). BET inhibition, which has distinct effects to BRD4 degradation (Winter et al., 2017), induces cell cycle arrest and apoptosis in *MLL*r cells following rapid down-regulation of MLL-FP target genes, and improves survival in MLL-AF4 and MLL-AF9 mouse models (Dawson et al., 2011; Zuber et al., 2011).

1.3.2.2 DOT1L

DOT1L is the only histone methyltransferase known to deposit mono-, di- and tri-methylation on lysine 79 on histone H3 (H3K79me) (Lacoste, Utley, Hunter, Poirier, & Côté, 2002), modifications associated with active transcription elongation (Steger et al., 2008). Unlike other histone methyltransferases, including MLL, DOT1L does not contain a SET domain and preferentially methylates nucleosomes (Feng et al., 2002). In *MLL*r leukaemia, DOT1L interacts with MLL fusion partners and is required for leukaemic transformation (Okada et al., 2005; M. J. Chang et al., 2010; Jo, Granowicz, Maillard, Thomas, & Hess, 2011). MLL-FP gene targets are also marked by abnormal H3K79me2, characterised by a wider distribution around the transcription start site (TSS) (Krivtsov et al., 2008; Bernt et al., 2011; Benito et al., 2015). H3K79me2/3 is thought to activate transcription by antagonising the activity of the histone deacetylase SIRT1 and preventing the deposition of the repressive mark H3K9me2 (C. W. Chen et al., 2015). Inhibition of DOT1L enzymatic activity reduces global H3K79me genome-wide but specifically down-regulates a subset of genes, including MLL-FP targets (Daigle et al., 2011). DOT1L inhibition also specifically inhibits the growth of *MLL*r cells

and triggers myeloid differentiation.

Although both BRD4 and DOT1L can interact with MLL-FPs and with P-TEFb, separation of these proteins by size exclusion chromatography suggests that they are in distinct complexes (Gilan et al., 2016). Together, BRD4 and DOT1L co-regulate a subset of genes, in addition to unique targets that are specifically altered with either BRD4 or DOT1L inhibition. Shared BRD4 and DOT1L targets are proximal to super-enhancers at which H3K79me2 serves to promote histone acetylation, and thereby an enrichment of BRD4, which together potentiates the expression of MLL-FP target genes.

Despite the encouraging evidence from pre-clinical models for the efficacy of BRD4 and DOT1L inhibition in *MLL*r leukemia, significant adverse effects and limited responses were observed in clinical trials (Shorstova, Foulkes, & Witcher, 2021). For example, in a clinical trial cohort of adult acute myeloid leukaemia (AML) patients, DOT1L inhibitors were able to reduce H3K79me levels yet only two out of 51 patients entered remission (Stein et al., 2018). Similarly, no objective response was noted in a clinical trial of a paediatric cohort (Shukla et al., 2016). Therefore, alternative ways of disrupting MLL-FP-driven transcription need to be explored in mechanistic detail.

1.3.2.3 ENL

Another therapeutic target in *MLL*r leukaemia, now under investigation in pre-clinical models, is the elongation factor ENL (also known as MLLT1), which, as well as being a member of the SEC, is the third most common MLL fusion partner in translocation events (Meyer et al., 2018). ENL interacts with MLL-FPs and recruits DOT1L and SEC components to MLL-FP target genes

(Yokoyama et al., 2010; H. Hu et al., 2022; Zeisig et al., 2005; Shen, Jo, Liao, Hess, & Nikolovska-Coleska, 2013). In addition to two bromodomains, ENL also contains a YEATS domain, which also recognises H3K27ac and histone H3 lysine 9 acetylation (H3K9ac) (Wan et al., 2017). The YEATS domain is essential for ENL's activity in sustaining transcription elongation in MLL-AF4 cells (Erb et al., 2017). The depletion of ENL from chromatin through inhibition with the molecule TDI-11055 inhibits transcription elongation at target genes, including key factors in the MLL-FP transcription repertoire such as *Myb* and the *HOX* cluster (Liu et al., 2022). At a subset of targets, DOT1L binding is also depleted following ENL inhibition. While ENL inhibition impaired leukaemia growth in clonogenic assays and in mouse models, normal haematopoiesis in mice was mildly affected, including a decrease in developing B cells (Liu et al., 2022). This suggests that ENL inhibition could be an effective and selective strategy to limit MLL-FP transcription, but further investigation is needed to identify its utility in the clinic and whether compensatory mechanisms can arise.

1.4 Menin as a clinical target in NPM1c acute myeloid leukaemia

The requirement for the Menin-MLL interaction to regulate *HOXA9* and *MEIS1* in *MLL*r leukaemia suggested that other leukaemias with inappropriate *HOX* gene activation and *MEIS1* over-expression may also be dependent on Menin. One such leukaemia subtype is acute myeloid leukaemia (AML) with *NPM1* mutations. NPM1 is a nucleolar chaperone protein, which is dynamically transported between the nucleus and cytoplasm (Yun et al., 2003). It is associated with various functions, including ribosome biogenesis,

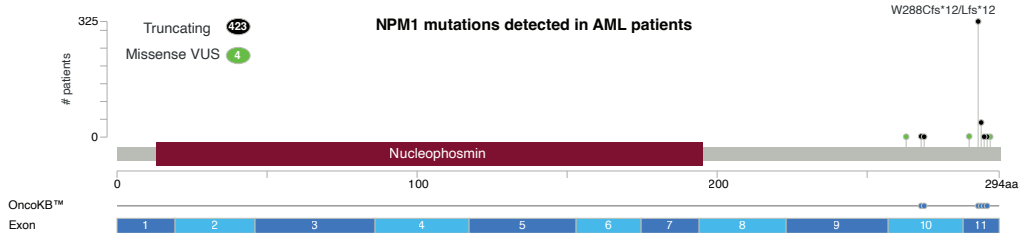


Figure 1.3: Mutations in *NPM1* detected in genomics studies of leukaemia, visualised using cBioPortal. OncoKB annotation refers to known oncogenic variants.

genome integrity, and cell growth (Grisendi, Mecucci, Falini, & Pandolfi, 2006; Falini et al., 2005). A visualisation of *NPM1* mutations detected in recent genomics studies of leukaemia is presented in Figure 1.3 (Hoadley et al., 2018; Tyner et al., 2018; Bottomly et al., 2022; Zhang et al., 2016; A. K. Andersson et al., 2015).

NPM1 is mutated in approximately one third of AML, which invariably causes the NPM1 protein to be mislocalised in the cytoplasm, therefore referred to as NPM1c (Falini et al., 2005). *NPM1* mutations are heterozygous, and often co-occur with other driver mutations, including *FLT3-ITD*, *DNMT3A*, *TET2* or *IDH1/2* mutations (Ranieri et al., 2022). This makes *NPM1* mutations the most common genetic aberration in AML, meaning that improved targeted therapy has the potential to benefit a large patient population, 50% of whom still do not achieve remission with standard treatment (Ranieri et al., 2022).

1.4.1 Mechanisms of gene regulation in NPM1c leukaemia

NPM1c leukaemias display increased expression of *HOX* genes and their co-factors *MEIS1* and *PBX3* (Alcalay et al., 2005). However, the mechanisms of activating and maintaining this transcriptional program are still being elucidated. NPM1c has been associated with the cytoplasmic mislocalisation

of additional proteins, including CTCF (A. J. Wang, Han, Jia, Chen, & Minden, 2020), the negative regulator of P-TEFb HEXIM1 (Gurumurthy et al., 2008), and PU.1 (X. Gu et al., 2018), but the significance of this has not been established in primary AML samples (Pianigiani, Betti, Bigerna, Rossi, & Brunetti, 2020). On the other hand, a CRISPR screen focused on protein-coding domains in the NPM1c cell line OCI-AML3 revealed that the Menin binding domains on MLL1 were essential for its survival (Kühn et al., 2016). These findings were confirmed by treating the cells with an inhibitor of the Menin-MLL interaction, which was also found to effectively down-regulate the *HOX* cluster and *Meis1* expression, which subsequently induced myeloid differentiation (Kühn et al., 2016).

More recently, a portion of the mutant NPM1c protein was shown to remain in the nucleus and directly associate with chromatin, including at *HOXA* and *HOXB* promoters. This process is dependent on its interaction with the nuclear export factor XPO1 (also known as CRM1) (Oka et al., 2019; Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). Targeted degradation of NPM1c was shown to rapidly decrease expression of its target genes, suggesting that NPM1c binding can directly promote transcription (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). NPM1c target genes were also found to be enriched for wild-type Menin-MLL1 complex binding, and a subset are sensitive to Menin inhibition, indicating that Menin binding may support NPM1c-dependent transcription (Uckelmann et al., 2023).

1.4.2 Disruption of NPM1c activity through XPO1 and Menin inhibition

The nuclear export protein XPO1 is involved both in transporting NPM1c to the cytoplasm (Bolli et al., 2007; Falini et al., 2006) and in tethering NPM1c to chromatin (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). Disruption of the NPM1c nuclear export signal, which is generated by a mutation in exon 12 in OCI-AML3 cells, causes NPM1c retention to the nucleus and down-regulation of *MEIS1* and *HOXA9* (Brunetti et al., 2018). This nuclear export signal is also important for generating a high-affinity interaction between NPM1c and XPO1 (Uckelmann et al., 2023), which is required for NPM1c enrichment at target genes (X. Q. D. Wang et al., 2023).

Given the altered NPM1c shuttling and cytplasmic localisation, the inhibition of nuclear export via XPO1 has been tested as a therapeutic strategy against NPM1-mutated leukaemia (Ranieri et al., 2022). However, strong inhibition of XPO1 is toxic and not specific to NPM1c, as XPO1 mediates nuclear export of multiple proteins, including Rb, p53, Topoisomerase IIα, and other tumour suppressors (Kudo et al., 1998; Gravina et al., 2014). While newer inhibitor design has aimed to limit toxicity through reversible XPO1 binding, early clinical trials with the inhibitor Selinexor revealed that it was poorly tolerated and yielded limited clinical benefit (Sweet et al., 2021; Fiedler et al., 2020; Bhatnagar et al., 2020). As a result, Menin inhibitors present a promising alternative method for inhibiting NPM1c-driven transcription. For example, Menin inhibition with VTP-50496 selectively disrupts wild-type MLL, NPM1c and XPO1 from a set of promoters, including *MEIS1* but not *HOXA9* (Uckelmann et al., 2023), which is consistent with the effects on MLL-AF9-driven AML (Krivtsov et al., 2019).

The mechanisms by which NPM1c maintains leukaemia and the significance of the balance of cytoplasmic versus nuclear mutant NPM1 remain unclear. For example, experiments which have shown that cytoplasmic NPM1c is required for AML survival and *HOX* expression maintenance have employed deletion of the NPM1c export signal, XPO1 inhibition, or NPM1c degradation (Brunetti et al., 2018), all of which could concurrently disrupt NPM1c chromatin binding and its interaction with XPO1. This makes it challenging to disentangle the functional relevance of cytoplasmic versus chromatin-associated NPM1c. Furthermore, it remains unclear to what extent the efficacy of Menin inhibition is linked to transcription regulation beyond NPM1c targets — whether Menin functions primarily to stabilise NPM1c or to also regulate transcription at additional loci.

1.5 Additional Menin inhibitor-sensitive leukaemias

Menin inhibitor efficacy is still being investigated in additional leukaemia subtypes with HOX cluster activation (Cuglievan et al., 2024). Two subtypes for which sensitivity to Menin inhibition has been demonstrated in pre-clinical models include AML with *NUP98* rearrangements (*NUP98*r) and AML with *UBTF* tandem duplications (UBTF-TD). Patients with *NUP98*r and UBTF-TD AML are now also eligible for clinical trials testing Menin inhibition in combination with other agents (*eg.* NCT05326516, NCT05360160, NCT04811560, NCT06177067, APAL2020K ITCC-101/COG/PEDAL) (Cuglievan et al., 2024).

1.5.0.1 Evidence for Menin dependency in *NUP98*-rearranged leukaemia

Patients with *NUP98* translocations account for 4-7% of paediatric AML (Struski et al., 2016; Bertrums et al., 2023). *NUP98* encodes Nucleoporin 98, a subunit of the nuclear pore complex, which facilitates transport between the nucleus and cytoplasm (Chatel, Desai, Mattheyses, Powers, & Fahrenkrog, 2012). Translocations of the N-terminal of NUP98 with over 30 known fusion partners give rise to oncoproteins, among which NUP98-KDM5A and NUP98-NSD1 are associated with aggressive disease in paediatric patients (Bertrums et al., 2023). NUP98-fusion proteins interact with the wild-type Menin-MLL1 complex to transcriptionally activate leukaemogenic gene expression, including at the *HOXA* and *HOXB* clusters (Xu et al., 2016).

Heikamp *et al.* have demonstrated that mouse cell line models of NUP98-HOXA9 and NUP98-JARID1A have a similar sensitivity to Menin inhibition to MLLr and NPM1c cells (Heikamp et al., 2022). Menin inhibition disrupted NUP98-fusion binding at a subset of Menin-bound sites and led to down-regulation of *MEIS1* and the *HOXA* cluster, plus up-regulation of differentiation marker CD11b. Menin inhibition also prolonged the survival of PDX models with NUP98-JARID1A leukemia (Heikamp et al., 2022).

1.5.0.2 Evidence for Menin dependency in AML with UBTF tandem duplications

Recurrent tandem duplications in exon 13 of the transcription factor UBTF (UBTF-TD) can be detected in approximately 3% of adult AML (Duployez et al., 2023) and 4-9% of paediatric AML (Umeda et al., 2022). UBTF is a member of the pre-initiation complex and can facilitate transcription of

ribosomal genes, histone cluster loci, and other highly transcribed genes (Sanij et al., 2015). UBTF-TDs are mutually exclusive with *NUP98* rearrangements, *MLL* rearrangements, NPM1c mutations, but they do co-occur with other genetic lesions found in *NUP98*r and NMP1c subtypes such as *FLT3-ITD* and *WT1* mutations, and with high *HOX* cluster expression. (Umeda et al., 2022).

The distinct transcriptional signature of AML with these mutations is driven by direct UBTF-TD binding to additional promoters compared to wild-type UBTF, the majority of which are also Menin-MLL targets (Barajas et al., 2024). For example, Barajas *et al.* showed that UBTF-TD binding was required for the maintenance of high *HOXA/B* cluster expression in cord blood CD34+ cells transformed with UBTF-TD (Barajas et al., 2024). Menin inhibition with SNDX-5613 reproduced effects seen in other inhibitor-sensitive cells, including down-regulation of canonical inhibitor-sensitive genes such as *MEIS1*, reduced growth of primary patient and PDX cells in vitro, and myeloid differentiation and increased survival in PDX mice (Barajas et al., 2024).

1.6 Molecular basis of Menin inhibition

Immunoprecipitation experiments by Yokoyama *et al.* (2005) and Caslini *et al.* (2007) revealed that Menin interacts with N-terminal sequences of MLL, which was termed a "high affinity Menin binding motif (hMBM)" (Yokoyama et al., 2005) and shown to be critical for leukaemic transformation and *Hoxa9* expression in the presence of MLL-FPs (Caslini et al., 2007). Caslini *et al.* (2007) demonstrated that expression of a peptide containing the region of Menin-MLL interaction could competitively bind Menin and therefore decrease

the endogenous interaction (Caslini et al., 2007). This effectively limited the growth of MLL-AF9 cells and reduced expression of *Meis1*, providing evidence that specifically inhibiting the Menin-MLL interaction could be a therapeutic intervention for *MLL*r leukaemia.

Further details of the Menin-MLL interaction provided by NMR spectroscopy (Grembecka, Belcher, Hartley, & Cierpicki, 2010) and X-ray crystallography (Huang et al., 2012) characterised the high affinity interaction site and led to the development of the first small molecule inhibitor developed by Grembecka *et al.* (2012) (Grembecka et al., 2012). The first inhibitor was identified through a high throughput screen and subsequently optimised to inhibit the Menin-MLL interaction at low nanomolar concentrations (Grembecka et al., 2012; A. Shi et al., 2012). The resulting compounds were capable of inducing the dissociation of Menin and MLL-AF9 from the *Meis1* and *Hoxa9* promoters, thereby triggering their down-regulation (Grembecka et al., 2012). These inhibitors also selectively impaired growth and triggered myeloid differentiation of cell lines with MLL fusions. This specificity indicated on-target activity, as cell lines lacking the Menin-MLL-FP dependency were unaffected (Grembecka et al., 2012).

Since the synthesis of the first Menin inhibitors, other compounds have been developed in the process of optimising potency and pharmacokinetics (reviewed in (Lei et al., 2021)). VTP-50469 (Figure 1.4) is a potent inhibitor which is both selective and bio-available, and is a close analogue of Revumenib, now in clinical trials (Krivtsov et al., 2019; Issa et al., 2023). This inhibitor is capable of eradicating leukaemia in *MLL*r and NPM1c patient-derived xenograft (PDX) models without affecting haematopoiesis (Krivtsov et al., 2019; Uckelmann et al., 2020). In *MLL*r cell lines, Krivtsov *et al.* showed that VTP-50469 depletes Menin from chromatin genome-wide, whereas MLL-

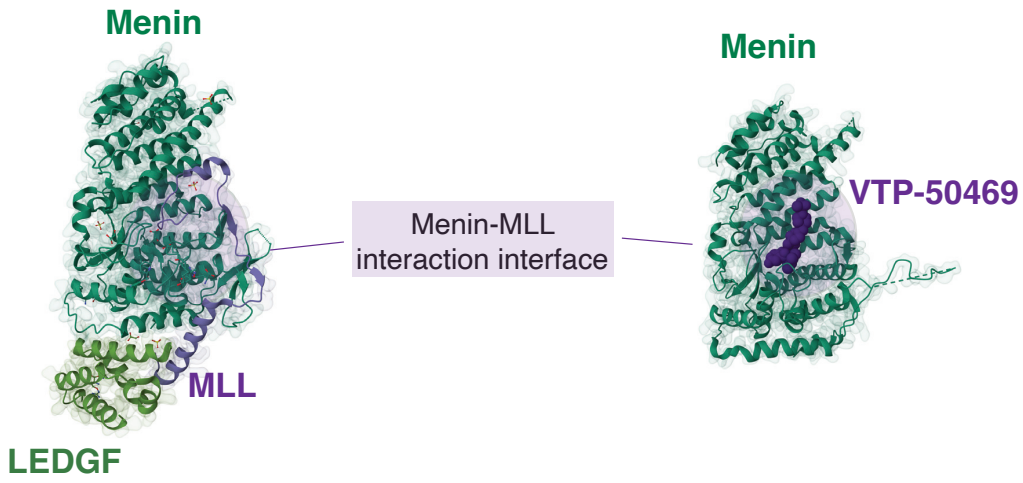


Figure 1.4: Structure of Menin interactions with MLL and LEDGF peptides (left) and Menin bound by inhibitor VTP-50469 (right). Images represent the RCSB PDB (RCSB.org) of PDB-ID 3U88 "Crystal structure of human menin in complex with MLL1 and LEDGF" (Huang et al., 2012) and of PDB-ID 6PKC "Inhibition of Human Menin by VTP-50469" (Krivtsov et al., 2019).

AF9 is specifically reduced at a subset of promoters and retained at others. Notably, *Hoxa9* and *Myb* retain MLL-AF9 binding, whereas *Mef2c* and *Meis1* are depleted of MLL-AF9 promoter binding and decrease in expression. The promoters that showed a decrease in MLL-AF9 binding upon treatment with VTP-50469 overlapped significantly with sites that lose MLL binding in the context of a Menin CRISPR-induced knock-out. Similarly, in NPM1c cells, VTP-50469 selectively disrupts NPM1c binding at a subset of targets, including *MEIS1*, and triggers down-regulation, while *HOX* cluster genes remain unaffected (Uckelmann et al., 2023, 2020; Kühn et al., 2016).

VTP-50469 was chosen for the experiments in this thesis because it has been extensively characterised in *MLL*r and NPM1c models and shown to closely recapitulate the phenotype of Menin knock-out cells (Uckelmann et al., 2023, 2020; Kühn et al., 2016). Moreover, it is a close analogue of Revumenib, from which patient response data was recently reported (Issa et al., 2023).

1.7 Menin inhibitors in the clinic

Several Menin inhibitors are in ongoing Phase I/II clinical trials. Patient enrolment is primarily focused on acute leukaemia, although some trials are also evaluating the safety and activity of Menin inhibitors in solid tumours (NCT05731947, NCT05631574). The results of the clinical trial with the Menin inhibitor Revumenib in patients with *MLL*r or NPM1-mutated leukaemia reported an overall response rate of 53%, of which 30% of patients achieved "complete remission or complete remission with partial haematologic recovery" (CR/CRh) (Issa et al., 2023). Of patients who achieved complete remission, 78% were also free of minimal residual disease. Paediatric patients had a similar response rate of morphologic remission to adults — 50% (4/8) and 54% (28/52), respectively. RNA-seq of patient bone marrow cells revealed the expected down-regulation of MLL-FP and NPM1c targets, including *MEIS1*, *HOXA9*, *PBX3*, *CDK6* and *FLT3*, together with up-regulation of myeloid markers such as CD11b and CD14, which indicated leukaemia differentiation. This was consistent with observations in pre-clinical models of *MLL*r and NPM1c (Uckelmann et al., 2020; Krivtsov et al., 2019).

These results were particularly encouraging given that the patient population involved in this trial had undergone a median of four previous therapies. A previous analysis by Issa *et al.* (2021) reported that *MLL*r patients who had relapsed achieve complete remission or remission with incomplete count recovery at a rate of 9% at or after their third line of therapy (Issa, Zarka, et al., 2021). However, the emergence of resistance to Menin inhibition in a subset of patients indicates that effective treatment is likely to require additional pathways to be targeted in combination with Menin inhibition in order to achieve a sustainable response.

1.7.1 Mechanisms of resistance to Menin inhibition

Using Next Generation Sequencing, Perner *et al.* evaluated material from a group of patients in the Revumenib trial (AUGMENT-101) who had relapsed despite an initial response to Menin inhibition (Perner, Stein, et al., 2023). A cluster of mutations in *MEN1* was characterised in 38.7% of patients, which had emerged post-treatment and were associated with clonal expansion. The mutational landscape of these patients was otherwise unchanged, suggesting that no new driver mutations had emerged. PDX models created from five AML patients, which were generated to assess long-term survival post Menin inhibition, also had a 48.8% (22/45 mice) rate of relapse and mutations in *MEN1* were detected in 68% of cases. These models included both *MLL*r and NPM1c patient samples. The mutations associated with resistance to Menin inhibition include M327I, M327V, G331R, G331D, T349M, and S160C. These residues, represented visually in Figure 1.5 (Perner, Stein, et al., 2023; Tang et al., 2023), are distinct from those affected in cases of Multiple Endocrine Neoplasia (Figure 1.1), which feature predominantly truncating variants (Lemos & Thakker, 2008).

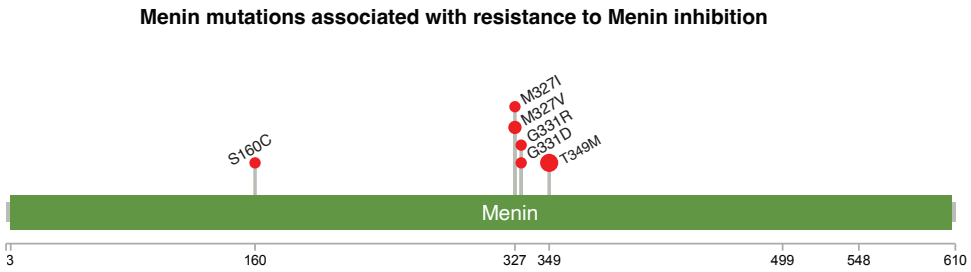


Figure 1.5: Mutations in *MEN1* detected by Perner *et al.* (Perner, Stein, et al., 2023) in patients and PDX mouse models with resistance to Menin inhibition.

X-ray crystallography revealed that the association between Revumenib and Menin is disrupted by these mutations as they cluster close to residue W346, which forms a strong hydrogen bond with Revumenib (Perner, Stein, et al., 2023). The mutations associated with resistance generate steric hindrance, which reduces the affinity between Menin and Revumenib. Critically, the mutations do not interfere with the binding of MLL to Menin. To assess the effects of these mutations *in vitro*, Perner *et al.* generated AML cell lines with M327I. These cell lines were also resistant to Menin inhibition. In the *MLLr* MV4;11 cells, treatment with VTP-50469 was ineffective in displacing $\text{Menin}^{\text{M327I}}$ from chromatin or inducing down-regulation of Menin targets.

The M327I mutation was also associated with reduced activity of two other Menin inhibitors, MI-3454 and DS-25 (Perner, Stein, et al., 2023). However, a recent report on the pre-clinical efficacy of the Menin inhibitor Bleximenib showed this inhibitor retains efficacy in the presence of these mutations through a distinct binding mechanism (Kwon et al., 2024). This suggests that further optimisation of Menin inhibitors has the potential to overcome genetic resistance.

Among PDX lines generated from MLL-AF9 AML in the study by Perner *et al.*, seven animals acquired resistance to Menin inhibition without *MEN1* mutations or any other discernible genetic cause (Perner, Stein, et al., 2023). RNA-seq in both a patient sample and a PDX model with acquired resistance in the absence of *MEN1* mutations nonetheless revealed decreased expression of Menin-MLL targets and up-regulation of myeloid markers, pointing to leukaemia survival despite a transcriptional response to Menin inhibition. These alternative epigenetic mechanisms of Menin inhibitor resistance, which allow leukaemia cells to no longer rely on cannonical Menin-MLL-FP target genes, remain to be elucidated.

1.8 Thesis Aim and Objectives

It remains unclear why inhibiting the Menin-MLL interaction is effective in some genetically distinct leukaemias, such as *MLL*r and NPM1c acute leukaemias, but not in others. To better predict which patients would benefit from Menin inhibition, we need a more comprehensive understanding of how Menin regulates transcription in both wild-type MLL and MLL-FP contexts. To address this, my thesis investigates the consequences of Menin inhibition on transcription in three leukaemia models, which represent clinically relevant contexts:

1. SEM cells, derived from a precursor B cell acute lymphoblastic leukaemia (ALL) harbouring an MLL-AF4 fusion protein (Greil et al., 1994), which are sensitive to Menin inhibition;
2. RCH-ACV cells, a precursor B cell ALL with wild-type MLL, driven instead by a E2A-PBX1 translocation (Jack et al., 1986), which are resistant to Menin inhibition and serve a control; and
3. OCI-AML3 cells, an AML cell line harbouring an NPM1 mutation and wild-type MLL1 (Quentmeier et al., 2005), which are sensitive to Menin inhibition.

My thesis focuses on exploring whether Menin has common or distinct functions in transcriptional regulation in MLL-AF4 ALL and NPM1c AML by addressing the following questions:

Chapter 3 — How does Menin inhibition affect transcription in leukaemias with distinct transcriptional drivers?

Chapter 4 — Does Menin interact with different protein complexes

in distinct leukaemia cell lines, and does this correlate with its role in transcription regulation?

Chapter 5 — Does Menin bind and regulate cis-regulatory elements outside of promoters?

2 | Materials and Methods

2.1 Cell Culture

SEM cells were grown in IMDM (Gibco 12440061) supplemented with 10 % FBS and 1 X GlutaMax (Gibco 35050061). RCH-ACV and OCI-AML3 cells were grown in RPMI (Gibco 11875093) supplemented with 10 % FBS and 1X GlutaMax.

2.2 CellTiterGlo

Cells were treated with a dilution series of VTP-50469 in triplicate wells in an opaque 96 well plate. After 48 h, CellTiterGlo 2.0 reagent (Promega G9241) was added to each well in equal volume to the media. The plate was shaken for 2 min and incubated for 5 min at room temperature, after which luminescence was recorded on a BMG FLUOstar OPTIMA plate reader. The luminescence signal was normalised to the DMSO-treated cells and averaged across replicates. Five replicates were performed for SEM and RCH-ACV cells, and three for OCI-AML3 cells.

The dose response curves were generated using the R package drc (Ritz, Baty, Streibig, & Gerhard, 2015).

2.3 Colony Assay

In duplicate liquid cultures, cells were treated with either 50 nM or 250 nM VTP-50469 or DMSO for 90 min, then added to MethoCult (StemCell Technologies H4100) containing 50 nM or 250 nM VTP-50469 or DMSO, and

aliquoted into three replicate 3 cm dishes. After 10-14 days, the number of colonies was counted under a light microscope and the counts across replicates were averaged.

2.4 Chromatin Immunoprecipitation followed by Sequencing (ChIP-seq)

For Menin, NPM1c or MLL-N ChIP, cells were fixed for 30 min with 2 mM Di(N-succinimidyl) glutarate (DSG) followed by 30 min with 1% formaldehyde. For H3K27ac, H3K4me3 and H3K4me1 ChIP, cells were fixed for 10 minutes with 1% formaldehyde. Fixed cells were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 with protease inhibitor cocktail) and the lysate was syringe-passaged through a 27 gauge needle. Cell lysates were sonicated using a Covaris ME220 (peak power 75, duty factor 25%, 500 cycles per burst) for 10 min for Menin, NPM1c or MLL-N ChIP or for 5 min for histone mark ChIP. The sonicated lysates were pre-cleared with a 1:1 mix of Protein A:Protein G beads for 15 minutes at 4°C, then incubated with antibody overnight. Antibodies are provided in Table 2.1. Five percent of the lysate was set aside as input.

The following day, a 1:1 mix of Protein A:Protein G beads was added to each IP and samples were rotated at 4°C for 3 hours. The beads were then washed three times with RIPA buffer (50 mM HEPES-KOH, pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-deoxycholate) and once with TE-50 mM NaCl. The chromatin was eluted from the beads with SDS lysis buffer for 30 min at 65°C, then treated with RNase A for 30 min at 37°C, followed by Proteinase K treatment combined with cross-link reversal overnight at

65 °C. DNA was purified using the ChIP DNA Clean & Concentrator kit (Zymo 5201). Libraries for sequencing were prepared using the NEBNext Ultra II library prep kit (E7645) and sequenced by 40 or 150 bp paired-end sequencing.

Table 2.1: Antibodies for ChIP.

Antibody	**Reference**	**Application**
Menin	Bethyl A300-105A	2 µg per 1×10^7 cells
MLL-N	Bethyl A300-086A	2 µg per 1×10^7 cells
H3K27ac	Diagenode C15410196	1.25 µg per 1×10^7 cells
H3K4me1	Diagenode C15410194	3 µg per 1×10^7 cells
H3K4me3	Diagenode C15410003	2.6 µg per 1×10^7 cells
NPM1c	TheroFisher PA1-46356	2 µg per 1×10^7 cells

2.5 ChIP-qPCR

Following ChIP as described above, DNA was purified using the QIAQuick PCR Purification kit (Qiagen 28104) and qPCR was performed using Fast SYBR Green Master Mix (ThermoFisher 4385612). Primer sequences are provided in Table 2.2. The enrichment of factors analysed by ChIP-qPCR was calculated as percentage of input.

$$\Delta CT = Ct(input) - Ct(ChIP)$$

$$\%Input = 2^{\Delta Ct} \div 5$$

Table 2.2: Primers for ChIP-qPCR.

Target	Sequence
Negative control	**For.** GGCTCCTGTAACCAACCACTACC **Rev.** CCTCTGGGCTGGCTTCATTC
CDK6	**For.**TCGAAGCGAAGTCCTCAACA **Rev.**GCTTGGGCAGAGGCTATGTA
MEIS1	**For.** TGACAACCTCGCCTGTGATTGAC **Rev.** CGCCCCTCAGACCCAACTACC
HOXA9	**For.** ATGCTTGTGGTTCTCCTCCAGTTG **Rev.** CCGCCGCTCTCATTCTCAGC

2.6 Transient Transcriptome RNA-sequencing (TT-seq)

TT-seq was performed as previously described (Schwalb et al., 2016). In triplicate, 4×10^7 - 5×10^7 cells were labelled with 500 µM 4-thiouridine (Sigma T4509) for 5 min. 50 ng spike-in RNA, consisting of three 4-thiouridine-labelled transcripts from a Cas9-encoding plasmid, was added to each replicate. Total RNA was extracted with either Trizol or RNeasy Maxi kit (Qiagen), DNase I treated, and fragmented by sonication on Covaris ME220 for 10 s (peak power 70 W, duty factor 20%, 1000 cycles/burst). 150-200 µg input RNA was labelled with EZ-Link HPDP-Biotin (Pierce 21341) for 90 min and purified by chloroform/isoamylalcohol extraction. Labelled RNA (*i.e.* nascent transcripts) was enriched using the uMACS Streptavidin Kit (Miltenyi 130-074-101) following the manufacturer's instructions and purified with the RNeasy MinElute Cleanup Kit (Qiagen 74204). cDNA synthesis and library preparation for sequencing were performed using the NEBNext Ultra

II Directional RNA Library Prep Kit (NEB E7760) following the protocol for purified mRNA. The libraries were quantified using the KAPA Library Quantification Kit (Roche KK4824) or High Sensitivity Qubit (ThermoFisher Q32851) and sequenced by 150bp paired-end sequencing (Illumina).

2.7 ATAC-seq

ATAC-seq was performed as described by Buenrostro *et al.* (Buenrostro, Wu, Chang, & Greenleaf, 2015). 50,000 cells were lysed in 50 µL cold lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 % IGEPAL CA-630) and centrifuged at 500 g for 10 minutes at 4°C. The pellet was resuspended in transposase buffer (1X Illumina Tagment DNA Buffer - 20034197) with 2.5 µL Tn5 Transposase enzyme and incubated for 30 min at 37°. Transposed DNA was extracted using the MinElute PCR Purification Kit (Qiagen 28004) following the manufacturer's instructions, then PCR-amplified using Nexterra indices and NebNext Ultra II Q5 MasterMix (NEB M0544S) for a total of 12 cycles. Amplified tagmented DNA was purified with the MinElute PCR Purification Kit and sequenced by 75 bp paired-end sequencing.

2.8 ChIPmentation

ChIPmentation was performed as previously described (Schmidl, Rendeiro, Sheffield, & Bock, 2015). Protein A magnetic beads were conjugated with 1.5 µg of antibody for 4 h at 4°C with rotation in 150 µg PBS 0.5 % BSA 1 x protease inhibitor cocktail (PIC). Antibodies are provided in Table 2.1. Cells were fixed as for ChIP-seq, detailed above. Fixed cells were lysed in 120 µL Lysis Buffer (50 mM Tris-HCl pH 8.0, 0.5% SDS, 10 µM EDTA, 1 x PIC) and

sonicated using Covaris ME220 using the same conditions as for ChIP-seq to generate 200-300 bp fragments. Triton-X100 was added to the sonicated chromatin to a final concentration of 1% and incubated for 10 min at room temperature to neutralize the lysis buffer SDS. The chromatin was pre-cleared with protein A beads for 30 min at 4°C with rotation. Antibody-conjugated beads were washed with 150 µL PBS 0.5% FBS, then added to the pre-cleared chromatin and incubated overnight at 4°C with rotation.

The following day the beads were washed three times with RIPA buffer (as in ChIP-seq), once with Tris-EDTA and once with 10 mM Tris-HCl pH 8.0. The beads were then resuspended in Tagmentation Buffer (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 10% dimethylformamide) with 1 µL transposase (Illumina). Samples were incubated at 37°C for 5 min, after which the transposition reaction was stopped with 150 µL RIPA buffer. Beads were washed with 10 mM Tris-HCl pH 8.0. PCR was performed with NEBNext Ultra II Q5 Mastermix and Nexterra indexed primers (125 nM final concentration) for 13 cycles. AMPure XP beads (Beckman A63881) in a 1:1 ratio were used for clean-up. Samples were sequenced with 150 bp paired-end sequencing (Illumina).

Patient material from an NPM1-mutated AML was generously provided by Dr. Marina Konopleva.

2.9 Sub-cellular fractionation

4×10^7 cells were lysed in 1 mL Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol) with 0.2% NP-40 and 1:200 PIC for 5 min on ice. Following a 5 min centrifugation at 500g and 4°C, the cytoplasmic fraction (supernatant) was removed, and intact nuclei (pellet)

were washed once in Buffer A with PIC. Nuclei were then resuspended in 1 mL No-Salt Buffer (3 mM EDTA, 0.2 mM EGTA) with PIC and incubated for 30 min on ice. Following a 5 min centrifugation at 1200g and 4°C, the nucleoplasm fraction (supernatant) was removed, and the pellet (chromatin) washed once in No-Salt Buffer with PIC. The chromatin was incubated in BC300 buffer (50 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM EDTA, 20% glycerol) with 0.5% NP40 and PIC for 20 min on ice. After centrifugation at 20,000g for 10 min and 4°C, the supernatant was retained as the salt-eluted chromatin fraction. Equal proportions of each fraction were adjusted to 1x NuPage LDS sample buffer (ThermoFisher Scientific NP0008) for SDS-PAGE followed by western blotting.

Whole cell lysate controls were prepared with lysis directly in BC300 buffer (50 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM EDTA, 20% glycerol) with 0.5% NP40 and PIC for 20 min on ice. Lysates were centrifuged at 20,000g for 10 min and 4°C. and the supernatant was mixed with LDS sample buffer for SDS-PAGE followed by western blotting.

The list of antibodies is provided in Table 2.3.

2.10 APEX2-Menin cell line generation

In duplicate, 5×10^7 cells were electroporated using a rectangle pulse EPI 2500 electroporator (10 ms, 350 V, Fisher) with 4 µg sgRNA/CAS9 plasmid (a modified version of pX458 [Addgene 48138] using mRuby in place of eGFP) and 6 µg plasmid containing the APEX2-containing region to be inserted flanked by approximately 500 bp homology arms. After 24 h, the cells were sorted for expression of mRuby from the CAS9 vector. mRuby-positive cells were expanded in culture for 1-2 weeks, after which they were sorted for

expression of mNeonGreen (indicating successful knock-in of the insert at the target locus). 24 h after sorting, the cells were plated in Methocult. Individual (clonal) colonies were then isolated when of sufficient size (approximately 2 weeks after seeding) and screened for expression of the APEX2-Menin fusion protein using western blotting. The list of antibodies is provided in Table 2.3.

2.11 Immunoprecipitation

4×10^7 cells were lysed in 1 mL Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol) with 0.2% NP-40 and Protease Inhibitor Cocktail (PIC) for 5 min on ice. Following a 5 min centrifugation at 500g and 4°C, the cytoplasmic fraction (supernatant) was removed and intact nuclei were washed once in Buffer A + PIC. The nuclei were resuspended in Nuclear Lysis Buffer (50 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40, PIC) with 250 U benzonase (Sigma E1014) and rotated for 1 h at 4°C. After centrifugation at 20,000 g for 10 min and 4°C, 2% of the supernatant was set aside as a nuclear input and the remainder was used for immunoprecipitation with 4 µg anti-Menin antibody (Bethyl A300-105A) overnight at 4°C. An equal amount of rabbit IgG antibody was used in parallel as a control. A 1:1 mix of Protein A:Protein G beads was added to each IP and rotated at 4°C for 3 h. The beads were washed three times with Nuclear Lysis Buffer and once with TE/50 mM NaCl. The beads were then resuspended in 35 µL 2X LDS buffer and proteins were eluted by boiling at 95°C for 5 min. For quality checks, approximately 4% of the sample was used for SDS-PAGE followed by Silver Stain (Pierce Silver Stain Kit, ThermoFisher 24612) and 8.5% was used for western blotting for LEDGF, a protein known to interact with Menin. The list of antibodies is provided in Table 2.3.

Table 2.3: Antibodies for IP and Western Blotting.

Antibody	Reference	Application
Menin	Bethyl A300-105A	IP: 1 µg per 1×10^7 cells Western Blot: 1:10,000
Rabbit IgG	Cell Signalling DA1E	1 µg per 1×10^7 cells
LEDGF	Bethyl A300-848A	Western Blot: 1:5,000
Anti-Mouse Secondary	Abcam ab216776	Western Blot: 1:15,000
Anti-Rabbit Secondary	Abcam ab216773	Western Blot: 1:15,000

2.12 Mass Spectrometry

Pepides were prepared for analysis by liquid chromatrography tandem mass spectrometry (LC-MS/MS) with the S-trap micro spin column digestion protocol (PROTIFI), following the manufacturer's instructions. Briefly, samples were reduced, alkylated and acidified, then purified with the S-Trap micro column. After 5 washes with the binding/wash buffer, 5 µg of Trypsin was added and columns were incubated at 37°C overnight. The peptides were then eluded, dried in a SpeedVac, and dissolved in 5% DMSO 5% formic acid. 200 ng of peptides were then separated on a 50 cm x 75 µm ID internal diameter, 2 µm particle size PepMap RSLC C18 column at 50°C. Solvent A was 5% DMSO, 0.1% formic acid in water and solvent B was 5% DMSO, 0.1% formic acid in acetonitrile. The gradient was increased from 5% to 35% solvent B over 60 min. Peptides were eluted onto a Thermo Q Exactive quadrupole-orbitrap hybrid mass spectrometer, and run in data independent acquisition mode with full MS scans with a resolution of 70,000, and top15 centroid fragment ion scans with a resolution of 17,500.

2.13 Micro-Capture-C (MCC)

Micro-Capture-C was conducted as previously described (Hamley, Li, Denny, Downes, & Davies, 2023; Hua et al., 2021). In triplicate, 5×10^7 SEM and OCI-AML3 cells were treated with 250 nM VTP-50469 or DMSO for 24 hours. Cells were fixed with 2% formaldehyde for 10 min, followed by quenching with glycine and a 1X PBS wash. Cells were then aliquoted into three technical replicates. 0.0005% digitonin (Sigma) was used to permeabilize the cells for 15 min, after which they were snap frozen on dry ice. After thawing, approximately 16×10^6 cells were resuspended in 10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ and divided into three aliquots for micrococcal nuclease (NEB M0247S) digestion with 7.5, 10 or 12.5 Kunitz U for 1 h at 37°C with shaking at 550 rpm in a Thermomixer (Eppendorf). MNase digestion was stopped with 5 mM EGTA and an aliquot was set aside to assess digestion by TapeStation. The cells were washed with PBS/5 mM EGTA and resuspended in DNA ligase buffer, 400 µM dNTPs, 5 mM EDTA, 100 U/µL DNA Polymerase 1, Large (Klenow) Fragment (NEB), 200 U/µL T4 PNK (NEB) and 300 U/µL T4 DNA lygase (Thermo Scientific). This ligation reaction was incubated at 550rpm at 37°C for 2 h followed by 20°C for 8 hours, then held at 4°C in a ThermoMixer. Cells were then washed with PBS and digested with Proteinase K at 65°C overnight to simultaneously reverse crosslinks. DNA was purified using phenol/chloroform/isoamyl alcohol (25:24:1) and digestion and ligation were assessed by TapeStation (Agilent D1000).

Ligated 3C libraries (two technical replicates per biological replicate) were sonicated to 200 bp on Covaris ME220. Indexing was performed in duplicate using Illumina paired-end adaptor, NEBNext Multiplex Oligos for Illumina and Herculase II (Agilent). Enrichment for promoters of interest was per-

formed twice with the HyperCapture Target Enrichment Kit (Roche), using M270 streptavidin beads (Invitrogen) for the pulldown. Biotinylated probes for capture were designed against accessible promoter regions in SEM and OCI-AML3 cells by Nicholas Denny from the Davies lab using an internal pipeline. Probe sequences are provided in Table B.1 and Table B.2. Samples were sequenced by 150 bp paired-end sequencing (Illumina).

2.14 Quality control for genomic material prior to sequencing

ChIP-seq, TT-seq, ChIPmentation and MCC libraries were quantified using high sensitivity Qubit reagents (ThermoFisher) and the expected fragment size was verified using D1000 DNA Screen Tape (Agilent) on an Agilent TapeStation.

2.15 Bioinformatic Analysis

2.15.1 ChIP-seq, ChIPmentation and ATAC-seq

Sequencing data was processed with the Seqnado pipeline developed by Catherine Chahrour and Alastair Smith in the Milne lab (https://github.com/alsmith151/SeqNado). Briefly, sequencing reads were trimmed using trim_galore and aligned to hg38 using bowtie2. deepTools was used to create bigwig files for visualisation on the UCSC genome browser (https://genome.ucsc.edu) and Homer was used call peaks. Normalisation for scaled bigwigs was performed as described in (Orlando et al., 2014). Heatmaps, correlation analyses, and metaplots were generated using deepTools. Promoters were

defined as ATAC peaks that overlap H3K27ac peaks and are within 2 kb from an annotated transcription start site (TSS). Enhancers were defined as ATAC peaks that overlap H3K27ac peaks and are over 2 kb away from an annotated TSS. ChIP-seq peaks were annotated with the Homer function annotatePeaks.pl to determine distance to TSS. Differential peak analysis was performed with the R package DiffBind (Stark & Brown, 2011) and differential peaks were annotated with the R package ChIPSeeker (G. Yu, Wang, & He, 2015). Heatmaps of differential peaks were generated with R package ComplexHeatmap (Z. Gu, Eils, & Schlesner, 2016).

For OCI-AML3 cells, FASTQ files for publicly available ATAC-seq were accessed from NCBI GEO : GSM5114291 (Schaefer et al., 2022) and analysed using the Seqnado pipeline.

2.15.2 TT-seq

Sequencing data was processed with the Seqnado pipeline developed by Catherine Chahrour and Alastair Smith in the Milne lab (`https://github.com/alsmith151/SeqNado`). In summary, TT-seq reads were aligned to hg38 using STAR, and gene expression levels were determined using the Subread package tool featureCounts by counting over transcripts. Bigwigs and counts data for the visualisation of strand-specific eRNA transcription were created using deepTools commands bamCoverage and computeMatrix.

Differential gene expression analysis was performed using edgeR (Robinson, McCarthy, & Smyth, 2010). TT-seq counts were normalised using the edgeR function for TMM normalisation, as spike-in library sizes did not differ consistently between conditions (shown in Appendix A.1) and introduced artificial statistical noise. In the analysis of SEM cells treated with DMSO

or Menin inhibitor for 6 hours, samples from two separate experiments were analysed together as two batches, for which batch correction was performed as per the EdgeR User's Guide.

2.15.3 Mass Spectrometry Analysis

The IP-MS data search was completed with DIA-NN (Demichev, Messner, Vernardis, Lilley, & Ralser, 2020) and data analysis was performed using R. Filtering for known contaminants was performed using the CRAPome database (Mellacheruvu et al., 2013), removing proteins which appear in 95% of all nuclear affinity purification experiments in human cells in the database, as well as excluding all keratin proteins. Imputation was performed by replacing missing values with randomly sampled values from a down-shifted normal distribution following methods described in (Tyanova et al., 2016), for which Frederik Lassen from the Kessler lab contributed code. Comparisons between conditions (IgG and Menin IPs) were performed with moderated t-tests in R, corrected for multiple comparisons using False Discovery Rate with the R package fdrtool. Comparisons between cell lines and volcano plots were generated using Genoppi (Pintacuda et al., 2021) and the R package ggplot. Enrichment analyses were performed with a hypergeometic test against datasets in the CORUM database (Tsitsiridis et al., 2023).

2.15.4 Micro-Capture-C

Analysis was performed using the Micro-Capture-C pipeline from the Davies lab (`https://github.com/jojdavies/Micro-Capture-C`). Peak calling was performed with deep-learning-based peak caller LanceOtron (Hentges et al., 2022). The peaks were filtered using a custom script from the Davies Lab based on size, width, and distance from the capture viewpoint. Peaks were excluded

if they were located more than 2 Mb away from a capture viewpoint or within 2.5 Kb of the viewpoint. Additionally, peaks with a width of under 50 bp and minimum peak score of less than 1 were excluded. Using the filtered peak list, unique junctions were counted at each peak site, then normalized using cis-unique ligation for the corresponding viewpoint as previously reported (Hua et al., 2021). The unique junction count analysis was performed by Joseph Hamley from the Davies lab using a custom script. Peak junctions were then compared between conditions using a Mann-Whitney U test and p-values were FDR-adjusted using the R package fdrtool. MCC peaks were intersected with Menin ChIP-seq peaks called with HOMER.

2.16 Introduction figures

The data used for preparing figures in **Chapter 1: Introduction** was accessed via cBioPortal.org (Gao et al., 2013; de Bruijn et al., 2023; Cerami et al., 2012). The studies from which data was aggregated included:

Acute leukaemia

1. Acute Lymphoblastic Leukemia (St Jude, Nat Genet 2015) (A. K. Andersson et al., 2015)
2. Acute Lymphoblastic Leukemia (St Jude, Nat Genet 2016) (Zhang et al., 2016)
3. Pediatric Acute Lymphoid Leukemia - Phase II (TARGET, 2018)
4. Acute Myeloid Leukemia (OHSU, Cancer Cell 2022) (Bottomly et al., 2022)
5. Acute Myeloid Leukemia (OHSU, Nature 2018) (Tyner et al., 2018)

6. Acute Myeloid Leukemia (TCGA, PanCancer Atlas) (Hoadley et al., 2018)
7. Pediatric Acute Myeloid Leukemia (TARGET, 2018)

Pancreatic Neuroendocrine Tumours

- Pancreatic Neuroendocrine Tumors (Multi-Institute, Nature 2017) (Scarpa et al., 2017)
- Pancreatic Neuroendocrine Tumors (Johns Hopkins University, Science 2011) (Jiao et al., 2011)
- Insulinoma (Shanghai, Nat Commun 2013) (Cao et al., 2013)
- Pancreatic Neuroendocrine Tumors (MSK, 2023)

Structures for Figure 1.4 were accessed through The Protein Data Bank (Berman et al., 2000).

3 | Distinct roles of Menin in transcription in MLL-AF4 and NPM1c leukaemia

3.1 Introduction

3.1.1 The role of Menin-MLL/MLL-FP complex in transcription regulation

The role of Menin in transcription was revealed through accumulating evidence that Menin interacted with transcription factors, such as JUND (Agarwal et al., 1999), Nf-κB (Heppner et al., 2001), Smad3 (Kaji et al., 2001), and FANCD (Jin et al., 2003), which implicated it in both gene activation (via Smad3) and repression (via JUND and Nf-κB). The purification of Menin as part of the MLL1 (which I hereafter refer to as simply "MLL") and MLL2 complexes also revealed a direct interaction with RNA Poll II, which pointed raised the possibility of a more general role in transcription regulation (Yokoyama et al., 2004; Hughes et al., 2004). However, gene expression analysis of Menin knock-out mouse embryos clarified that Menin was specifically required to activate transcription at a subset of genes, including the *Hox* cluster genes, by directly associating with these loci (Maillard et al., 2009; B. E. Li et al., 2013). This function then emerged as critical for MLL-FP-driven transformation (Yokoyama et al., 2005; Y. X. Chen et al., 2006; Caslini et al., 2007), which motivated the development of Menin-MLL inhibitors (Grembecka et al., 2012).

While Menin inhibitors generally target the interaction between Menin and MLL, some differences have been reported in the precise MLL-FP target genes down-regulated by different molecules. For example, treatment with

the first class of inhibitors (MI-2-2) reported by Grembecka *et al.* (2012) and their second-generation optimised versions MI-503 and MI-463 caused down-regulation of *HOXA9* (Grembecka et al., 2012; Borkin et al., 2015), whereas VTP-50469 leads to down-regulation of HOX co-factors *MEIS1* and *PBX3* without affecting HOX cluster expression (Krivtsov et al., 2019). Which genes specifically require the Menin-MLL/MLL-FP interaction for transcription thus remains to be clarified in different leukaemia contexts.

In B lymphopoiesis, Menin is required only at a small number of MLL targets (B. E. Li et al., 2013). Similarly, Krivtsov *et al.* (2019) used ChIP-seq for the N-terminus of MLL to show that MLL-AF9 is not reduced genome-wide upon Menin inhibition, but rather selectively depleted from a group of genes in MOLM-13 cells (Krivtsov et al., 2019). For example, *MEF2C*, *MEIS1* and *JMJD1c* were depleted of both Menin and MLL-AF9, whereas the *HOXA* cluster and *MYB* retained MLL despite Menin loss. Genes which selectively lost MLL binding were characterised by higher levels of Menin and DOT1L occupancy, suggesting that some MLL-FP target genes have a stronger requirement for Menin binding.

While Menin inhibitors have been extensively characterised in pre-clinical models and advanced to phase I/II clinical trials, most of the literature to date has centred on models of AML, typically driven by MLL-AF9 fusions. Krivtsov *et al.* observed that MLLr ALL cells undergo apoptosis at early treatment time points whereas MLLr AML cells undergo differentiation following 4-6 days of inhibitor treatment, suggesting that Menin regulates alternative pathways in cells of distinct lineages (Krivtsov et al., 2019). Further, the slower response of AML cells to inhibition could imply that Menin is more critical for transcription generally in B-cell leukaemias with MLL-FPs.

3.1.2 Menin is required for a subset of genes in NPM1-mutated leukaemias

Although Menin inhibitors were originally developed to target the stability of MLL-FPs, the co-localisation of both MLL and Menin at the *HOX* cluster during haematopoiesis (B. E. Li et al., 2013; Y. X. Chen et al., 2006) in addition to MLL-FP-driven leukaemogenesis (Yokoyama et al., 2004) hinted that Menin inhibitors may be able to target high HOX expression in additional leukaemia subtypes.

This implicated NPM1-mutatated AML, which exhibits high *HOX* gene expression (Mullighan et al., 2007; Alcalay et al., 2005) in the presence of wild-type MLL, as another therapeutic opportunity. Using a domain-tiled CRISPR screen to specifically mutate the Menin-LEDGF binding motif on MLL, Kühn *et al.* (2016) showed that the Menin-MLL interaction was essential for NPM1c OCI-AML3 cells (Kühn et al., 2016). Similarly to MLL-FP cell lines, treatment of OCI-AML3 cells with Menin inhibitor for 4 days also triggered down-regulation of *MEIS1*, as well as the up-regulation of differentiation marker CD11b. The DOT1L inhibitor EPZ4777 was also able to reduce *MEIS1* expression and proliferation in NPM1c cells. Together, this data indicated that the integrity of the wild-type MLL-Menin complex was also necessary to sustain the NPM1c transcriptional program.

The mechanism of gene regulation via Menin in NPM1c cells was clarified when Uckelmann *et al.* and Wang *et al.* independently reported that a portion of the NPM1c protein is retained in the nucleus and associates with chromatin at promoters with Menin-MLL enrichment (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). They showed that inducible NPM1c degradation using the dTag system triggered down-regulation of genes with high NPM1c

promoter occupancy, implicating NPM1c in direct transcriptional regulation of its target genes. NPM1c chromatin occupancy and expression of its target genes could be disrupted with either XPO1 inhibition or Menin inhibition, highlighting the requirement for its protein-protein interaction with XPO1 and the pre-existing chromatin state associated with MLL-Menin binding. However, Uckelmann *et al.* showed that OCI-AML3 cells require at least a 48 h treatment with Menin inhibitor for the down-regulation of *MEIS1*, whereas NPM1c degradation triggers down-regulation of target genes within 30 minutes (Uckelmann et al., 2023). This delay suggests that it is unclear whether Menin binding is generally required to a similar degree for transcriptional activation at all NPM1c loci, or whether a delayed transcriptional response is indicative of indirect or secondary consequences of Menin inhibition.

3.1.3 Use of E2A-PBX1 cells as a model of insensitivity to Menin inhibition

In order to compare Menin activity in Menin-dependent leukaemias to an insensitive leukaemia model, I chose to assess the response of RCH-ACV cells to Menin inhibition. RCH-ACV cells harbour a t(1;19) translocation, which accounts for about 25% of paediatric pre-B ALL (Malouf & Ottersbach, 2017). The resulting fusion protein, E2A-PBX1, combines the transactivation domain of E2A with the DNA binding and homeobox domains of PBX1, thereby inappropriately activating PBX1 targets. Wild-type E2A and PBX1 are both important for B-cell development and haematopoiesis respectively (Bain et al., 1994; Ficara et al., 2013; Dimartino et al., 2001). While PBX1 can interact with MEIS1, a Menin target gene and a vulnerability in both MLL-AF4 and NPM1c leukaemias, the interaction domain on PBX1 is lost in the E2A-PBX1 fusion (C.-P. Chang et al., 1997). Still, E2A-PBX1 can

recruit co-activator complexes and histone acetyl-transferases, including the SAGA complex and EP300/CBP, to facilitate transcription at its gene targets (Holmlund, Lindberg, Grander, & Wallberg, 2012; Scheele et al., 2009; Bayly et al., 2004). I therefore chose this leukaemia model as a B-lineage leukaemia driven by a transcription activation mechanism via a fusion protein to contrast against MLL-AF4 SEM cells.

While the mechanisms of RCH-ACV insensitivity to Menin inhibition will not be explored in detail in this chapter, the cell line has several features which may be implicated in its insensitivity. Briefly, RCH-ACV cells do not express the *HOXA* cluster, which could account for their insensitivity to Menin inhibition. Additionally, according to the mutation data available in the DepMap database, the cell line has a likely deleterious mutation in *EZH2*, the gene encoding the catalytic component of Polycomb Repressive Complex 2 (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.). EZH2 and Menin were shown to be co-dependencies in the regulation of bivalent genes, which are loci characterised by both activating and repressive histone marks (Sparbier et al., 2023). Menin inhibition has been shown to phenocopy EZH2 inhibition in cases of EZH2 gain of function in diffuse large B-cell lymphoma (Sparbier et al., 2023; X. Chen et al., 2023). The ability of RCH-ACV cells to survive with a putative loss of function in EZH2 could suggest a decreased reliance on its genetic interaction with Menin.

3.1.4 Remaining questions about Menin activity in transcription

It remains unclear whether Menin has identical or distinct roles in transcription regulation across these leukaemias of different lineage and with varying driver

mutations. While MLL-FPs are an initiating event and sufficient to drive leukaemia (A. K. Andersson et al., 2015), NPM1c tends to arise in already pre-malignant cells and push existing clonal haematopoiesis into further transformation (Loberg et al., 2019). Despite these differences in the timing and context of these mutations, both MLL-FPs and NPM1c have been characterised as transcriptional activators capable of recruiting additional co-activators and the Pol II complex to target genes. Menin has been proposed to stabilise both MLL-FP complexes and NPM1c at a subset of genes important for leukaemia maintenance (Krivtsov et al., 2019; Uckelmann et al., 2023; X. Q. D. Wang et al., 2023).

However, multi-day treatments of NPM1c cells with Menin inhibitor (Krivtsov et al., 2019; Uckelmann et al., 2023; Kühn et al., 2016) and steady state RNA-seq readouts of gene expression changes reported in the literature do not reveal the early effects of Menin inhibition on active transcription. Furthermore, reports of Menin inhibition to date have primarily focused on linking the decrease of MLL-FP or NPM1c binding at promoters to decreases in gene expression. It has recently been demonstrated that Menin inhibition can trigger the redistribution of chromatin complexes to novel sites (Sparbier et al., 2023; Soto-Feliciano et al., 2023). This led me to hypothesise that gain of MLL-AF4 or NPM1c at new gene promoters may be implicated in gene activation in response to Menin inhibition.

3.1.5 Rationale for the use of VTP-50469

I chose to evaluate the function of the Menin-MLL interaction in these distinct leukaemia subsets using the Menin inhibitor VTP-50469 (Krivtsov et al., 2019), hereafter referred to as "MENi" in figures. Menin inhibition was favoured over Menin knock-down, knock-out or degradation for several

reasons. Firstly, an analogue of VTP-50469, known as Revumenib, is now being trialled in patients (NCT04065399) (Issa et al., 2023), and thus, use of this compound more closely resembles the effects in a clinical setting than knock-out or degradation. Secondly, Menin inhibition allows for assays that can detect short-term effects on transcription and the chromatin landscape. Because Menin is essential in MLL-AF4 and NPM1c cells, generating stable knock-out lines is not feasible for techniques such as ChIP-seq and Transient Transcriptome RNA-sequencing, which can require up to 50 million cells per replicate. Finally, Menin inhibition has been shown to have high on-target activity. The emergence of Menin mutations in the Revumenib/VTP-50469 binding pocket abrogate the effects of Menin inhibition both in cell lines, PDX models, and patients, which suggests that there are minimal, if any, off-target effects (Perner, Stein, et al., 2023).

3.1.6 Chapter aims

In this chapter, the questions I pose are: Is Menin required for transcription of its target genes to the same extent in MLL-AF4 and NPM1c cells, and how does decreased Menin chromatin occupancy affect MLL and NPM1c distribution and activity? To address these, the aims of this chapter are to:

1. Characterise the early transcriptional response to Menin inhibition in MLL-AF4, NPM1c and E2A-PBX1 leukaemia cell lines.
2. Define the alterations in MLL-AF4 and NPM1c genome-wide enrichment following Menin inhibition.
3. Link Menin, MLL-AF4 and NPM1c binding to transcriptional changes in response to Menin inhibition.

3.2 Results

3.2.1 Distinct in vitro sensitivity to Menin inhibition in MLL-AF4 and NPM1c cells

To compare differences in the response to Menin inhibition with VTP-50469 in the cell line panel, a combination of assays was used to assess both a short-term response and long-term survival and leukaemic potential. First, CellTiterGlo (CTG), which uses intracellular ATP as a read-out of metabolically active, viable cells, was used to measure viability after 48 hours of exposure to increasing doses of Menin inhibitor (Figure 3.1A). At this time point, only SEM cells, harbouring the MLL-AF4 fusion, showed a dose-dependent sensitivity with an IC-50 of 45.5 nM. RCH-ACV cells, an MLL-wild-type (MLLwt) ALL cell line intended as a negative control for Menin essentiality, were insensitive to Menin inhibition even at high micromolar doses. Despite their known dependence on Menin, OCI-AML3 cells, harbouring the mutant NPM1c, showed a similar response to the RCH-ACV cells after 48 h of treatment and did not approach 50% cell death even at the highest concentration of inhibitor (62.5 µM) tested.

Next, to measure proliferative capacity in the presence of Menin inhibitor, cells were pre-treated with Menin inhibitor for 90 min and then plated in semi-solid media to assess colony formation (Figure 3.1B). In this assay, both SEM and OCI-AML3 cells did not form colonies in the presence of Menin inhibitor. RCH-ACV cells had a mildly reduced colony-forming capacity, which did not differ between the two concentrations of inhibitor tested (50 nM or 250 nM), confirming their insensitivity to Menin inhibition.

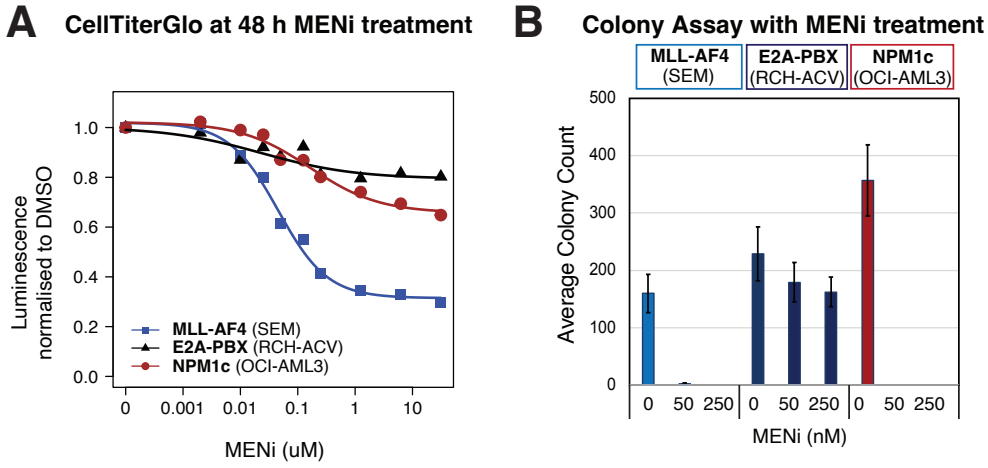


Figure 3.1: Sensitivity of SEM, RCH-ACV and OCI-AML3 cells to Menin inhibition. **A.** Dose response curves to increasing concentrations of Menin inhibitor recorded at 48 h using CellTiterGlo (n = 5 for SEM and RCH-ACV cells, n = 3 for OCI-AML3 cells). **B.** Colony forming units recorded after culture in Menin inhibitor (n=6). Error bars represent standard error.

The distinct effects of Menin inhibition on short-term viability, as measured by the CTG assay, and clonogenic potential, as measured by the colony forming assay, in SEM and OCI-AML3 cells suggest that Menin is essential to different survival mechanisms in these cells, or that the inhibitor acts over different time-frames. These results are in line with previous reports that *MLL*-rearranged ALL cell lines tend to undergo apoptosis at an early treatment time point (*e.g.* 2 days in Krivtsov *et al.* (2019)) compared to Menin-dependent AML cell lines, which tend to undergo differentiation rather than apoptosis (Krivtsov et al., 2019; Uckelmann et al., 2020; Kühn et al., 2016; Adriaanse et al., 2024).

3.2.2 Differential effects of Menin inhibition on transcription in MLL-AF4 and NPM1c cells

The distinct responses to Menin inhibition in MLL-AF4 (SEM), E2A-PBX1 (RCH-ACV), and NPM1c (OCI-AML3) cells indicated that Menin may be differentially required for transcriptional activation in these different cell types. To test whether differences in Menin enrichment at active genes could explain the difference in inhibitor sensitivity, ChIP-seq was used to map Menin binding at promoters. Menin target genes were defined as genes that contained a Menin peak within 2 kb of the transcription start site (TSS), characteristic of promoter binding. In all three cell lines, regardless of sensitivity to Menin inhibition, Menin was enriched at highly-expressed genes (Figure 3.2A), consistent with the known localisation of MLL complexes at active genes (Kerry et al., 2017). The mean expression of Menin-bound genes was significantly higher (K-S test ρ-value $<$ 2.2e-16) than non-Menin targets in each cell line. Therefore, Menin enrichment at highly expressed genes could not account for differences in the viability of SEM and OCI-AML3 cells, or the insensitivity of RCH-ACV cells, to Menin inhibition.

To evaluate the effects on transcription following Menin inhibition, cells were treated with 250 nM VTP-50469 for 24 hours. This concentration and treatment duration were sufficient to cause Menin depletion from chromatin genome-wide in each cell line (Figure 3.2B). This confirmed that the inhibitor disrupts Menin association with chromatin to a similar extent, and insensitivity to inhibition is not linked to retention of Menin on chromatin.

To compare the effects on transcription, I used Transient Transcriptome RNA-seq (Schwalb et al., 2016), which involves labelling nascent RNA for 5 minutes with 4-thiouridine (4-SU) and isolating labelled transcripts. This method

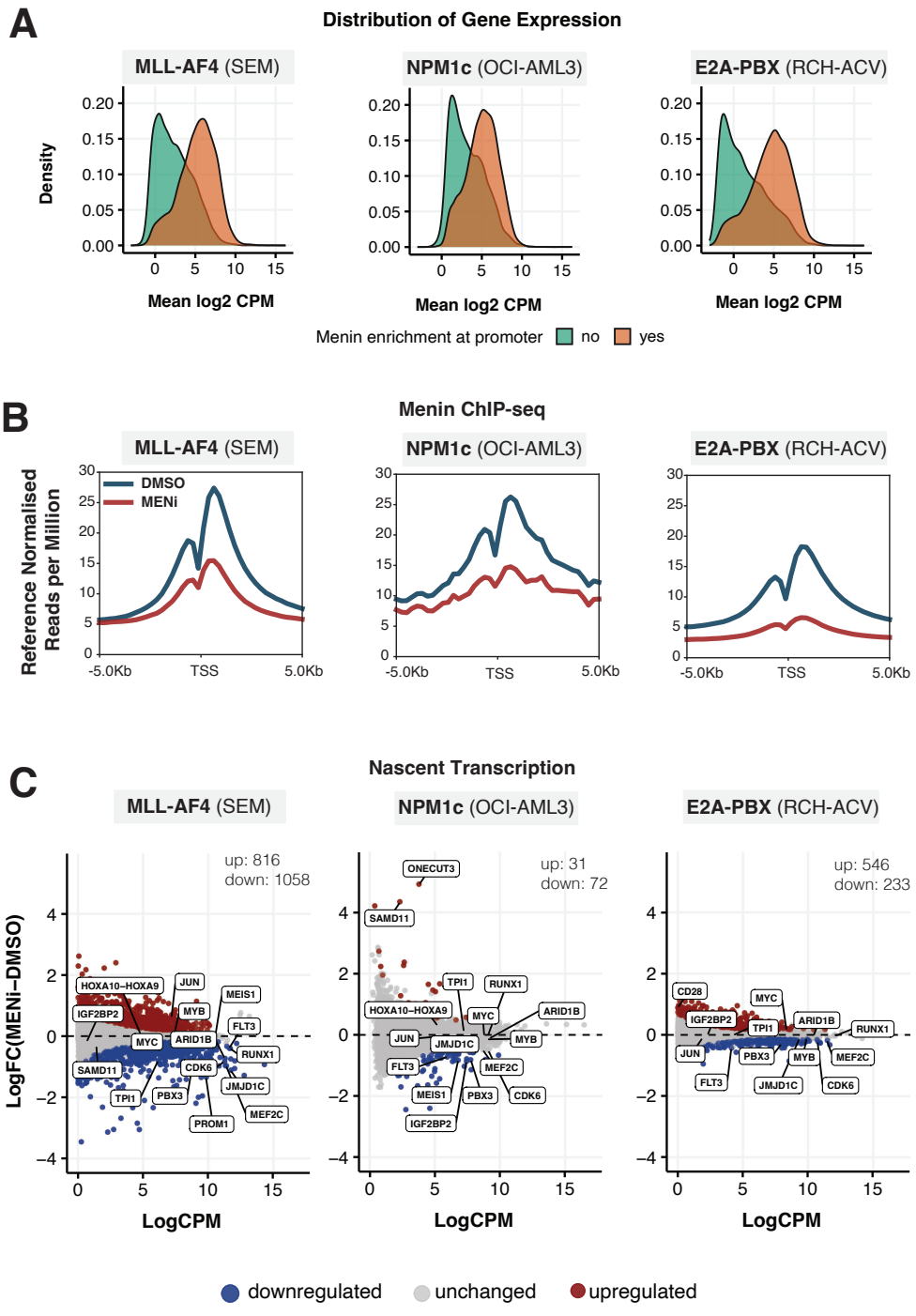


Figure 3.2: *(see next page)*

Figure 3.2 *(previous page)*: Distinct effects of Menin inhibition on transcription. **A.** Density plots of gene expression of Menin and non-Menin target genes. The distributions are statistically different in each cell line (Kolmogorov-Smirnov test ρ-value $<$ 2.2e-16). **B.** Metaplots displaying the average Menin enrichment at genes after 24 h of treatment with DMSO or Menin inhibitor at 250 nM. **C.** MA plots of transient transcriptome RNA-seq after 24 h of treatment with DMSO or Menin inhibitor at 250 nM. Up-regulated transcripts (FDR $<$ 0.05, $\log_2$ fold change $>$ 0) are coloured in red and down-regulated (FDR $<$ 0.05, $\log_2$ fold change $<$ 0) transcripts are coloured blue.

effectively captures active transcription rather than steady-state RNA levels, allowing me to test the effects of Menin inhibition specifically on transcription. Despite the comparable expression of Menin-bound genes and the loss of Menin from chromatin with inhibition, the effects on transcription varied across cell lines (Figure 3.2C). In the MLL-AF4 SEM cells, 1874 genes were differentially transcribed, with a majority of these being down-regulated (n = 1058). Among the down-regulated transcripts were classical MLL-fusion target genes such as *CDK6*, *MEF2C*, and *FLT3* (Krivtsov et al., 2019), although *HOXA9* and *MEIS1* were not differentially expressed at this treatment time point. In the inhibitor insensitive RCH-ACV cells, differentially expressed genes were primarily up-regulated with small $\log_2$ fold changes, indicating that Menin is not required to maintain the transcriptional program of the E2A-PBX1 fusion protein. NPM1c cells, despite requiring Menin for long-term proliferation and survival, did not show a strong transcriptional response to 24 h inhibition, with only 103 differential transcripts, of which 72 were down-regulated. The *MEIS1* gene, considered the main Menin-dependent vulnerability in these cells, was down-regulated at this time point (Borkin et al., 2015; Grembecka et al., 2012; Krivtsov et al., 2019).

Next, Menin ChIP-seq was used to evaluate whether there was a correlation

between transcription changes and Menin binding at target genes. Pearson's chi-squared test indicated a significant association between Menin enrichment at promoters and differential gene expression in SEM and RCH-ACV cells, with down-regulated genes more likely to be bound by Menin (Figure 3.3). This was not the case for NPM1c cells, in which Menin binding did not correlate with a specific class of differentially expressed genes.

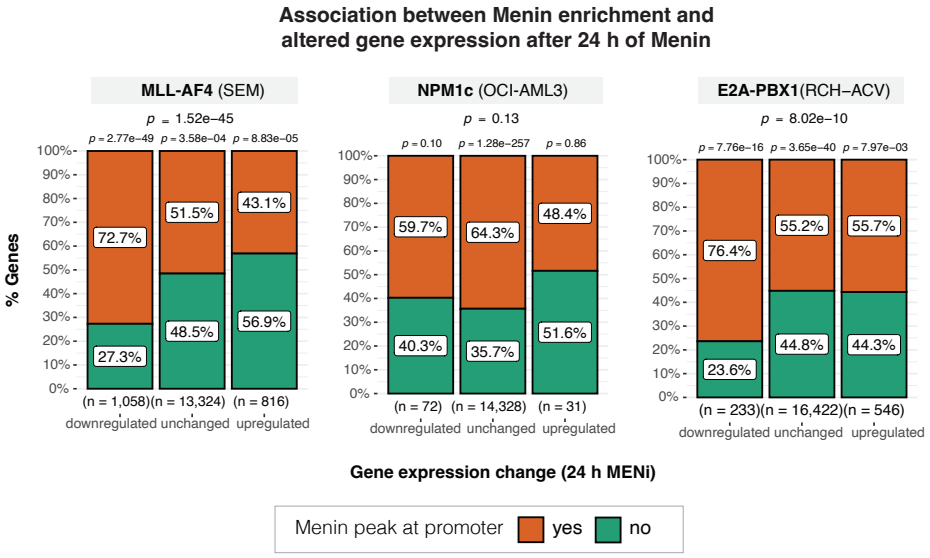


Figure 3.3: Menin inhibition-responsive genes are primarily Menin targets in lymphoid lineage MLL-AF4 (SEM) and E2A-PBX1 (RCH-ACV) cells but not myeloid NPM1c (OCI-AML3) cells. Reported ρ-values are from a Pearson chi-squared test.

The magnitude of the transcriptional responses to 24 h of Menin inhibition in the cell lines correlated with their viability as detected by the CTG assay at 48 h (Figure 3.1A) — only the MLL-AF4 SEM cells displayed broad transcriptional dysregulation, whereas the NPM1c cells were mildly affected by this short-term exposure to the inhibitor. This contrast in transcriptional responses points to distinct roles for Menin in regulating the expression of highly active genes in these two cell lines, which both require Menin for

long-term leukaemic potential (Figure 3.1B).

3.2.3 Menin binding regulates distinct targets

In order to assess the chromatin features of Menin inhibitor-responsive genes, I extracted the differentially expressed genes from each cell line and analysed Menin, MLL/MLL-AF4, and NPM1c (in OCI-AML3 cells) enrichment at promoters (Figure 3.4). The MLL antibody used recognises a sequence close to the N-terminus of the protein, and thus detects both wild type MLL as well as MLL-AF4 binding. Although this represents a mixture of both MLL and MLL-AF4 proteins in SEM cells, siRNA knock-downs of MLL-AF4 suggest the largest part of the signal is due to MLL-AF4 (Kerry et al., 2017). I therefore use MLL and MLL-AF4 interchangeably when refering to data in SEM cells. For the chromatin immunoprecipitation of NPM1c, I used an antibody which specifically targets the C-terminal region of mutant NPM1, thereby selectively detecting NPM1c over wild-type NPM1.

In NPM1c (OCI-AML3) cells, Menin enrichment was similar at down-regulated genes and unchanged genes (Figure 3.4A), which was not observed in the other two cell lines. However, this may be an averaging affect due to the small number (n = 73) of down-regulated genes in OCI-AML3 cells. In the lymphoid lineage cells — E2A-PBX1 (RCH-ACV) and MLL-AF4 (SEM) (Figure 3.4 B and C) — the down-regulated genes had more Menin enrichment compared to unaffected genes. This analysis, which utilised average ChIP-seq signal at promoters, was in concordance with the proportion of Menin target genes in each group, and the χ^2 association test between Menin enrichment and differential gene expression (Figure 3.3), which relied on peak calling.

MLL enrichment (encompassing MLL-AF4 in SEM cells) correlated with

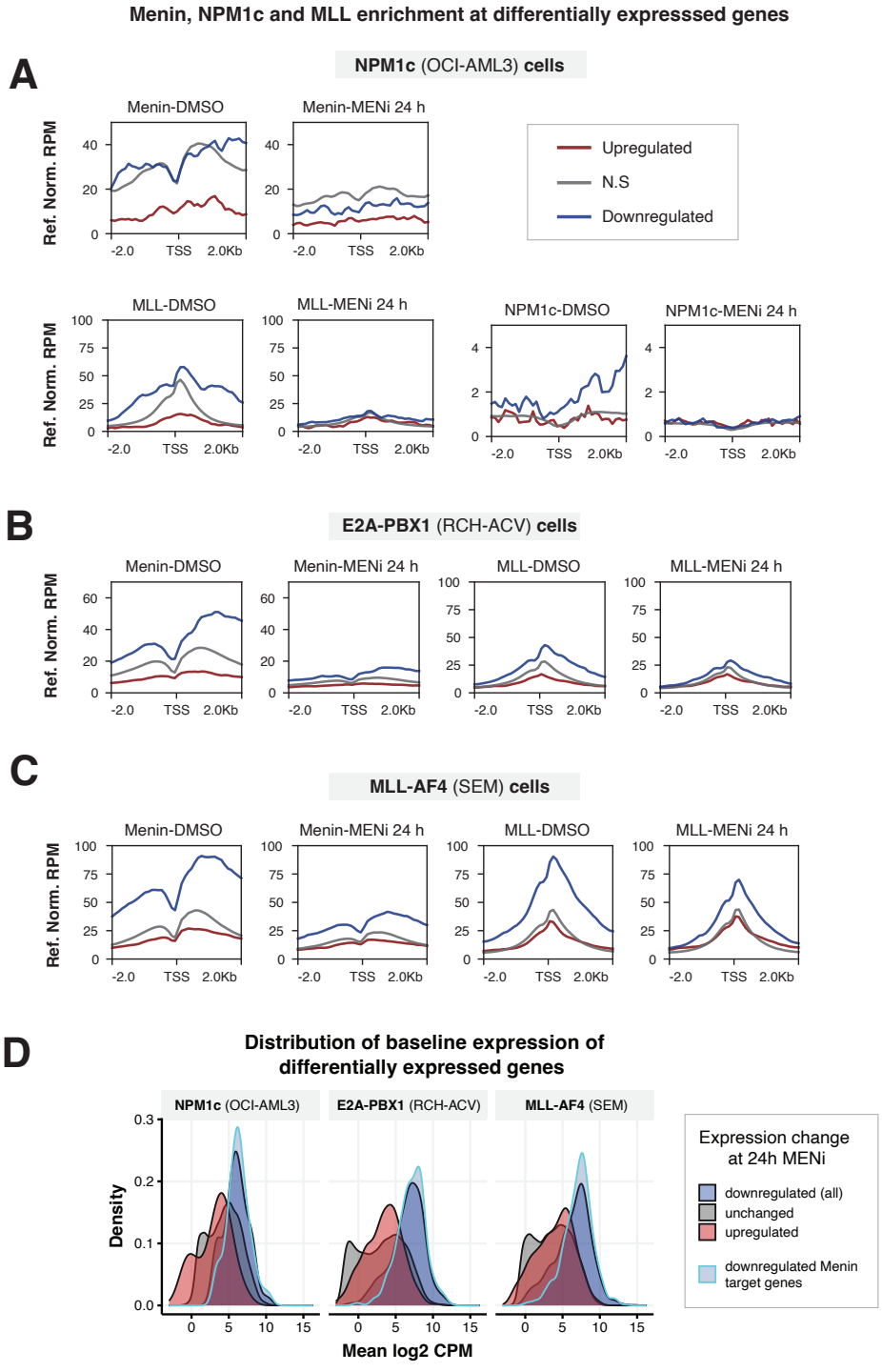


Figure 3.4: *(see next page)*

Figure 3.4 *(previous page)*: Menin loss triggers decrease of MLL and NPM1c binding and down-regulation of highly expressed genes. Metaplots of Menin, MLL and NPM1c binding in **A.** OCI-AML3 cells, **B.** RCH-ACV cells and **C.** SEM cells at unchanged and differentially expressed gene promoters, in either control (DMSO) or Menin inhibitor treatment (MENi) conditions. **D.** Density plot of baseline expression ($\log_2$ counts per million) of differentially expressed genes. Menin target genes that decrease in expression are highlighted in cyan.

Menin enrichment at each gene set, except in NPM1c cells, where the average MLL signal was broader at down-regulated genes despite similar Menin levels at down-regulated and unaffected genes. NPM1c was specifically enriched at down-regulated genes in OCI-AML3 cells. However, out of 62 genes with an NPM1c promoter peak, 9 were down-regulated, pointing to a selective sensitivity of some NPM1c target genes to Menin inhibition.

In each cell line, the down-regulated gene set was comprised of highly expressed genes in the untreated condition (Figure 3.4D). Together, this data shows that the common features of Menin inhibitor-responsive genes across the cell line panel are high levels of Menin and MLL enrichment, plus NPM1c enrichment in OCI-AML3 cells, and high baseline expression. However, despite the overall correlation of Menin binding with high gene expression across cell lines, the more potent transcriptional response to inhibition in SEM cells suggests that some genes may require Menin only in the MLL-AF4 context.

To focus on putative direct Menin targets, I extracted the set of 1058 genes down-regulated in SEM cells (MLL-AF4) and compared Menin binding across cell lines and patient samples (Figure 3.5A). Of these genes, 769 are bound by Menin in SEM cells (Figure 3.3). To characterise Menin binding in patients, I used small cell number ChIPmentation to generate a Menin binding profile in an adult NPM1c AML sample, and used previously published Menin

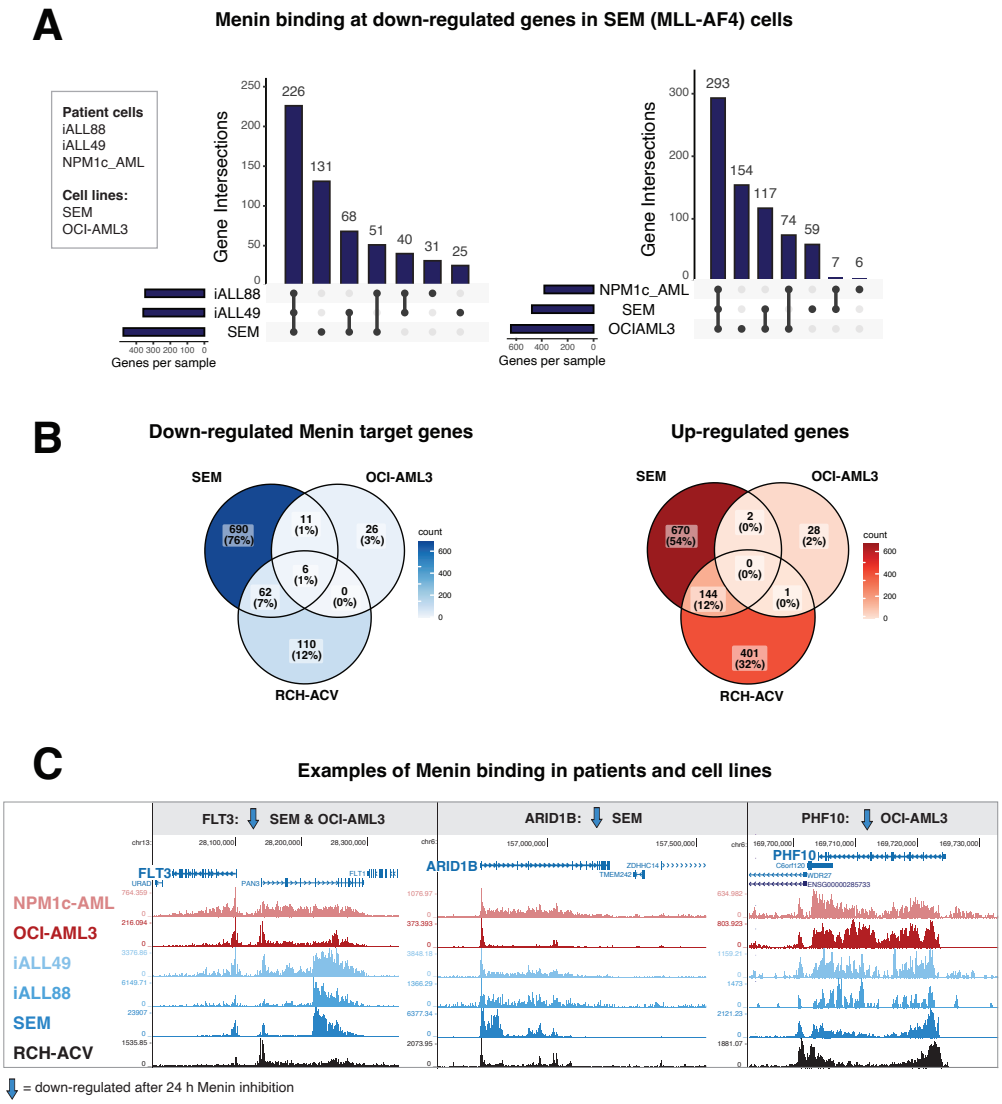


Figure 3.5: Menin regulates distinct targets. **A.** Upset plots representing intersections of Menin-bound genes from the gene set that is down-regulated in SEM cells (MLL-AF4) upon 24 h of Menin inhibition. iALL88, iALL49 and NPM1c_AML represent patient samples. **B.** Venn diagrams showing intersections of down-regulated Menin target genes and all up-regulated genes in the cell line panel. **C.** Examples of Menin binding at *FLT3*, *ARID1B* and *PHF10* in cell lines and patient samples. The blue arrow indicates down-regulation after 24 h Menin Inhibition.

ChIPmentation from two t(4,11) infant ALL patients (iALL) (Crump et al., 2023). In the ALL samples, the largest set of genes (226) contained a Menin peak in both SEM cells and the patient samples, while the second largest set (131 genes) were Menin-bound in SEM cells but not in the patients. Similarly, when intersecting these down-regulated genes with Menin targets in OCI-AML3 cells and the NPM1c patient sample, the largest gene set (293) shared Menin binding across samples, with only 59 genes unique to SEM cells.

Despite the shared Menin enrichment at 410 genes down-regulated in SEM cells in both SEM and OCI-AML3 cells, only 11 of these were also down-regulated in OCI-AML3 cells (Figure 3.5B). For example, *FLT3* is bound by Menin in both SEM and OCI-AML3 cells and down-regulated upon Menin inhibition in both (Figure 3.5C). On the other hand, ARID1B is uniquely down-regulated ($\log_2$ fold change = -0.73, FDR = 2.2e-6) in SEM cells, despite having some Menin enrichment at the promoter in OCI-AML3 cells. Conversely, *PHF10* is uniquely down-regulated ($\log_2$ fold change = -0.65, FDR = 1.3e-5) in OCI-AML3, while also bound by Menin in SEM and in RCH-ACV cells. This indicates that the majority of shared Menin targets are uniquely dependent on Menin binding in MLL-AF4 cells for transcription.

Previously reported (Uckelmann et al., 2023; Crump et al., 2023) key Menin targets *RUNX2*, *SATB1*, and *FLT3* were among the shared down-regulated genes. Thus, while transcription is disrupted on a broader scale in SEM cells, Menin inhibition in OCI-AML3 cells may trigger the down-regulation of a small number of essential genes, causing a slower, delayed fitness response. Furthermore, the largest intersections of down-regulated (n = 62) or up-regulated (n = 144) genes were between SEM and RCH-ACV cells, which suggests that the transcriptional response of ALL cells, despite differential sensitivity to Menin inhibition, is more similar than the responses of inhibitor-

sensitive cells of different lineages (lymphoblastic vs myeloid).

3.2.4 Menin inhibition leads to a redistribution of MLL and NPM1c chromatin binding

The transient transcriptome RNA-sequencing following Menin inhibition indicated that Menin has a more general co-activator function in the MLL-AF4 complex in SEM cells in contrast to its function in the NPM1c-MLLwt complex in OCI-AML3 cells, with the small number of down-regulated genes in the latter pointing to a highly specific requirement for Menin at select gene targets. Both MLL-AF4 and NPM1c have been shown to recruit RNA polymerase II and transcription elongation factors (Milne, Dou, et al., 2005; Okuda et al., 2014; X. Q. D. Wang et al., 2023). Menin is in turn thought to stabilise both MLL-AF4 and NPM1c at target genes and thus facilitate transcription (Issa, Ravandi, et al., 2021). The delay between Menin inhibition and gene expression changes in OCI-AML3 cells led me to hypothesise that wild-type MLL and NPM1c binding may be altered more gradually compared to MLL-FPs. To test for a temporal connection between transcriptional changes and differential MLL-AF4 or NPM1c binding, I performed ChIPmentation for these factors at two time points of Menin inhibition. I assessed MLL binding after 6 h and 24 h of Menin inhibition in SEM cells, whereas in OCI-AML3 cells, I assessed both MLL-wt and NPM1c binding at 24 h and 72 h of Menin inhibition. Gene expression changes in response to Menin inhibition have previously been reported at this later time point in OCI-AML3 cells (Uckelmann et al., 2023).

In SEM cells, following Menin inhibition, differential (ρ-adj < 0.05) MLL (*i.e.* MLL-AF4) peaks clustered in two groups — MLL loss (cluster 1) or MLL

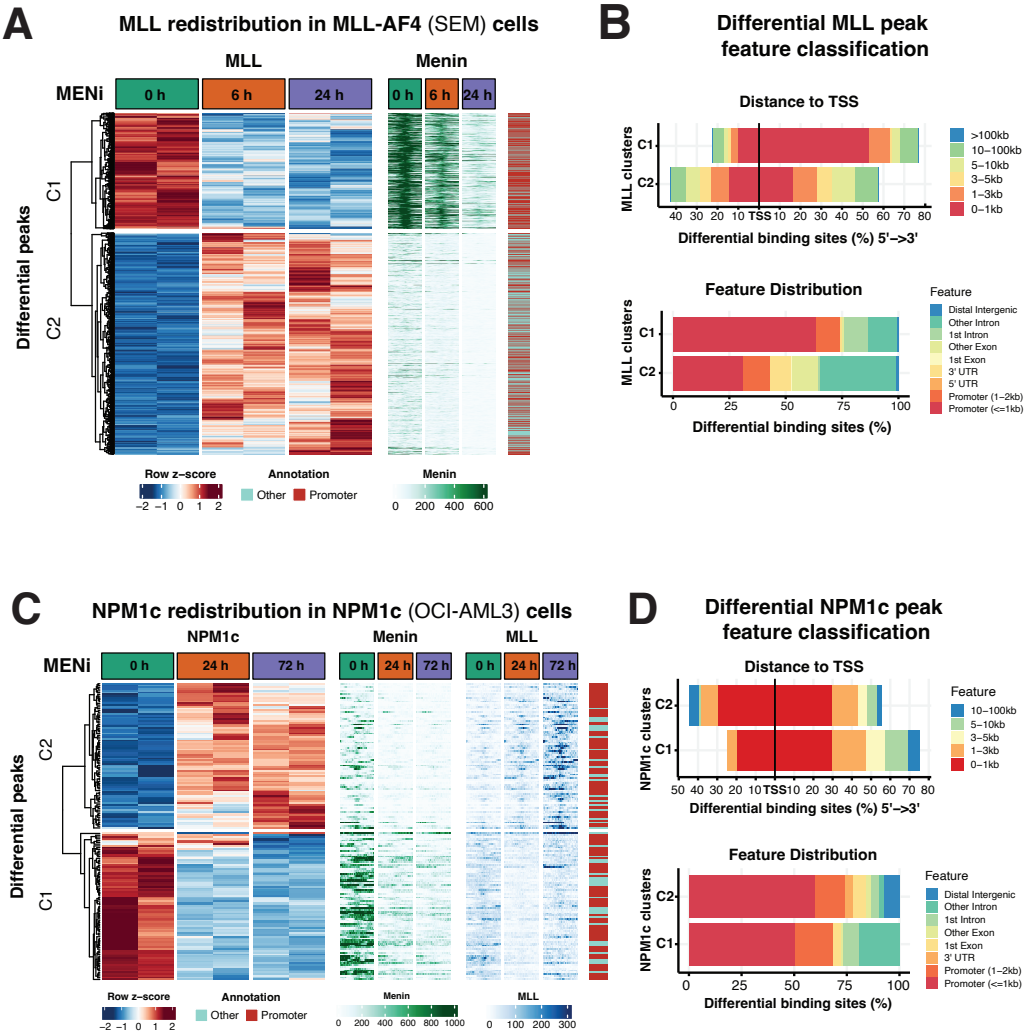


Figure 3.6: Menin inhibition causes re-distribution of MLL and NPM1c. **A.** Heatmap showing differential (p.adj <0.05) MLL peaks in SEM cells with Menin inhibition at 6 h and 24 h (n=2). A heatmap of Menin binding is shown at the same sites, spanning a 2kb region centred on MLL peaks. Peaks are annotated as promoter if within 2 Kb of a TSS or "other" if more than 2 Kb away from a TSS. **B.** Annotation of genomic features at differential MLL peaks. **C.** Heatmap showing differential (p.adj <0.05) NPM1c peaks in OCI-AML3 cells with Menin inhibition at 24 h and 72 h (n=2). Heatmaps of Menin and MLL binding are shown at the same sites, centred on NPM1c peaks and spanning 2kb. Peaks are annotated as promoter if within 2 Kb of a TSS or "other" if more than 2 Kb away from a TSS. **D.** Annotation of genomic features at differential NPM1c peaks.

gain (cluster 2) (Figure 3.6A). The MLL binding profiles were similar at the 6 h and 24 h treatment time points, demonstrating that Menin inhibition rapidly alters MLL binding. Regions depleted of MLL binding (cluster 1) were initially also bound by Menin, whereas regions that gained MLL binding (cluster 2) upon Menin inhibition had much lower Menin enrichment in the untreated (DMSO) condition. A portion of the cluster 2 peaks correspond to increased signal in gene bodies (Figure 3.6B), whereas MLL binding decreased predominantly at promoters within 1 kb of a TSS (Figure 3.6B).

In OCI-AML3 cells, ChIP-mentation for NPM1c at 24 h and 72 h of Menin inhibition also revealed two clusters of differential peaks (ρ-adj < 0.05) — NPM1c loss and NPM1c increase (Figure 3.6C). While some peaks in cluster 1 (NPM1c decrease) show a decrease at 72 h but not at 24 h, they are in proximity to promoters which show a decrease of NPM1c binding at 24 h as well. Similarly to MLL in the SEM cells, decreased NPM1c binding was associated with Menin loss, whereas regions that gained NPM1c binding lacked Menin binding in the DMSO condition. NPM1c gain also correlated with subtle increased MLL enrichment. Differential NPM1c regions were primarily promoters within 1 kb of the TSS (Figure 3.6D). Decreased NPM1c binding sites also included intronic and 3' UTR regions. This suggested that the sites most likely to have destabilised NPM1c binding following Menin inhibition had intragenic NPM1c spread, which has been previously reported by Wang *et al.* (X. Q. D. Wang et al., 2023).

Visual inspection of differential MLL peaks in SEM cells demonstrates that the loss of MLL enrichment at promoters is more substantial compared to subtle increases in MLL enrichment within the intragenic regions of loci where MLL is retained (Figure 3.7A). MLL ChIP-seq data showed a similar pattern to the ChIPmentation used for differential analysis, validating the observation

that the "Cluster 2" peaks of increased MLL more likely represent sites where MLL-AF4 binding is insensitive to Menin inhibition. Given that the metaplot analysis in Figure 3.4C shows no change in average MLL signal at up-regulated or unchanged genes after 24 h of Menin inhibition, it is unlikely that MLL gain is driving gene activation. For example, *JUN* and *TPI1* both harboured an increased MLL peak, but only *JUN* was concordantly up-regulated, whereas *TPI1* was down-regulated (Figure 3.7B). In contrast, known MLL-AF4 targets *CDK6*, *PROM1* and *TAPT1* showed a concordant loss of MLL-AF4 promoter binding and expression.

In OCI-AML3 cells, decreased NPM1c was observed at the down-regulated *IGF2BP2* gene (Figure 3.8A), which agrees with observations by Uckelmann *et al.* that NPM1c binding directly regulates the expression of a subset of Menin-bound genes (Uckelmann et al., 2023). In contrast to the subtle increases in MLL-AF4 within gene bodies in SEM cells, the largest gain in NPM1c enrichment following Menin inhibition in OCI-AML3 cells corresponded to the emergence of a new promoter binding site for both NPM1c and MLL-wt at *ONECUT3* (Figure 3.8). *ONECUT3* was also the most up-regulated gene following 24 hours of Menin inhibition (Figure 3.2C, Figure 3.8B), suggesting that NPM1c gain could be associated with the induction of transcription.

To test whether changes in MLL and NPM1c binding correlated with the timing of transcriptional changes, I generated TT-seq data at the same time points. I first compared the changes in transcription at Menin-bound genes at each time point to DMSO controls. As expected, more genes were differentially expressed at the later treatment time point in both SEM and OCI-AML3 cells (Figure 3.9). In SEM cells, a greater proportion of Menin-bound genes became down-regulated at 24 h compared to 6 h, whereas in OCI-AML3 cells, Menin-bound genes were up- or down-regulated in approximately equal

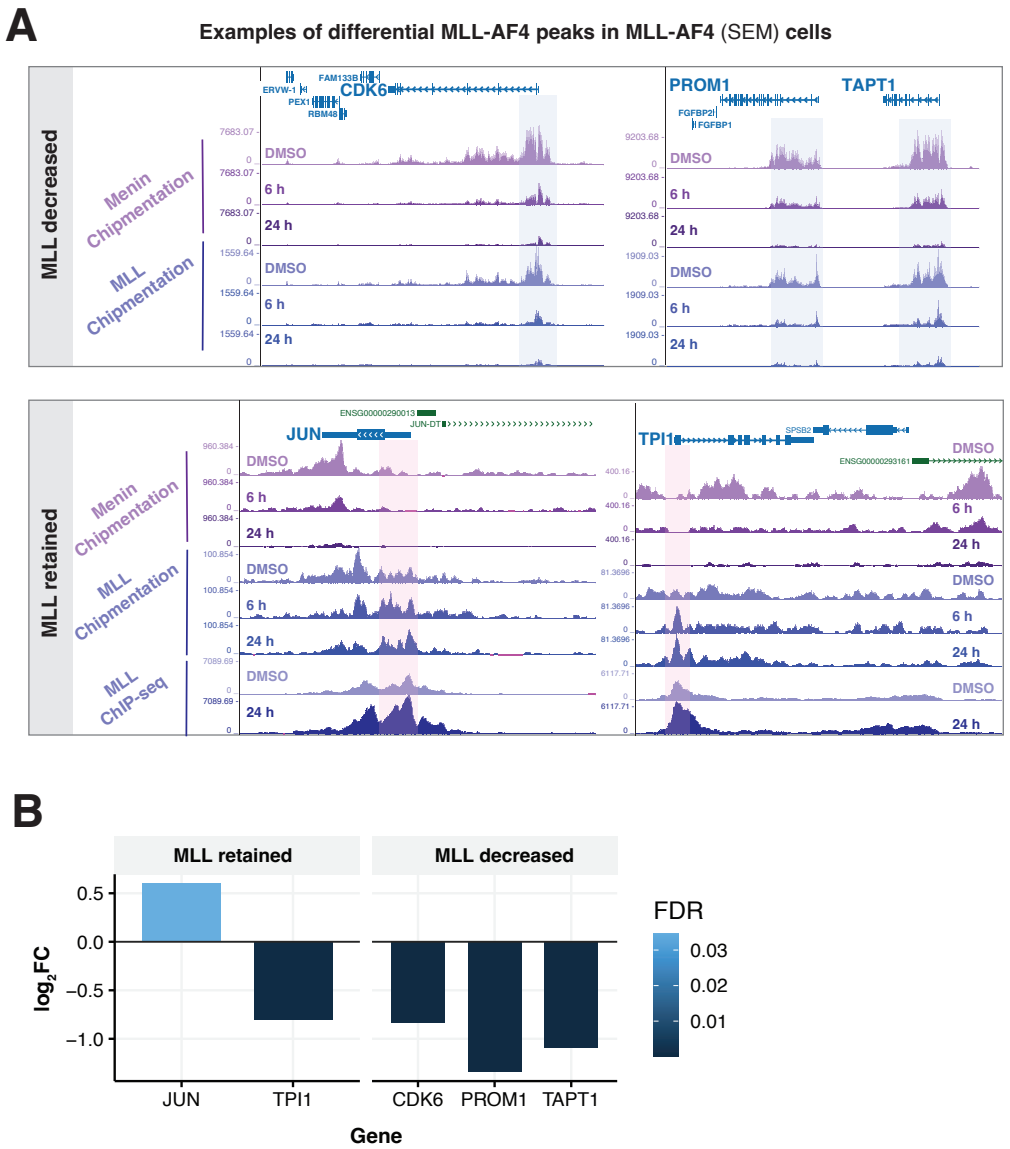


Figure 3.7: Examples of altered MLL-AF4 binding in SEM cells. **A.** Examples of MLL-AF4 loss from promoters (top) and MLL retention with increased signal (bottom). MLL ChIP-seq in addition to ChIPmentation is shown for increased peaks. **B.** Transcription changes at the genes shown in **A.** at 24 h of Menin inhibition.

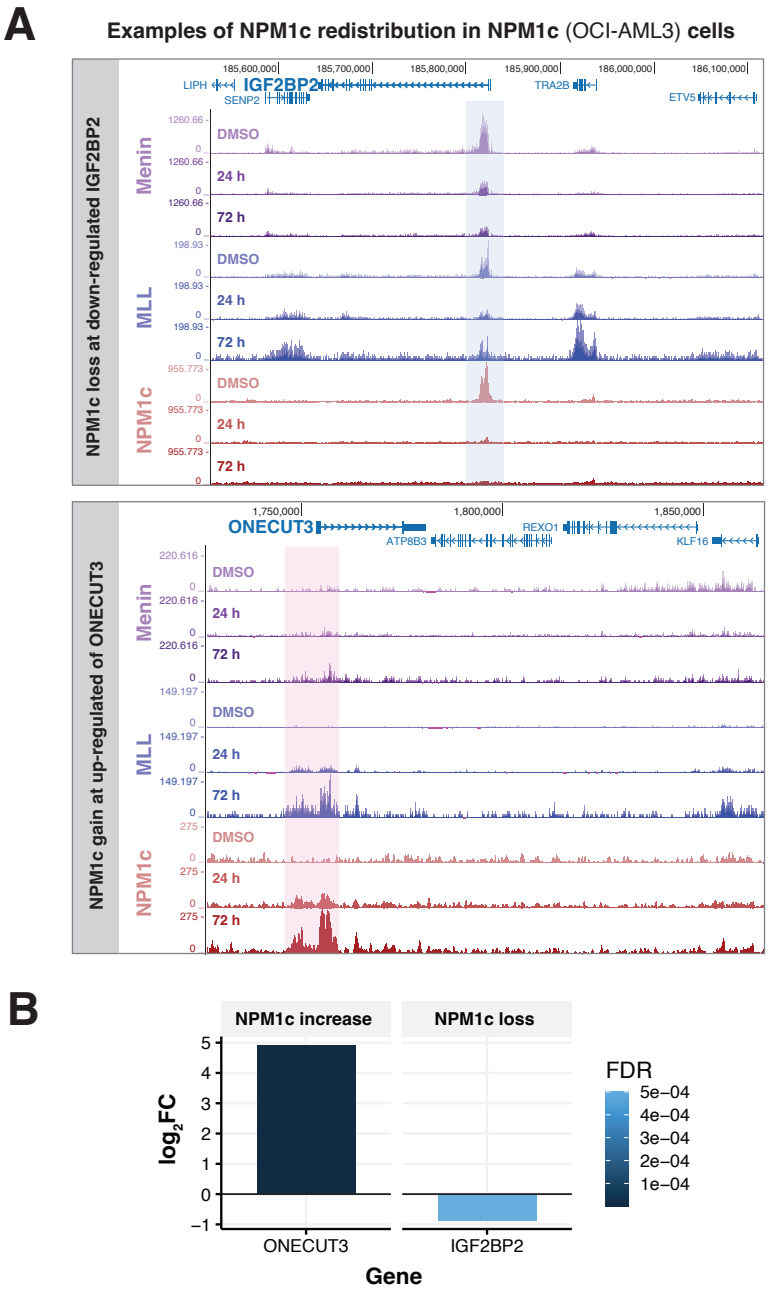


Figure 3.8: *(see next page)*

Figure 3.8 *(previous page)*: Examples of NPM1c loss at *IGF2BP2* (top) and novel binding at *ONECUT3* (bottom) with Menin inhibition. Tracks represent ChIPmentation data. The gene expression status (up- or down-regulated) refers to 24 h of Menin inhibition compared to DMSO. **B.** Transcription changes at the genes shown in **A.** at 24 h of Menin inhibition.

proportions at 72 h compared to 24 h. This indicates that Menin is required for transcription activation at a greater proportion of its targets in the MLL-AF4 cells compared to in NPM1c cells.

I next compared the gene sets with altered MLL-AF4 or NPM1c promoter enrichment to differentially expressed genes (FDR < 0.05). At both 6 h and 24 h of Menin inhibition in SEM cells, correlating the fold changes in gene expression and MLL-AF4 binding revealed that the two are linked at a relatively small subset of loci compared to the total differentially expressed genes (Figure 3.10A). At 6 h of Menin inhibition, there were 627 decreased MLL promoter peaks and 463 down-regulated genes in total, of which 144 (28.7% of down-regulated genes) had a correlated decrease in expression and MLL binding. At 24 h, there were 629 decreased MLL promoter peaks and 1058 down-regulated genes, of which 176 (16.1% of down-regulated genes) exhibited both decreased MLL binding and expression. The genes that gained both expression and MLL promoter binding were much fewer — 12 and 29 at 6 h and 24 h respectively — indicating that increases in transcription at these loci is likely to be regulated independently of MLL.

Overall, however, there is an association between transcription and MLL redistribution at a larger total number of targets in SEM cells compared to transcription and NPM1c redistribution in OCI-AML3 cells. In total, NPM1c binding was decreased at 31 and 35 promoters at 24 h and 72 h of Menin inhibition respectively; 9.7% of down-regulated genes (8 / 72) had decreased

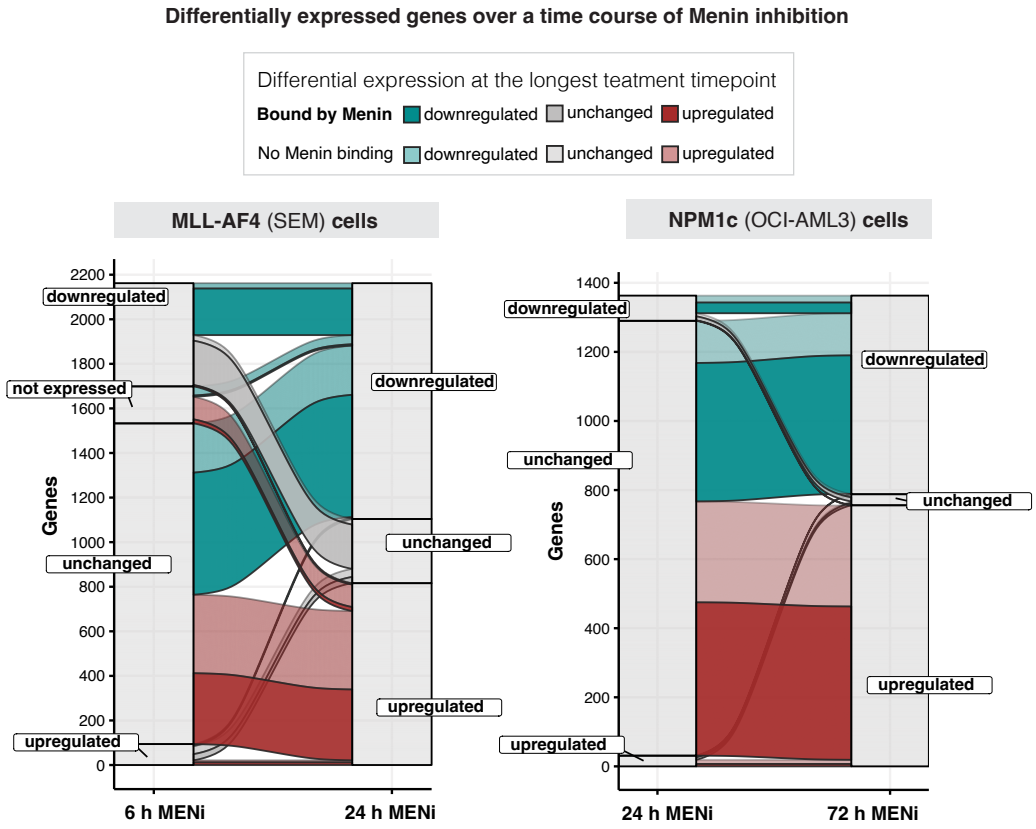


Figure 3.9: Gene expression changes at two time points of Menin inhibition in SEM and OCI-AML3 cells distinguish early and delayed transcription effects. The alluvial diagrams represent the change in transcription status relative to DMSO control at two time points for each cell line. The thickness of the flows between categories is proportional to the size of the gene set which changes expression status. The colour of the flows corresponds to the gene expression status (down-regulated in cyan, up-regulated in red) at the longest treatment time point (24 h in SEM and 72 h in OCI-AML3). The shading of the flows corresponds to Menin binding status, where darker flows represent Menin-bound genes and lighter flows represent genes without Menin binding.

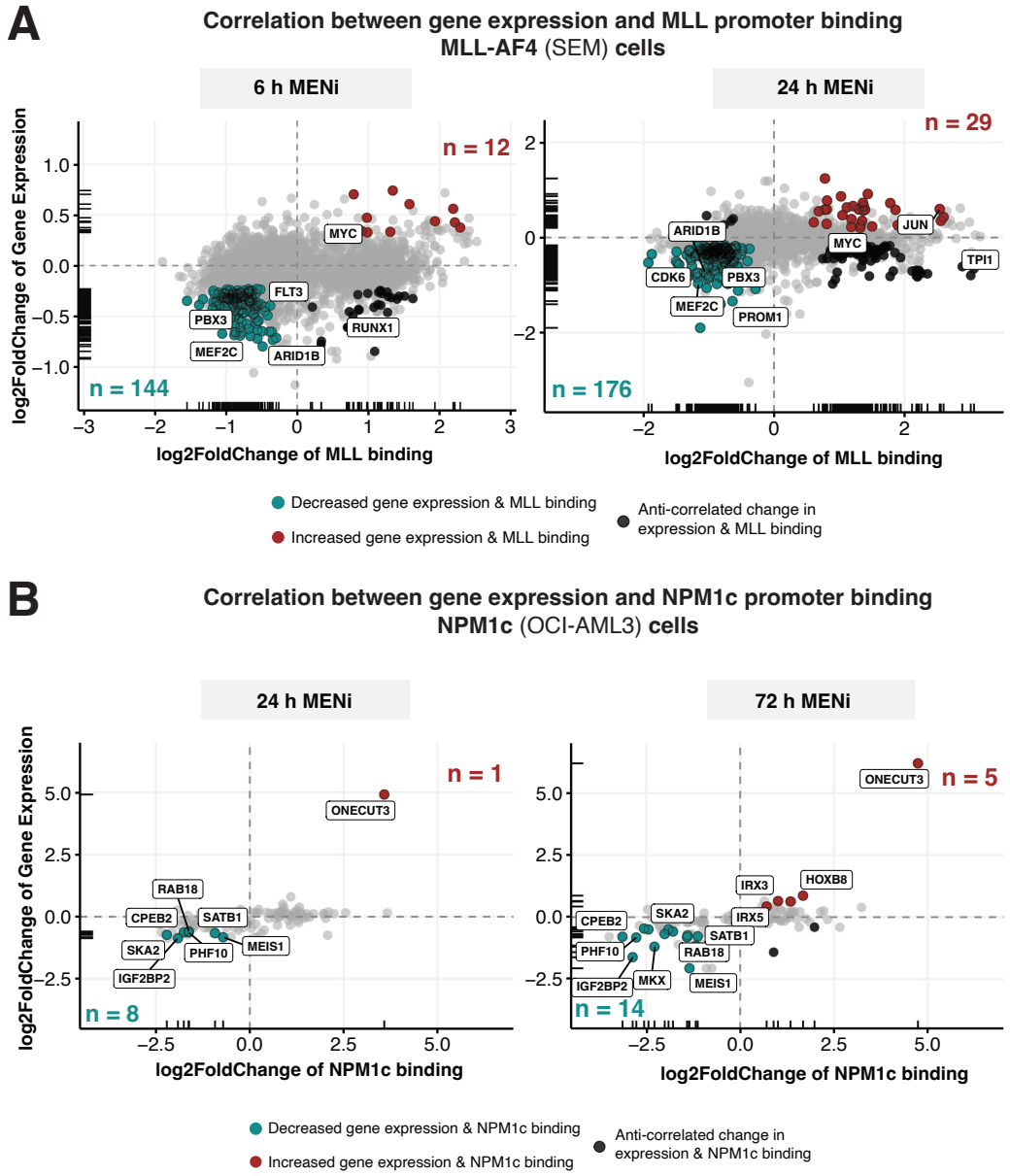


Figure 3.10: *(see next page)*

Figure 3.10 *(previous page)*: Gene expression correlates with MLL or NPM1c binding at a subset of genes. **A.** Scatter plots of changes in gene expression compared to changes in MLL (*i.e.* MLL-AF4) binding at 6 h (left) and 24 h (right) of Menin inhibition in SEM cells. **B.** Scatter plots of changes in gene expression compared to changes in NPM1c binding at 24 h (left) and 72 h (right) of Menin inhibition in OCI-AML3 cells. In both **A** and **B**, genes are coloured if they display a significant alteration in either expression or MLL/NPM1c binding, whereas non-significant genes are shown in grey. Only expressed genes with called MLL or NPM1c peaks are shown.

NPM1c binding at 24 h and 2.4% (14 / 572) at 72 h (Figure 3.10B). The lack of a correlation between NPM1c binding redistribution and transcription changes supports the hypothesis that the NPM1c-Menin-MLL complex is only required for transcription at highly specific target genes, whereas the MLL-AF4-Menin complex regulates a broader set of genes. NPM1c gain also activates highly specific targets, such as the dramatic up-regulation of the One Cut Homeobox 3 (*ONECUT3*) gene at 24 h of Menin inhibition, which was also accompanied by gain of wild-type MLL.

3.3 Discussion

Together, epigenetic and transcriptional profiling of the response of Menin inhibitor-sensitive MLL-AF4 (SEM) and NPM1c (OCI-AML3) cells, plus insensitive E2A-PBX1 cells (RCH-ACV), showed distinct requirements for Menin at its target genes to facilitate transcription. While Menin was bound at the promoters of highly expressed genes in both inhibitor-sensitive cells and insensitive cells, only SEM cells showed a combination of short-term, expansive transcriptional dysregulation and cell death upon loss of Menin from chromatin. In contrast, while Menin inhibition led to down-regulation of target genes in E2A-PBX1 cells after 24 h of treatment, there was no fitness detriment to the cells. NPM1c cells, which lost long-term proliferative capacity in response to Menin inhibition, showed only a mild transcriptional response at a small number of gene targets and no short-term detriment to cell viability at 24 h.

The distinct effects of Menin loss on transcription at its target promoters in MLL-AF4 cells versus NPM1c cells may be linked to the regulatory activity of these oncoproteins. Both NPM1c and various MLL fusions have been linked to transcription activation through their ability to interact with RNA Pol II and elongation factors through multivalent interactions (X. Q. D. Wang et al., 2023; Milne, Dou, et al., 2005; Okuda et al., 2014; Crump et al., 2023). When MLL-FPs are exogenously expressed, they have been shown to induce self-renewal and expression of haematopoiesis genes (Krivtsov et al., 2006), and to activate novel enhancer elements (Pan et al., 2023). The interaction between MLL-FPs and Menin is known to be critical for leukaemic transformation (Caslini et al., 2007; Yokoyama et al., 2005) and for MLL-FP complex stability at promoters (Kerry et al., 2017).

On the other hand, the role of NPM1c on chromatin is less well understood. It remains unclear to what extent NPM1c enhances existing transcription, and thereby drives leukemogenesis by abberantly sustaining the gene expression program in the leukaemic cell of origin, or whether it can also initiate transcription. Wang *et al.* (2023) showed that introducing NPM1c into mouse bone marrow progenitor cells which were immortalized with HOXB8 did not alter the cells' chromatin state or induce transcription of *Hoxa9* and *Meis1*; instead, it led to the up-regulation of genes that were already expressed prior to NPM1c knock-in (X. Q. D. Wang et al., 2023). On the other hand, Uckelmann *et al.* (2020) found that when NPM1c was introduced into sorted mouse Granulocyte-monocyte progenitors (GMP) and LSK cells, *Hoxa9* expression was induced *in vitro* and the cells demonstrated increased self-renewal capacity in colony forming assays (Uckelmann et al., 2020).

The greater disruption to transcription triggered by Menin inhibition in SEM cells compared to OCI-AML3 cells indicates that in complex with the more potent transcriptional activator MLL-AF4, Menin is co-opted as a transcriptional co-activator to a greater extent than when in complex with NPM1c. If the predominant role of NPM1c is to enhance existing transcription at Menin-MLLwt-bound loci, as suggested by Wang *et al.* (2003) (X. Q. D. Wang et al., 2023), destabilising this complex at promoters may be leading to a more gradual dampening of leukaemia-essential genes compared to loss of MLL-AF4, explaining the delay in gene expression changes. Furthermore, Menin inhibition for 24 h was insufficient to trigger the rapid down-regulation of the majority of NPM1c target genes in OCI-AML3 cells, which was observed within 30 - 60 min with NPM1c degradation using the dTag system (Uckelmann et al., 2023). This indicates that a depletion of NPM1c protein levels may be necessary to trigger a short-term transcriptional response

in OCI-AML3 cells, as opposed to solely destabilisation from promoters via Menin inhibition.

Menin inhibition led to a shift in chromatin binding of both MLL and NPM1c in SEM and OCI-AML3 cells. In addition to the expected depletion of MLL and NPM1c binding at some target genes, which has been proposed as the major mechanism by which Menin inhibition causes leukaemia cell death and differentiation (Issa, Ravandi, et al., 2021), I also observed increases in MLL and NPM1c at a fraction of loci. In SEM cells, a mildly increased signal was primarily detected at sites which retained MLL binding, which agrees with observations from Krivtsov *et al.* (2019) that Menin inhibition affects only a subset of MLL-FP target genes (Krivtsov et al., 2019). In OCI-AML3 cells, NPM1c and MLL-wt displayed *de novo* promoter binding at *ONECUT3*, which was associated with its up-regulation. This indicates that MLL and NPM1c binding could be restricted by Menin anchorage at specific loci and in the absence of Menin, changes in affinity for target promoters could lead to differential binding at sites which lack abundant Menin enrichment under normal conditions.

Wild-type MLL gain at new promoters upon Menin inhibition has previously been reported by Sparbier *et al.* (2023) in an investigation of regulators of bivalent genes. They showed that inhibiting Menin causes wild-type MLL binding to be depleted from a subset of active genes and newly acquired at Polycomb-repressed bivalent genes, which triggered their up-regulation (Sparbier et al., 2023). However, in SEM and OCI-AML3 cells, only a small proportion of up-regulated genes showed a corresponding gain of MLL or NPM1c. It is unclear what factors stabilise MLL and NPM1c at these loci in the absence of Menin, or whether these represent lower-affinity binding sites that are increasingly occupied when MLL or NPM1c is released from

Menin-bound genes. It is also uncertain whether this redistribution is important for the cells' response to Menin inhibition. The work by Sparbier *et al.* (2023) showed that MLL gain can augment pluripotent stem cell differentiation (Sparbier et al., 2023), which could point to a related activation of differentiation pathways resulting from MLL, and potentially NPM1c, gain at new promoters in AML Menin-inhibitor induced differentiation.

4 | Identification of Menin protein-protein interactions in distinct leukaemia subtypes

4.1 Introduction

In Chapter 3, I showed that Menin regulates distinct gene sets in NPM1c and MLL-AF4 leukaemia cells, and that the transcriptional response to Menin inhibition is faster and affects more genes in MLL-AF4 cells. In both cell lines, Menin is known to be a component in a complex — interacting with either MLL-AF4 or NPM1c and wild-type MLL1 — of transcriptional activators, enriched at gene promoters. The differences in the transcriptional response to Menin inhibition led me to hypothesise that unique interactions between Menin and additional transcription activators may be implicated in the magnitude of transcriptional disruption.

Protein-protein interactions between Menin and other transcription factors (TFs) have been demonstrated to be important for Menin's function in gene regulation in a context-specific manner (Matkar et al., 2013). Menin can function as either a transcriptional activator or repressor by associating with distinct complexes and thereby regulating their genomic targets (Huang et al., 2012). I therefore reasoned that the specific protein-protein interactions of endogenous Menin in NPM1c AML cells compared to in MLL-AF4 ALL cells may explain the distinct requirements for Menin in transcription regulation.

A caveat to the data that have elucidated some Menin interactions to date is that many experiments were conducted using over-expression systems in HEK293T cells or using in-vitro translated proteins (Poisson, Zablewska, & Gaudray, 2003). Furthermore, the mechanism of some Menin interactions,

such as the suppression of TGF-β and SMAD3 activity (Kaji et al., 2001) have been studied in the context of endocrine tissues, in which Menin has a tumour suppressor function. As a result, further study of the relevant protein-protein interactions between Menin and key leukaemia regulators can resolve its function in specific contexts, such as NPM1c or MLL-FP leukaemia cells. For example, one possibility is that while Menin interactions with RNA Pol II and other members of core transcription complexes may be conserved, the cell-type specific expression of transcription factors may give rise to unique Menin-TF interactions in distinct leukaemias. Alternatively, if Menin has distinct roles in regulating transcription, its association with complexes that regulate transcription may vary. To examine this, complexes which associate with Menin-MLL1/MLL-FPs through weaker or transient interactions also need to be characterised in leukaemia.

4.1.1 The Menin complex in MLL-AF4 cells

Menin-MLL-FP complexes recruit additional transcription factors and co-activators to activate and sustain gene expression. Several of these interactions occur via the MLL fusion partner, which were revealed through affinity purifications of tagged fusion proteins or fusion partners (Lin et al., 2010; Biswas et al., 2011; Yokoyama et al., 2010). For example, multiple fusion partners, including AF4, AF9 and ENL, can recuit AF4 (wild-type) and P-TEFb, both members of the Super Elongation Complex, to promote transcription elongation. In addition, Biswas *et al.* showed that AF4 and AF9 can also associate with TFIID, whereas AF4 uniquely associates with Mediator components (Biswas et al., 2011). TFIID facilitates binding to promoters through the TATA box-binding protein (TBP) subunit and to transcription start sites through the TAF subunits (X. Chen et al., 2021), which implicates the

MLL-AF4 fusion in transcription initiation as well as elongation (Biswas et al., 2011). The Mediator complex can regulate multiple steps in transcription, including initiation via association with TFIID and elongation through the kinase activity of the Mediator subunit CDK8 (Richter et al., 2022; Malik & Roeder, 2010). Mediator is also enriched at enhancers and can facilitate the accumulation of a high concentration of transcription regulators through multiple interactions between the intrinsically disordered domains of Mediator components and other transcription factors (Richter et al., 2022; Zamudio et al., 2019; Boija et al., 2018). Together, these data point to a large assembly of complexes at MLL-AF4 target genes, which suggests that characterising weaker or more transient complex-complex associations, and how they are stabilised, can further elucidate the necessary components of transcription activation in MLL-AF4 cells.

Although the fusion partners of MLL were considered the "effectors" of transcription regulation (Yokoyama et al., 2010; Yokoyama, 2017), key interactions with transcription regulations can also be facilitated by Menin. For example, Myb, a critical factor in normal haematopoiesis (Emambokus et al., 2003) and B-cell development (Thomas, Kremer, Ravichandran, Rajewsky, & Bender, 2005), associates with MLL-FP complexes by interacting directly with Menin (Jin et al., 2010). The interaction between Myb and acetyl-transferases EP300 and CBP is required for the regulation of Myb targets in haematopoiesis (Kasper et al., 2002). This indicates that Menin may also serve as a link between MLL-FPs and EP300 activity via Myb. Similarly, Menin links MLL1 and MLL-FPs with LEDGF (Yokoyama & Cleary, 2008), a co-activator of transcription (Ge, Si, & Roeder, 1998b). The PWWP domain of LEDGF, which can recognise modified H3K36me3 (Maurer-Stroh et al., 2003; Eidahl et al., 2013), was found to be required for leukaemic transformation by MLL-

FPs and for maintaining *Hoxa9* expression (Yokoyama & Cleary, 2008). The interaction of the Menin-MLL complex with LEDGF was shown to depend on the pre-established association between Menin and MLL, implicating Menin as the key link between MLL-FPs and LEDGF. Disrupting the interaction between Menin and MLL-AF4 with Menin inhibitors may therefore also lead to reduced interactions between the MLL-AF4 complex and other co-activators, thereby reducing their activity in transcription activation. Therefore, defining the complexes that interact more transiently with the MLL-AF4 complex, but are stabilised by the association with Menin, can shed light on the mechanisms of gene dysregulation in response to Menin inhibition.

4.1.2 The Menin complex in NPM1c cells

In NPM1c cells, experiments by Uckelmann *et al.* suggest that the interaction between the Menin-MLL1 complex and chromatin-associated NPM1c is unstable. Uckelmann *et al.* (2023) demonstrated that nuclear NPM1c co-immunoprecipates with XPO1, the nuclear export factor which is also enriched at *HOX* loci and required for NPM1c binding at chromatin (Uckelmann et al., 2023). However, MLL1 did not immunoprecipitate with NPM1c, which the authors interpreted as a "transient or indirect interaction" between the two proteins (Uckelmann et al., 2023). Further, NPM1c degradation did not reduce XPO1 or Menin-MLL enrichment at NPM1c-bound genes (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023), indicating that NPM1c is likely recruited to loci where XPO1 and Menin-MLL are already enriched. The synergistic inhibition of XPO1 and Menin further implicates both factors as key stabilizers of NPM1c chromatin binding (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). Menin has previously been shown to interact with XPO1 in mouse embryonic fibroblasts (MEFs) and 293T cells to regu-

late the nuclear levels of β-catenin (Cao et al., 2009), which could suggest that XPO1 can link Menin-MLL1 and NPM1c at NPM1c target genes. In addition to its interaction with Menin-MLL1 and XPO1, the combination of ChIP-seq and the LacO array imaging used by Uckelmann *et al.* and Wang *et al.* respectively revealed that NPM1c associates with RNA Pol II and promotes CDK9 enrichment at its target genes (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). The reduction of H3K27ac at NPM1c target genes associated with NPM1c degradation could also point to a decreased binding of acetyltransferases EP300/CBP at NPM1c loci (Uckelmann et al., 2023). Examining the composition of the Menin interactome in NPM1c cells could clarify why fewer genes respond to Menin inhibition in the short term compared to MLL-AF4 cells.

4.1.3 Chapter aims

Wang *et al.* proposed that NPM1c can facilitate the assembly of transcription activators at its target genes through multivalent interactions with its intrinsically disordered domains and nucleolar localisation sequences (X. Q. D. Wang et al., 2023). MLL-AF4 has been proposed to function similarly to recruit co-activators and link promoters to enhancers (Crump et al., 2023). It is uncertain to what extent Menin's function as a scaffold stabilises multivalent interactions between protein complexes involved in transcription in NPM1c (OCI-AML3) versus MLL-AF4 cells (SEM). In Chapter 2, I showed that while Menin was generally enriched at highly transcribed genes in both NPM1c and MLL-AF4 cells, NPM1c has fewer direct target genes than the MLL-AF4 complex. Consequently, I hypothesised that Menin interacts with distinct protein complexes in NPM1c versus MLL-AF4 cells, and that the Menin complex in MLL-AF4 cells is more enriched for transcription activators than

in NPM1c cells.

To investigate this hypothesis, the aims for this chapter were to:

1. Identify Menin interactors in MLL-AF4, NPM1c and E2A-PBX1 cells.
2. Compare the enrichment of transcription regulators in MLL-AF4 and NPM1c cells.

4.2 Results

4.2.1 Detecting Menin localisation with sub-cellular fractionation

In order to capture proteins associated with Menin, I wanted a system that could detect Menin interactions both on chromatin and under the conditions of Menin inhibition, when Menin is no longer localised on chromatin. To characterise the localisation of Menin under normal and inhibitor conditions, I optimised a cell fractionation protocol to separate chromatin-associated, nucleoplasmic, and cytoplasmic proteins (details in Chapter 2). In untreated cells, Menin is primarily in the chromatin fraction (Figure 4.1A). However, in the presence of Menin inhibitor, Menin shifts from the chromatin fraction to the nucleoplasm, although a portion remains chromatin-associated (Figure 4.1A, C). LEDGF also remains associated with chromatin, indicating that Menin inhibition does not disrupt LEDGF chromatin binding globally. Menin remains stable in the presence of inhibitor — the overall abundance is only decreased to approximately 60% after 24 h of inhibition, indicating that the protein is not degraded upon chromatin dissociation (Figure 4.1B). There was almost no Menin protein in the cytoplasm in either normal or inhibitor conditions (Figure 4.1A), indicating that this fraction could be discarded to reduce noise in immunoprecipitation methods. ChIP-qPCR, a more sensitive method for detecting proteins at specific chromatin loci than western blotting, showed that Menin levels at key genes were lower at 6 h and 24 h relative to 2 h of inhibition.

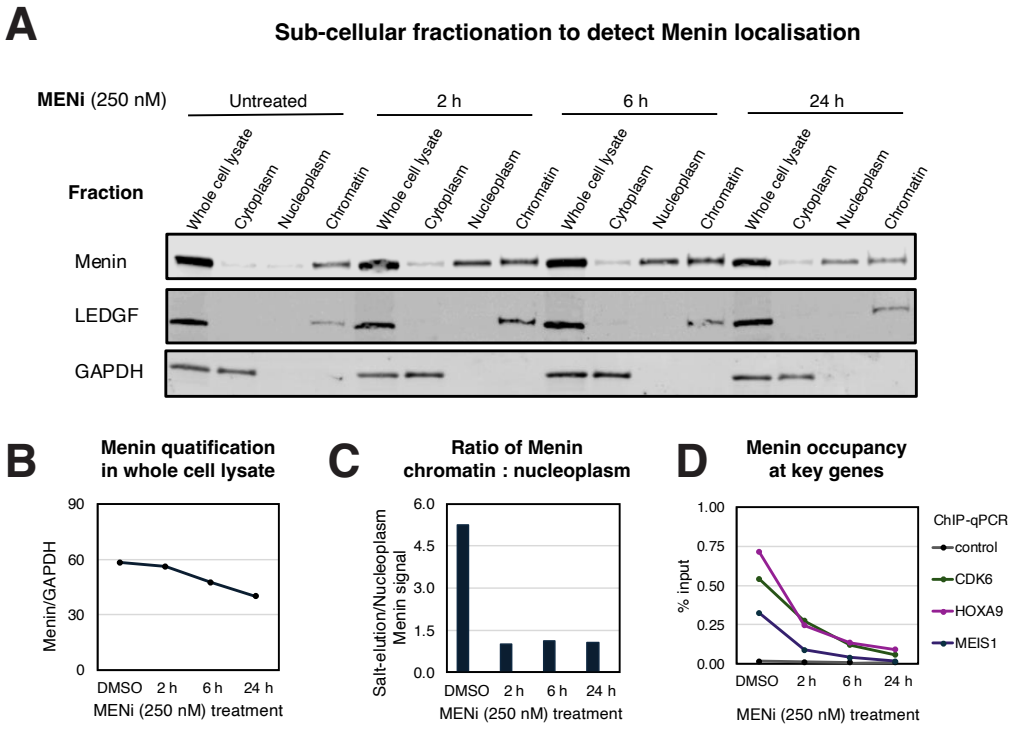


Figure 4.1: Sub-cellular fractionation to detect Menin localisation. **A.** Western blot following fractionation to separate proteins in the cytoplasm, nucleoplasm, and chromatin fractions in SEM cells. LEDGF is shown as a control for a chromatin-bound protein and Menin interactor, and GAPDH as a cytoplasmic control. **B.** Quantification of the Menin signal, normalised to GAPDH signal, in the whole cell lysates in the western blot in **A**. **C.** Quantification of the ratio of Menin signal in the chromatin fraction to the nucleoplasm fraction in the western blot in **A**. **D.** Menin ChIP-qPCR at key Menin targets in SEM cells.

4.2.2 APEX2 proximity labelling for capturing interactions

High purity MLL1/2 complexes containing Menin has been previously described in K562 (Yokoyama et al., 2004), HeLa, and HEK293T cells (Hughes et al., 2004). To build on this, I aimed to capture weaker and more transient interactions, which could reveal additional complexes associated with the MLL-Menin complex.

The first strategy to capture transient protein-protein interactions that I tested was APEX2 proximity labelling. APEX2 is a 27 kDa engineered peroxidase domain which can be fused to a protein of interest, either using gene editing to target the endogenous gene or by transgene expression (Lam et al., 2014). In the presence of H_2O_2, APEX2 catalyses the oxidation of biotin-phenol to a biotin-phenoxyl radical. This short-lived radical reacts with nearby tyrosine residues, resulting in biotinylation of proximal proteins within an estimated radius of approximately 20 nm (Tan et al., 2020). Labelled proteins can subsequently be enriched using streptavidin beads and analysed by mass spectrometry. Unlike classical immunoprecipitation, APEX2 proximity labelling allows for the capture of weak protein-protein interactions in the endogenous context of the cell. The short labelling time of 1 minute also allows for the capture of dynamic processes, such as transient protein-protein interactions.

I used a CRISPR-Cas9-based gene editing strategy (Figure 4.2A) to insert the APEX2 tag in the *MEN1* locus, testing both N-terminus and C-terminus targeting. I chose this approach over exogenous APEX2-tagged Menin overexpression to avoid non-physiological interactions driven by artificially elevated levels of Menin in the cell. In addition, given that Menin is essential in SEM

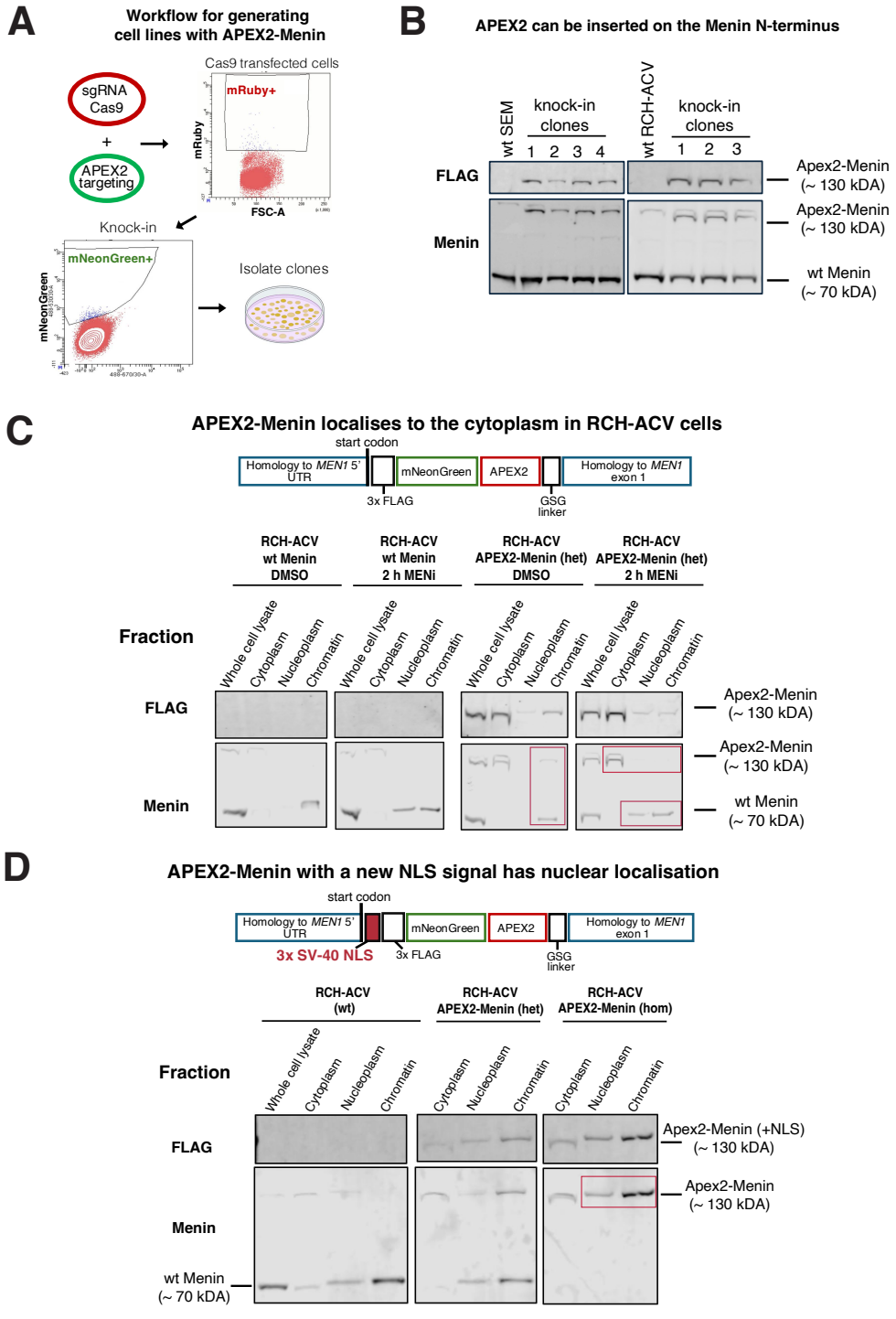


Figure 4.2: *(see next page)*

Figure 4.2 *(previous page)*: Tagging Menin with APEX2 disrupts protein function. **A.** Workflow for inserting the APEX2 domain at the *MEN1* locus to generate APEX2-tagged Menin. FACS plots are from a representative sort with RCH-ACV cells. **B.** Western blot demonstrating both wild-type Menin and APEX2-tagged Menin in heterozygous clones in RCH-ACV and SEM cells. **C.** Diagram of APEX2-Menin N-terminus knock-in strategy and western blot demonstrating wild-type Menin and APEX2-Menin localisation in sub-cellular fractions upon Menin inhibition. Red boxes highlight the comparison between the wild-type Menin (∼70kDa) and APEX2-Menin (∼130kDa) within the same cells. **D.** Modified APEX2-Menin knock-in strategy to include nuclear localisation signal sequences. Western blot of fractionated RCH-ACV cells with wild-type Menin, a heterozygous clone with one copy of APEX2-Menin with additional NLS, and a homozygous clone with two copies of APEX2-Menin with additional NLS.

and OCI-AML3 cells, isolating viable clones with endogenously tagged Menin would indicate that the protein is functional in the presence of the APEX2 tag. I was able to isolate N-terminal, but not C-terminal, tagged clones in SEM and RCH-ACV cells, which were, unexpectedly, all heterozygous (*i.e.* only one *MEN1* allele was successfully edited). The absence of homozygous clones suggested that modifying Menin introduced a fitness detriment to the cells, likely because the addition of the APEX2 domain disrupted an essential wild-type Menin function.

To test the response of the APEX2-Menin protein to Menin inhibition, I treated a heterozygous RCH-ACV (containing the E2A-PBX1 fusion) clone with Menin inhibitor for 2 h, which is sufficient to detect the shift of Menin from the chromatin to the nucleoplasm (Figure 4.1A). Sub-cellular fractionation revealed that most of the tagged Menin protein was mislocalized in the cytoplasm even in the absence of Menin inhibition (Figure 4.2C). Altering the construct to include a triple SV40 nuclear localisation signal (NLS) restored the association with chromatin of the APEX2-Menin protein and

homozygous clones were obtained in RCH-ACV cells (Figure 4.2D). However, only RCH-ACV cells, which are insensitive to Menin inhibition, could be successfully edited. The absence of edited SEM clones, in which Menin is essential, indicated that the APEX2 tag was likely affecting normal Menin function and conferring a survival disadvantage to edited cells. I therefore concluded that the APEX2 system would not be appropriate for assaying the protein-protein interactions of Menin, as normal Menin function had been altered by the introduction of the tag.

4.2.3 Optimisation of immunoprecipitation of endogenous Menin for mass spectrometry

The APEX2 strategy indicated that tagging Menin would be problematic for Menin localisation, which ruled out using an affinity purification method with a tag such as FLAG or HA. Instead, I opted for immunoprecipitation (IP) of the endogenous Menin protein from cell nuclei using an anti-Menin antibody. The cytoplasmic fraction was discarded as it does not contain Menin in either untreated or inhibitor-treated cells, and may have introduced non-specific antibody or Menin interactions in a whole cell lysate.

I tested both a more stringent IP condition with 300 mM KCl and 1% Triton-X100 washes, as well as less stringent IP in 150 mM NaCl and 0.5% NP-40 washes in SEM cells. In the high-salt buffer (300 mM KCl), I was unable to co-immunoprecipitate LEDGF, a known Menin interactor (Yokoyama & Cleary, 2008), whereas in the low-salt nuclear lysis buffer, LEDGF was efficiently co-immunoprecipitated with Menin (Figure 4.3A). These IP and wash conditions were therefore chosen for subsequent experiments and mass spectrometry analysis. As an additional quality check prior to mass spectrometry analysis,

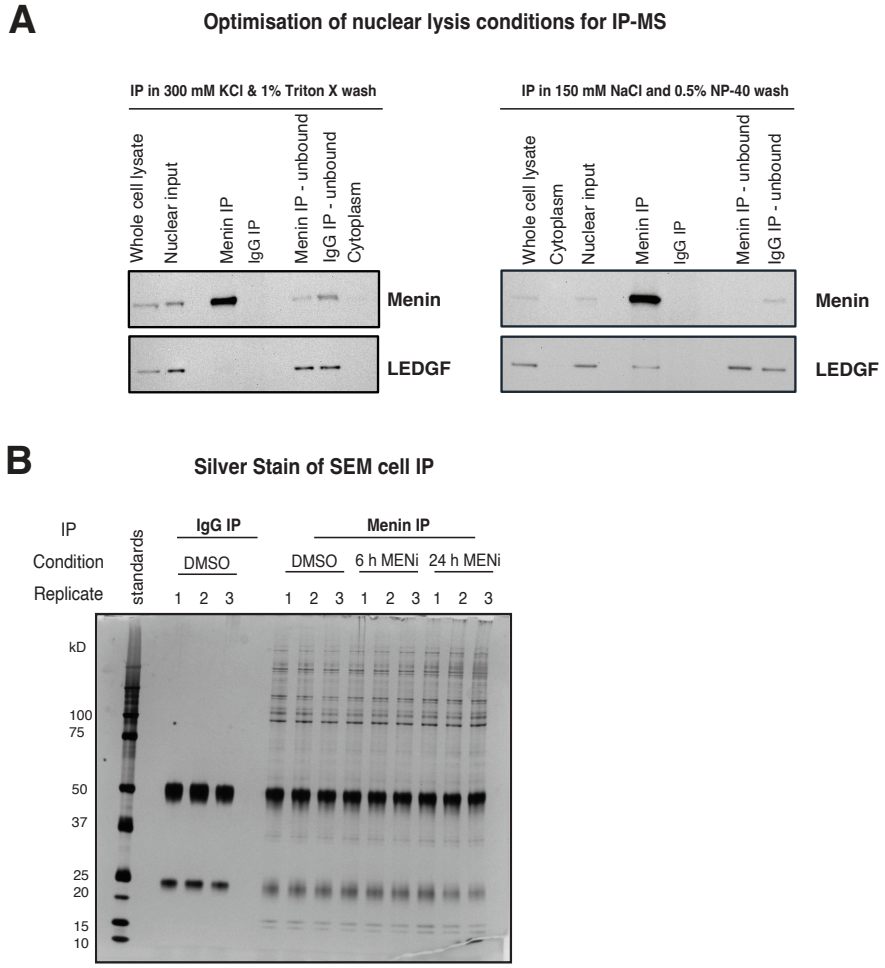


Figure 4.3: Optimisation of Menin IP conditions in SEM cells. **A.** Co-IP of known Menin interactor LEDGF from SEM cells in two different IP buffer conditions. **B.** Silver stain performed as a quality control prior to mass spectrometry analysis.

silver staining was used to confirm the presence of multiple proteins in the endogenous Menin IP, which were absent in the IgG control IP, indicating effective co-immunoprecipitation (Figure 4.3B).

4.2.4 Chromatin complexes and transcriptional machinery are enriched in Menin IP-MS

I conducted the Menin IP in triplicate in MLL-AF4 (SEM), E2A-PBX1 (RCH-ACV), and NPM1c (OCI-AML3) cell lines and analysed the protein content by mass spectrometry (IP-MS). Firstly, to compare Menin interactions across cell lines, I focused on the interactions with Menin under basal conditions, in the absence of inhibitor. To identify high confidence interactors, I used stringent cut-offs for imputation and enrichment over IgG controls. Proteins were excluded from analysis if imputation was required for more than one replicate per Menin IP. A significance threshold of FDR < 0.01 was applied to proteins carried forward for comparison between between cell lines. I identified 2729 proteins in at least one cell line (Figure 4.4A). The majority (1406) of enriched proteins were found in all three cell lines, whereas the second largest set (428) was composed of proteins shared between B-ALL cell lines SEM and RCH-ACV (Figure 4.4B).

In order to compare the protein complexes involved in transcriptional regulation that co-purified with Menin in each cell line, I performed an enrichment analysis for protein complexes available in the CORUM database (Tsitsiridis et al., 2023). Reassuringly, known interactions with the MLL complex and RNA Polymerase II were detected in all three cell lines (Figure 4.4C). While some additional transcription regulators such as the Mediator complex were also enriched in all three cell lines, the FACT (Facilitates Chromatin Tran-

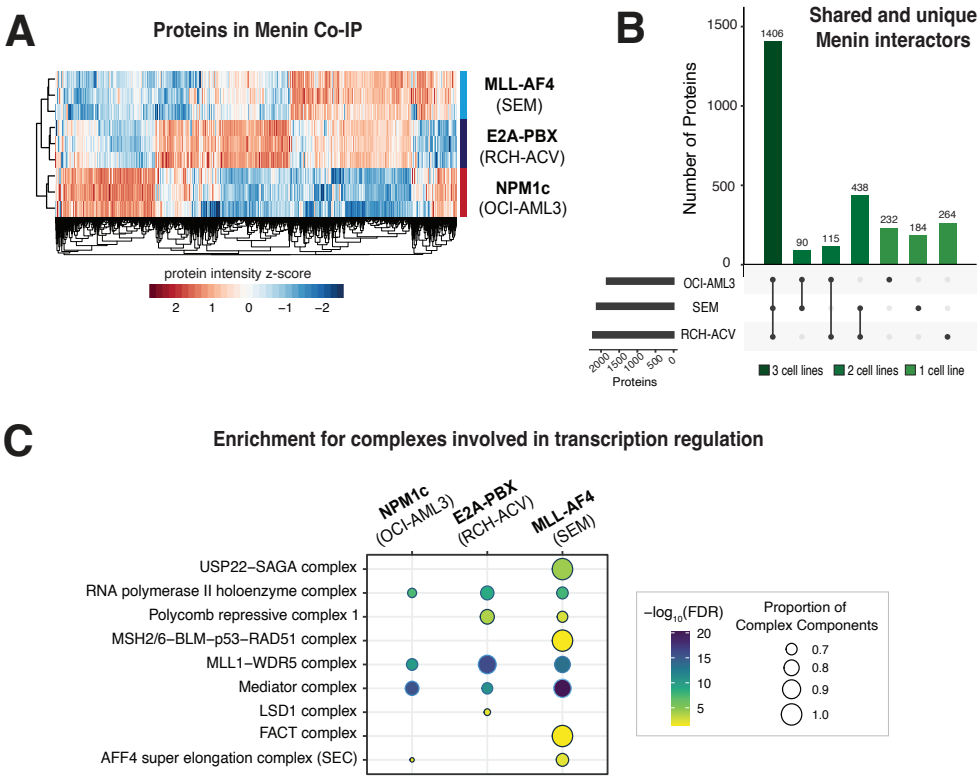


Figure 4.4: Menin associates with transcriptional regulators in different leukaemia cells. **A.** Heatmap of statistically enriched Menin interactors in SEM, RCH-ACV, and OCI-AML3 cells. **B.** UpSet plot representing shared and unique proteins co-immunoprecipitated in the cell line panel. **C.** Global enrichment of select complexes involved in transcription.

scription) complex, SAGA co-activator complex, and the DNA mismatch repair MSH2/6 complex were uniquely enriched in the Menin IP from MLL-AF4 SEM cells. This selective enrichment indicated additional Menin activity in transcriptional activation and elongation, as well as genome maintenance, specifically in the presence of MLL-AF4, which correlates with the stronger transcriptional response to Menin inhibition.

To further evaluate differences in the enrichment of individual factors between cell lines (Figure 4.4B), I performed pairwise comparisons between the fraction of statistically enriched proteins in SEM cells, which showed the strongest transcriptional response to Menin inhibition, and those enriched in RCH-ACV cells and OCI-AML3 cells. This highlighted a greater abundance of IKZF family proteins and EP300 in the SEM Menin interactome (Figure 4.5). Additionally, the transcription factor RUNX2 was similarly enriched in the Menin inhibitor-sensitive SEM (MLL-AF4) and OCI-AML3 (NPM1c) cells, but not absent in the inhibitor-insensitive RCH-ACV cells. Together, this indicates that Menin may interact with transcription factors involved in haematopoietic development and differentiation in a cell-type specific manner.

4.2.5 Menin associates with enhancer-bound proteins

Given recent observations from the Milne lab that MLL-AF4 binds enhancer elements (Crump et al., 2023), I further assessed the Menin IPs specifically for the enrichment of proteins known to bind and activate enhancer elements, summarized in Table 4.1. Enhancer-associated proteins were enriched in the immunoprecipitations across all three cell lines (Figure 4.6), however, EP300, which deposits H3K27-acetylation at promoters as well as enhancers, was specifically enriched in SEM cells. I therefore decided to further explore Menin function at enhancer elements as a possible explanation for the difference in

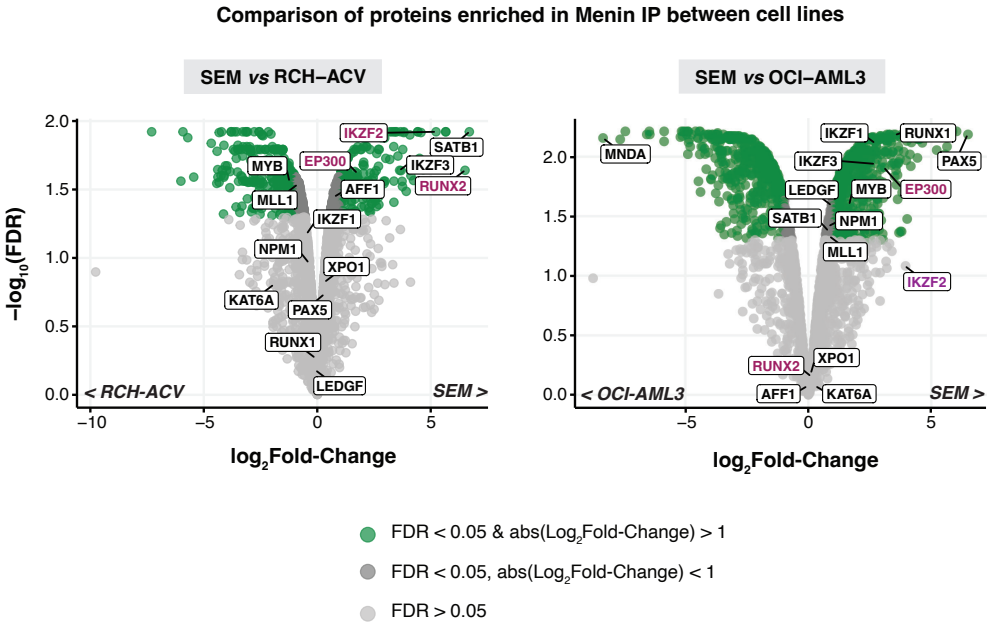


Figure 4.5: Comparison of Menin interactors between cell lines reveals cell-line specific enrichment of transcription factors. Volcano plots show the comparison of statistically enriched proteins SEM cells versus RCH-ACV cells and OCI-AML3 cells. Proteins highlighted in green have an absolute $\log_2$fold change > 1 and FDR < 0.05.

Menin sensitivity in MLL-AF4 ALL cells (SEM) compared to NPM1c AML cells (OCI-AML3), which is described in Chapter 5.

Table 4.1: Enhancer-associated proteins.

Protein	Activity
MLL3 (KMT2C); MLL4 (KMT2D); UTX (KDM6A)	Members of the MLL3/4 complex, which deposits H3K4me1 — a modification enriched at enhancers and depleted at promoters (C. Wang et al., 2016; Lee et al., 2013; D. Hu et al., 2013).
EP300; CREBBP (CBP)	Histone acetyltransferases that deposit H3K27ac, which has been shown to activate poised enhancers (Creyghton et al., 2010).
BRD4	Reader of H3K27ac and enriched at super-enhancers (Qian et al., 2023).
C/EBPα; C/EPBδ	Enhancer-binding proteins that regulate myeloid lineage differentiation (Paz-Priel & Friedman, 2011).
MED1	Component of the Mediator complex, enriched at super-enhancers (Whyte et al., 2013).

4.2.6 Effects of Menin inhibition on Menin interactions

Finally, I aimed to assess the effect of Menin inhibition, when Menin localisation shifts to the nucleoplasm, on the Menin interactome. Cells were cultured with 250 nM Menin inhibitor, and 1 µM inhibitor was added to all IP buffers to minimise dissociation of the Menin and the inhibitor during the IP. However, comparing DMSO and MENi conditions using a moderated t-test did not yield statistically significant differences (data not shown). As an alternative, qualitative comparison, I inspected the raw intensity values of known Menin-MLL complex components in SEM cells in order to investigate

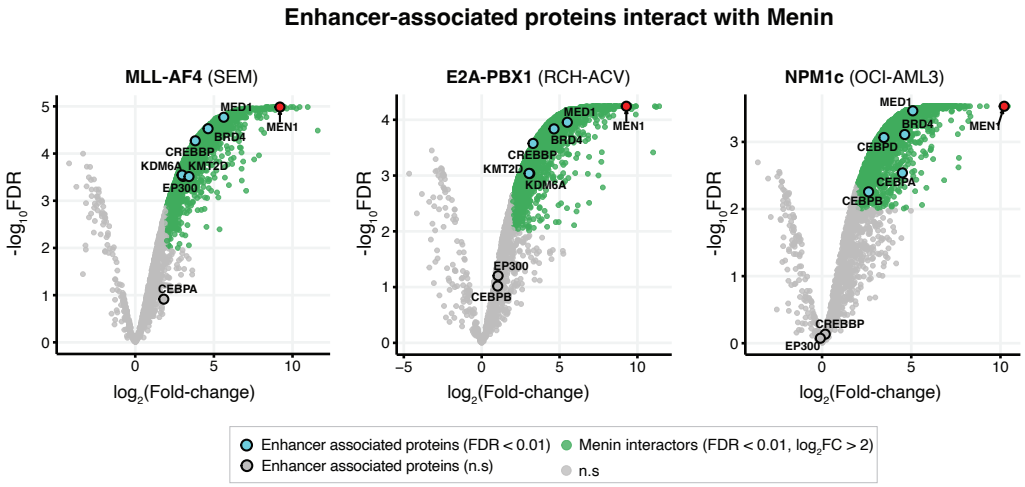


Figure 4.6: Menin associates with enhancer-binding proteins. Volcano plots show labelled enhancer-associated proteins of interest in blue if significantly enriched compared to IgG controls (FDR < 0.01 and log_2 fold change > 2).

whether the expected reduction in interactions could be detected. I focused this analysis specifically on SEM cells, because they exhibit the strongest transcriptional response to Menin inhibition.

I first compared z-scored raw intensity values of proteins annotated as the "Menin-associated histone methyltransferase complex" in the CORUM database (Figure 4.7A) and was able to detect the expected decrease in interaction with these core components in the presence of inhibitor. Comparison of the normalised raw $\log_2$-intensity values (Figure 4.7B) also revealed decreases in MLL1, MLL2 and AF4 in the presence of Menin inhibitor, pointing to weaker interactions with Menin. To compare against this expected decrease in interactions, I focused on a subset of factors that were highly enriched in SEM cells, including enhancer associated proteins EP300, CREBBP (CBP), and KDM6A (UTX), and transcription factors RUNX2 and IKZF2. In contrast to MLL and AF4, although more variable between replicates, the average intensities of enhancer-associated proteins EP300, CREBBP, and KDM6A

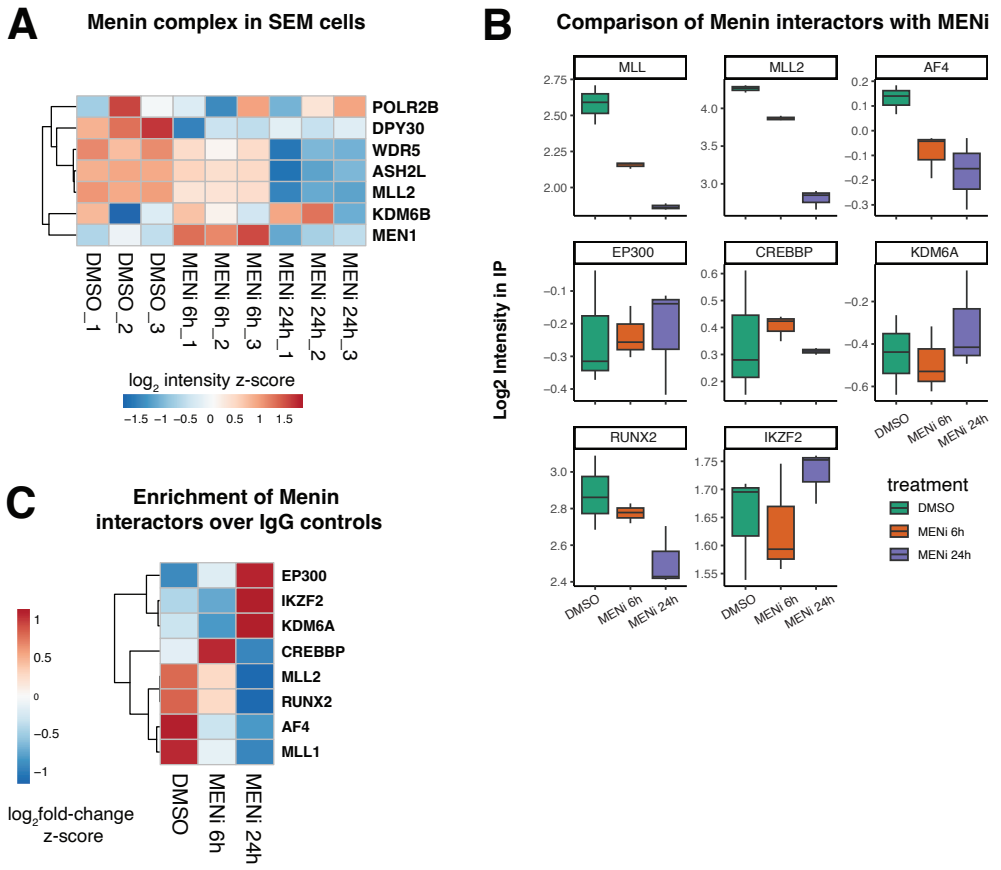


Figure 4.7: Qualitative comparisons of Menin interactors in the presence of inhibitor revealed decreased interaction with MLL/MLL-AF4 complex components and retained association with EP300. **A.** Z-scored $\log_2$ intensity values of Menin complex (CORUM) components in SEM cells treated with DMSO, MENi for 6 h and MENi for 24 h. Three biological replicates per condition are shown. **B.** Normalised raw $\log_2$ intensity values (no z-score) for proteins of interest in SEM cells, grouped by treatment condition. **C.** Z-scored $\log_2$ fold changes for proteins of interest in the IP versus IgG.

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did not decrease (Figure 4.7B). RUNX2 intensity values decreased with Menin inhibition, indicating reduced IP enrichment, whereas IKZF2 values increased, suggesting increased pull-down in the IP. In order to assess enrichment over IgG controls, I also compared the $\log_2$ fold changes, which corroborated greater enrichment in the IP in the presence of inhibitor for EP300, KDM6A, and IKZF2, in contrast to the decreased enrichment of MLL1/2 and AF4 (Figure 4.7C). This could indicate that a subset of factors can continue to associate with Menin in the nucleoplasm in the absence of the MLL-AF4-Menin interaction.

4.3 Discussion

Using endogenous Menin co-immunoprecipitation, I set out to test whether different protein-protein interactions were associated with the differential sensitivity to Menin inhibition of MLL-AF4 (SEM), NPM1c (OCI-AML3) and E2A-PBX1 (RCH-ACV) cells.

The Menin interactomes revealed that transcriptional activators, including FACT (Facilitates Chromatin Transcription) and SEC (Super-elongation complex), were more enriched in the presence of MLL-AF4. Both of these complexes are known to interact with the fusion protein (Crump et al., 2023; Lin et al., 2010). In addition, EP300 was uniquely enriched in SEM cells, which may be due to the association of Menin-MLL-AF4 complex with very transcriptionally active genomic loci. The MLL-AF4 complex has also been shown to bind enhancer elements in SEM cells (Crump et al., 2023), which could explain the greater enrichment of EP300 relative to the cell types with wild-type Menin-MLL. The enrichment of additional transcriptional activators correlated with the rapid and extensive transcriptional dysregulation observed in SEM cells with Menin inhibition. In OCI-AML3 cells, on the other hand, the weaker immediate transcriptional response to Menin inhibition could be attributed to weaker interactions between the Menin-MLL-NPM1c complex and other transcriptional activators, suggesting that Menin has a less direct role in gene activation.

Of the uniquely enriched proteins identified through comparisons between cell lines, the transcription factor RUNX2 was enriched in both SEM and OCI-AML3 cells, whereas IKZF2 was unique to SEM cells. These proteins were of particular interest because other RUNX family and IKZF family

proteins have been implicated in related leukaemias. For example, RUNX1 was demonstrated to be both essential to the MLL-AF4 gene regulatory network and enriched at MLL-AF4-bound enhancers in SEM cells (Harman et al., 2021; Crump et al., 2023). Furthermore, RUNX1 has been previously reported to interact with the wild-type MLL complex and the reciprocal AF4-MLL fusion (Wilkinson et al., 2013). On the other hand, a CRISPR screen in the MLL-AF9 cell line MOLM13 revealed that IKAROS (IKZF1) loss, as well as degradation, synergises with Menin inhibition (Aubrey et al., 2022). However, the regulatory roles for RUNX2 and IKZF2 in MLLr or NPM1c contexts have not been clearly defined. RUNX2 has previously been shown to interact with Menin in the bone marrow cell line ST2, wherein Menin can regulate the activity of RUXN2 in the process of differentiation to osteoblasts (Sowa et al., 2004). Further investigation of whether this regulatory relationship is conserved in leukaemia could examine whether a subset of RUNX2 target genes are also sensitive to the loss of Menin from chromatin and exhibit decreased RUNX2 binding as well.

Based on the assumption that Menin interacts with these TFs when bound to chromatin, using ChIP-seq to test whether RUNX2 and IKZF2 are also re-distributed with Menin inhibition, similarly to MLL-AF4 and NPM1c, could reveal whether there is a functional connection between these factors and Menin binding. While the RUNX2 protein was expressed and enriched in the Menin IP in both SEM and OCI-AML3 cells, the IKZF2 protein was not detected in the OCI-AML3 cells' nuclear proteomes, indicating that the protein is not expressed in this cell line. With a newly available IKZF2-specific degrader (Bonazzi et al., 2023), IKZF2 could be explored as an MLL-AF4 ALL-specific vulnerability.

The immunoprecipitations did not achieve sufficient resolution to distinguish

differential interactions in the presence of Menin inhibitor compared to DMSO with statistical significance. However, a qualitative assessment of the $\log_2$fold changes compared to IgG controls, as well as raw intensity values, for a subset of proteins nonetheless revealed expected decreases in the abundance of MLL1/2 complex components. Therefore, it is possible that an increase in a protein's enrichment under inhibitor conditions, as was the case for EP300, could be reflective of it continuing to associate with Menin in the nucleoplasm, away from its chromatin context. To further explore the possibility that Menin continues to interact with a subset of proteins in the presence of Menin inhibitor, western blotting for candidate proteins, such as EP300, on fractionated lysates could be a targeted approach to detect shifts in the ratio of nucleoplasmic to chromatin-associated protein. However, if other Menin interactors behave like MLL/MLL-AF4 and NPM1c, it is more likely that there is a redistribution of chromatin proteins to new binding sites as opposed an extensive loss of chromatin binding, the former being difficult to detect through a high-throughput screen. Ultimately, a combination of validation with western blotting and ChIP-seq/ChIPmentation with multiple replicates, in order to identify differential binding events with statistical power, would be needed to validate whether Menin inhibition triggers the redistribution of factors other than MLL/MLL-FPs and NPM1c.

4.3.1 Technical caveats to endogenous co-IPs

The endogenous IP conditions used in the co-IP experiments were relatively gentle to ensure weaker interactions were captured, allowing for the pull-down of multiple complex components. Since the more aggressive 300 mM KCl/1% Triton-X100 condition disrupted the known interaction between Menin and LEDGF, I reasoned that using a lower stringency can better retain

physiological interactions. However, this choice could also be considered a technical limitation, as it may have introduced more noise in the mass spectrometry data compared to more stringent lysis conditions by permitting low affinity, non-specific interactions.

The use of rabbit IgG IPs as a negative control was another technical limitation in this experiment, since the off-target binding of the anti-Menin antibody may not replicate by bulk IgG. Ideally, superior negative controls would have been to use the same antibody in syngenic cell lines lacking Menin expression, either through a CRISPR-induced knock-out or an inducible degron system such as dTAG ((Nabet et al., 2018). Alternatively, affinity purification with a FLAG- or HA-tagged Menin protein would have allowed for the use of cell lines containing the wild-type, untagged protein as a negative control. Either options would have allowed me to filter off-target antibody binding by looking for enrichment compared to proteins identified in the absence of Menin. However, my unsuccessful attempts to generate cell lines with APEX2-tagged Menin indicated that Menin was not amenable to tagging in SEM cells, where it is essential. This excluded the option of generating cell lines with FLAG- or FKBP-tagged Menin. However, since these caveats apply to a similar extent in all three cell lines, the strength of this analysis lies in providing qualitative observations about the relative enrichment of protein complexes, as opposed to direct, physical interactions with Menin, in the Menin interactome of each cell line.

4.3.2 Future work

The cell-line specific Menin interactomes could be informative for designing targeted CRISPR screens for cell line-specific transcription factors and chromatin proteins in order to identify mechanisms of resistance or synthetic lethality

with Menin inhibition in MLL-AF4 ALL or NPM1c AML. Initial data from the clinical trial with the Menin inhibitor Revumenib reported that resistance arose in approximately 38% of patients via mutations in Menin that affected the drug's binding pocket (Perner, Stein, et al., 2023). However, resistance to Menin inhibition in the absence of Menin mutations, or mutations in known driver mutations detected in the targeted sequencing panel used by Perner *et al.* (Perner, Stein, et al., 2023; Ross et al., 2019), was also observed, pointing to additional non-genetic resistance mechanisms in patients. CRISPR screens have already revealed how functional interplay between Menin and other chromatin regulators, such as the UTX-MLL3/4 complex (Soto-Feliciano et al., 2023) and the histone acetyltransferase KAT6A (Perner, Rahnamoun, et al., 2023), can mediate sensitivity to Menin inhibitors. MLL3/4 components were identified in all three cell lines, suggesting a conserved interplay between Menin and these H3K4me1 methyltranserase complexes.

In addition to resistance mechanisms, CRISPR screens that specifically target Menin interactors could point to co-dependencies between Menin and other transcriptional regulators that can bring about synergistic benefits if targeted in combination. Various combination treatments with other chromatin targets, such as EP300 and BRD4, both identified in in my interactome dataset, have been explored in AML (Fiskus et al., 2023). However, the context of the cell lineage (AML *vs* ALL), the oncogenic driver stabilized by Menin (an MLL-FP or NPM1c), and the extent of the transcriptional response to Menin inhibition may all influence the extent of synergy. As such, integrating context-specific proteomic data on complex interactions could inform investigations on co-dependencies and synergy with Menin loss.

5 | Regulation of Menin targets via enhancers

5.1 Introduction

5.1.1 Features of enhancers

Oncogenic enhancer activation is a common driver of transcription de-regulation in leukaemias of various subtypes. Enhancers are non-coding, accessible regions of DNA which harbour a high-density of transcription factor binding and can regulate transcription activation at distal genes (Panigrahi & O'Malley, 2021). Gene regulation via enhancers is particularly important to fine-tune expression at key stages in development or in response to stimuli (Buecker & Wysocka, 2012). Transcription factors and co-activators, which include EP300, CBP, elongation factors BRD4 and P-TEFb, and proteins that make up the pre-initiation complex, assemble at enhancer DNA and the activation of distal genes is achieved by the looping of intervening DNA sequence, which brings enhancers in proximity to their target promoters (Heinz, Romanoski, Benner, & Glass, 2015; Deng et al., 2012; Bulger & Groudine, 1999; J. H. Yang & Hansen, 2024). In addition to the enrichment of co-activators, active enhancer elements are characterized by H3K4me1, H3K27ac, and bi-directional transcription of short, bidirectional, unstable RNAs termed eRNAs (R. Andersson & Sandelin, 2020; Haberle & Stark, 2018). Clusters of enhancers occupied by transcription factors, Mediator and BRD4 are considered "super-enhancers" due to their size and ability to activate higher transcriptional levels than other enhancers (Whyte et al., 2013; Lovén et al., 2013; Hnisz et al., 2013). Loops of enhancers and their target promoters are organized into dynamic, higher order structures known as topologically associating domains (TADs),

which are maintained by the proteins CTCF and cohesin (Dixon et al., 2012; Bintu et al., 2018).

5.1.2 Oncogenic enhancer activity in leukaemia

Reprogramming of enhancer elements in haematological malignancies has been reported through multiple mechanisms (Bhagwat, Lu, & Vakoc, 2018). At the level of DNA sequence, different mutations can alter enhancer activity. For example, chromosomal translocations can bring oncogenes into new proximity with enhancers in multiple myeloma and T cell acute lymphoblastic leukaemia (T-ALL). In multiple myeloma, a plasma cell malignancy, chromosomal rearrangements can place the oncogene *MYC* in proximity to super-enhancers, such as those regulating expression of the *IGH* locus, and trigger increased *MYC* expression (Affer et al., 2014). In T-ALL, which is a T cell malignancy, t(1;14) translocations can place the transcription factor *TAL1* under the regulatory control of T-cell receptor enhancers (Begley et al., 1989). Alternatively, copy-number variation can amplify enhancer sequences; an example is the amplification of a distal enhancer cluster which comes into contact with *MYC*, 1.7 Kb away, found in AML (J. Shi et al., 2013). Sequence variants can create new binding sites for transcription factors. In T-ALL, novel MYB binding sites have been reported as a result of indels 8 kb upstream of *TAL1* (Mansour et al., 2014) and a SNP 4 kb upstream of the *LMO1* TSS (Z. Li et al., 2017). Both of these events lead to the formation of a novel enhancer that up-regulates *TAL1* or *LMO1*, respectively.

Beyond DNA sequence alterations, an alternative mechanism for enhancer deregulation is perturbation of proteins that regulate chromatin. For example, mutations in the DNA methyltransferase *DNMT3A* or the demethylase *TET2* in AML can trigger oncogenic enhancer activation or decreased enhancer

activity regulating tumour suppressor genes, respectively (L. Yang et al., 2016; Rasmussen et al., 2015). Finally, disruption of TAD organization due to mutations in cohesin proteins can also rearrange chromatin accessibility and the genomic distribution of key TFs involved in haematopoiesis (Kon et al., 2013).

Beyond the recognition that co-opted enhancer elements can be a driver of leukemogenesis, differences in enhancer usage can have clinical implicates within leukaemia subtyptes. For example, genome-wide enhancer profiling has also been used to classify AML into subgroups, which then identified a sensitivity to an RARα agonist, a therapy typically specific to acute promyelocitic leukaemia, resulting from a *RARA* super-enhancer in a subset of patients (McKeown et al., 2017). Adaptive enhancer remodeling has also emerged as a key mechanism driving clonal selection, which triggers disease relapse (Fennell et al., 2022; S. Li et al., 2016). In development, enhancer switching is a process whereby distinct enhancers regulate the same genes in stem or progenitor cells compared to differentiated cells (Kieffer-Kwon et al., 2013). This developmental process can be hijacked in leukemia to drive therapy resistance in the absence of new genetic alterations. For example, the activation of an enhancer controlling the expression of *ABCB1*, a drug efflux pump, as part of a stress-response pathway has been implicated in resistance to chemotherapy in AML (Williams et al., 2019). In contrast, Fong *et al.* (2015) showed that non-genetic resistance to BET inhibition can emerge in the absence of drug efflux, whereby beta-catenin sustains the expression of BRD4 targets such as *Myc* in the absence of BRD4 binding, suggesting that alternative transcription factors can compensate for loss of co-activator activity in MLL-AF9 cells (C. Y. Fong et al., 2015).

Enhancer switching can also be therapeutically modulated with the goal

of overcoming drug resistance. Bell *et al.* (2019) showed that inhibition of the histone demethylase LSD1 generated novel BRD4-bound enhancers, which re-sensitized resistant AML to BET inhibition (Bell et al., 2019). This indicates that understanding the regulation of enhancers and enhancer usage can uncover vulnerabilities in relapsed or treatment-resistant leukemia.

Given that non-genetic adaptations are suspected in up to 40% of relapsed or therapy-resistant cancer cases (Marine, Dawson, & Dawson, 2020), novel therapies that can inhibit or revert the advantage of transcriptional plasticity are being explored. However, our understanding of how transcription regulation via enhancers is regulated remains incomplete. Specifically, it is unclear what mechanisms drive and maintain enhancer-promoter proximity and to what extent this is important for the maintenance of transcription. For example, inactivation of co-activators BRD4 or Mediator has minimal effects on enhnacer-promoter interactions. BET inhibition, despite abrogating transcription, has no impact on enhancer-promoter contacts (Crump et al., 2021), while depletion of Mediator causes only a mild reduction in interactions (Ramasamy et al., 2023). Additionally, inducible degradation of CTCF, which is generally important for maintaining TAD structure, was shown to deregulate only a few hundred genes in the MLL-AF4 SEM cell line despite the disruption of TAD boundaries (Hyle et al., 2019). Notably, the *MYC* gene was especially susceptible to the loss of proximity to a distal enhancer cluster, specifically in SEM cells. Identifying key genes that are sensitive to the loss of enhancer proximity, and methods to disrupt this, could therefore be a potential therapeutic strategy.

5.1.3 Prior evidence for MLL-AF4 as a regulator of enhancer activity

Aberrant enhancer activation has been implicated as a key driver of *MLL*r leukaemia. In addition to promoter binding, the MLL fusion proteins MLL-AF9 and MLL-AF4 have been shown to bind enhancers and co-operate with transcription factors, such as RUNX1, at these sites to activate gene expression (Prange et al., 2017; Crump et al., 2023). Milan *et al.* (2022) demonstrated that the enhancers which emerged upon transformation in an MLL-AF9 model were also present in human CD34+ haematopoietic stem and progenitor cells (Milan et al., 2022). Additionally, Pan *et al.* (2023) showed that introducing the MLL-AF10 oncogene into mouse cells triggered the emergence of novel enhancers, which were linked to the activation of AML-associated genes such as *Myb* (Pan et al., 2023). These observations extend the hypothesised mechanism of action of MLL-FPs beyond promoting transcriptional elongation through recruitment of the SEC complex to potentially activating distal regulatory sites. As a result, modulating inappropriate enhancer activation is emerging as a potential therapeutic avenue.

The Milne lab has demonstrated that the activity of MLL-AF4-bound enhancers is sensitive to the loss of various MLL-AF4 complex components. Godfrey *et al.* (2019) identified a subset of H3K79me-marked enhancer elements, many of them intragenic, in MLL-AF4 cells, which lost H3K27ac and promoter proximity upon DOT1L inhibition (Godfrey et al., 2019). Subsequently, Crump *et al.* (2023) showed that the MLL-AF4 oncoprotein is also enriched at both intragenic and intergenic enhancers, at which it recruits ENL, and the FACT and PAF1 complexes (Crump et al., 2023). Similarly to DOT1L inhibition, loss of either FACT or PAF1 complex components

through inducible degradation resulted in decreased enhancer-promoter proximity and H3K27ac. DOT1L-catalysed H3K79me2/3, FACT and PAF1 have traditionally been associated with transcription elongation (Mueller et al., 2007; Orphanides, LeRoy, Chang, Luse, & Reinberg, 1998; Kim, Guermah, & Roeder, 2010), but this data suggests that they can gain enhancer-activating properties in presence of MLL-AF4. As such, I hypothesised that MLL-AF4 may also co-opt Menin for driving enhancer activation.

5.1.4 A role for Menin in regulating enhancers

The absence of an MLL-FP does not necessarily preclude the possibility of a regulatory role for Menin at enhancers in the context of wild-type MLL, as in NPM1c cells. Some evidence for this has emerged in breast and prostate cancers. Dreijerink *et al.* (2017) detected Menin binding at a subset of enhancers in the ER+ breast cancer cell line MCF-7, at which it likely stabilizes FOXA1 binding (Dreijerink et al., 2017). When comparing naïve and treatment-resistant prostate tumours, Terranova *et al.* (bioRxiv 2021) observed the emergence of enhancers marked by H3K4me1 that were sensitive to Menin inhibition with MI-503 (Terranova et al., 2021). Transcription factors FOXA1, HOX13, and AR, which drive prostate tumourigenesis, were also enriched at these enhancers. While Menin is not thought to be a part of the MLL3/4 complex, which catalyses H3K4me1 at enhancer elements (Nuland et al., 2013), this disruption of H3K4me1-marked enhancers by Menin inhibition may not result from a direct inhibition of MLL3/4 complex activity, but rather a re-distribution of MLL3/4. The indirect dysregulation of MLL3/4 by Menin inhibition with MI-503 has been described in an MLL-AF9 model of AML, in which redistribution of MLL3/4-UTX activated genes regulating cell cycle and senescence (Soto-Feliciano et al., 2023). Whether Menin can

regulate enhancer activity directly or indirectly in NPM1c cells has not yet been explored.

5.1.5 Chapter aims

The susceptibility of MLL-AF4-bound enhancers to the loss of multiple MLL-AF4 complex components as well as H3K79-methylation suggests that a high density of multivalent interactions may be necessary to support enhancer activity in MLL-AF4 cells (Crump et al., 2023; Ruthenburg, Li, Patel, & David Allis, 2007). Wang *et al.* (2023) also propose a model whereby NPM1c assembles transcriptional regulators at its target genes through multivalent interactions (X. Q. D. Wang et al., 2023). NPM1c retains the intrinsically disordered domains and one of the two nucleolar localisation signals of the wild-type protein, which have been shown to promote the formation of condensates similar to transcriptional hubs (Mitrea et al., 2018; Cho et al., 2018). Wang *et al.* showed that NPM1c, Menin and RNA Pol II can be precipitated from condensates and that an NPM1c-LacI fusion could recruit the C-terminus of RPB1, an RNA Pol II subunit, to an integrated LacO locus (X. Q. D. Wang et al., 2023). These observations led the authors to propose that NPM1c may act as a scaffold protein, stabilising transcriptional hubs at the promoters of its target genes. NPM1c was also detected at some distal loci, though its function at enhancers or its co-operation with Menin at these sites was not explored.

Given that multivalency may be a key mechanism for transcription regulation in both MLL-AF4 and NPM1c cells, and Menin's ability to stabilise complexes on chromatin, I set out to test whether Menin played a regulatory role at enhancer elements in these cells. Given differential transcriptional responses to Menin inhibition and the enrichment for EP300 in the statistically significant

Menin interactome in MLL-AF4 but not NPM1c cells, I hypothesised that Menin may have distinct activity at enhancers in these two contexts. To test this, I used a combination of ChIP-seq, RNA-seq and high resolution Micro-Capture-C to evaluate the effects of Menin inhibition at enhancers.

The aims for this chapter were to:

1. Evaluate the effects of Menin inhibition on enhancer H3K27ac and eRNA in MLL-AF4 (SEM) and NPM1c (OCI-AML3) cells.
2. Evaluate the effects of Menin inhibition on enhancer-promoter proximity in MLL-AF4 (SEM) and NPM1c (OCI-AML3) cells.

5.2 Results

5.2.1 Menin is enriched at enhancer elements

To determine if Menin is enriched at enhancers in SEM, RCH-ACV and OCI-AML3 cells, I used a combination of ATAC-seq and ChIP-seq to define active enhancers and promoters in each cell line. Active promoters were designated as regions with open chromatin and H3K27ac within 2 kb of an annotated TSS, whereas enhancers were defined as regions with open chromatin and H3K27ac more than 2 kb away from a TSS. With this characterisation method, there was a strong correlation with expected patterns of H3K4me3 enrichment at promoters and H3K4me1 enrichment at enhancers in all three cell lines (Figure 5.1). As observed in Chapter 3, regardless of sensitivity to Menin inhibition, Menin was enriched at active promoters. To a lesser extent, Menin binding was also observed at intergenic and intragenic enhancers in all three cell lines, regardless of sensitivity to Menin inhibition. SEM cells, however, had higher average Menin enrichment at intragenic enhancers, relative to the signal at intergenic enhancers within the same cells, compared to OCI-AML3 and RCH-ACV cells.

Overall, the genome-wide correlation between Menin and H3K27ac was also highest in SEM cells (Spearman $r = 0.84$) (Figure 5.2). Given that changes in transcription following Menin inhibition are not detrimental to RCH-ACV cell viability, I focused my remaining analysis on the differences between SEM and OCI-AML3 cells, with the aim of exploring whether Menin's role at enhancer elements could be a possible difference in the mechanisms by which Menin regulates genes in these cells. Comparing the correlation between H3K27ac and Menin specifically at putative enhancers revealed a similar

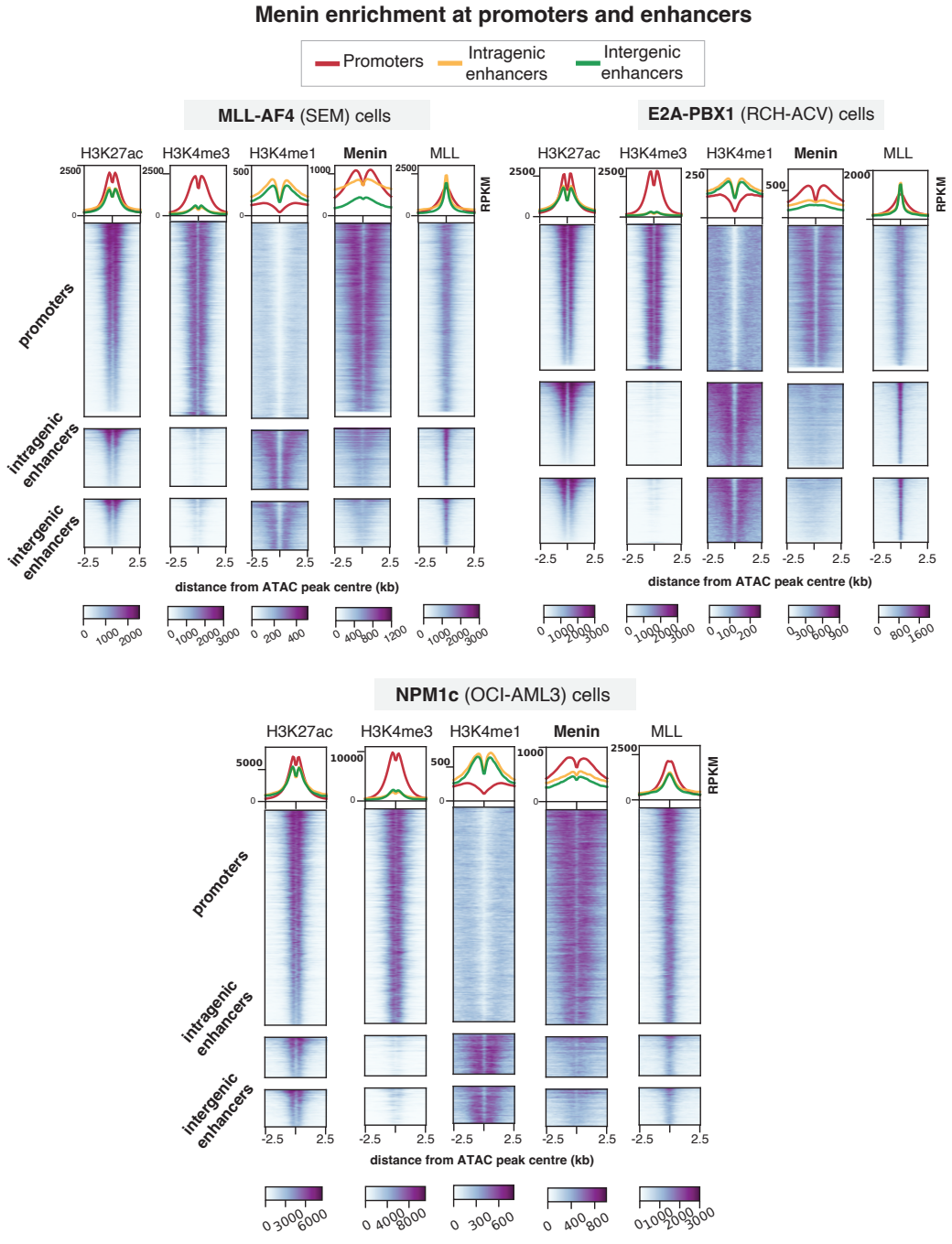


Figure 5.1: *(see next page)*

Figure 5.1 *(previous page)*: Menin is enriched at intragenic and intergenic enhancers as well as promoters. **A.** Heatmaps of Menin and MLL ChIP-seq signal alongside histone marks enriched at promoters and enhancers in SEM, RCH-ACV and OCI-AML3 cells. Regions are sorted by H3K27ac signal for each cell line.

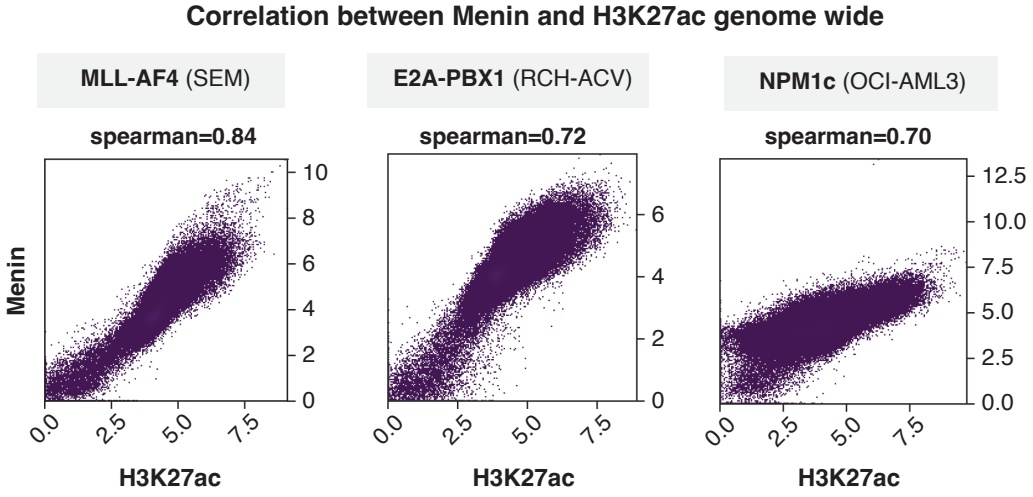


Figure 5.2: Spearman correlations of genome-wide, binned Menin and H3K27ac signal in SEM, RCH-ACV and OCI-AML3 cells.

trend in SEM and OCI-AML3 cells. At Menin-bound enhancers, there was a stronger correlation with H3K27ac in both cell lines, although this was slightly higher in SEM cells at intragenic enhancers (Figure 5.3A).

In SEM cells, the average Menin enrichment at intragenic enhancers was higher than at intergenic enhancers and more similar to the signal at promoters (Figure 5.1). On the other hand, OCI-AML3 cells had more similar Menin levels at intragenic and intergenic enhancers, both of which were lower than at promoters. Given these differences, I hypothesised that there could be an association between Menin enrichment at intragenic enhancers and the transcriptional response to Menin inhibition at the corresponding gene.

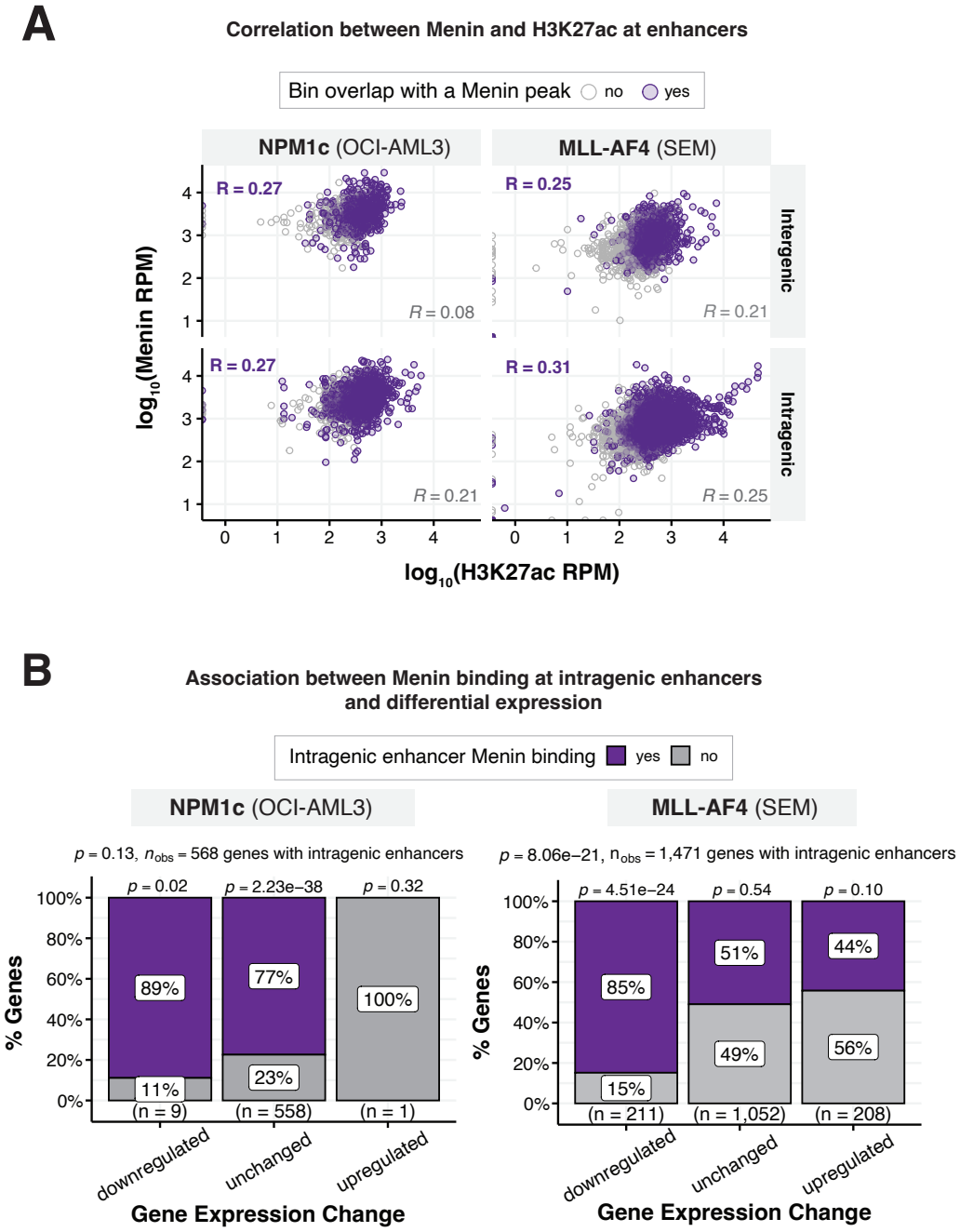


Figure 5.3: *(see next page)*

Figure 5.3 *(previous page)*: Menin-bound intragenic enhancers are associated with down-regulated genes following Menin inhibition in SEM cells. **A.** Correlations between Menin and H3K27ac binned ChIP-seq signal at enhancer elements in OCI-AML3 and SEM cells. Data in purple corresponds to enhancers that overlap with a Menin peak. R values represent the Spearman correlation, in purple at Menin peaks and in grey for non-Menin peaks. **B.** Genes with intragenic enhancers in SEM and OCI-AML3 cells annotated with Menin binding at the intragenic enhancer and sorted by differential expression at 24 h of Menin inhibition. ρ-values are reported from a Pearson chi-squared test.

To test this, I intersected the list of all genes harbouring intragenic enhancers with differentially expressed genes after 24 h of Menin inhibition in the inhibitor-sensitive cells. Menin-bound enhancers were specifically enriched in the down-regulated gene set in SEM cells (Figure 5.3B). In OCI-AML3 cells, Menin-bound intragenic enhancers were also common among genes that did not alter expression in response to Menin inhibition.

To confirm whether these patterns of intragenic Menin binding were also found in MLL-AF4 and NPM1-mutated leukaemia patients, I assessed Menin binding patters in the infant ALL patients with MLL-AF4 and the adult NPM1c AML patient (Figure 5.4A.) Proportionally to promoter peaks within the same sample, the MLL-AF4 infant patient cells tended to have more Menin peaks in intragenic regions 3 kb or more away from a TSS compared to SEM cells, in which a higher proportion of Menin peaks were found within 2 kb of promoters. OCI-AML3 cells and the NPM1c AML patient sample had similar Menin binding profiles, with most peaks at promoters or proximal intragenic regions.

As ATAC-seq data was unavailable for the infant ALL samples, H3K27ac ChIP-mentation was instead used to identify active promoters and putative

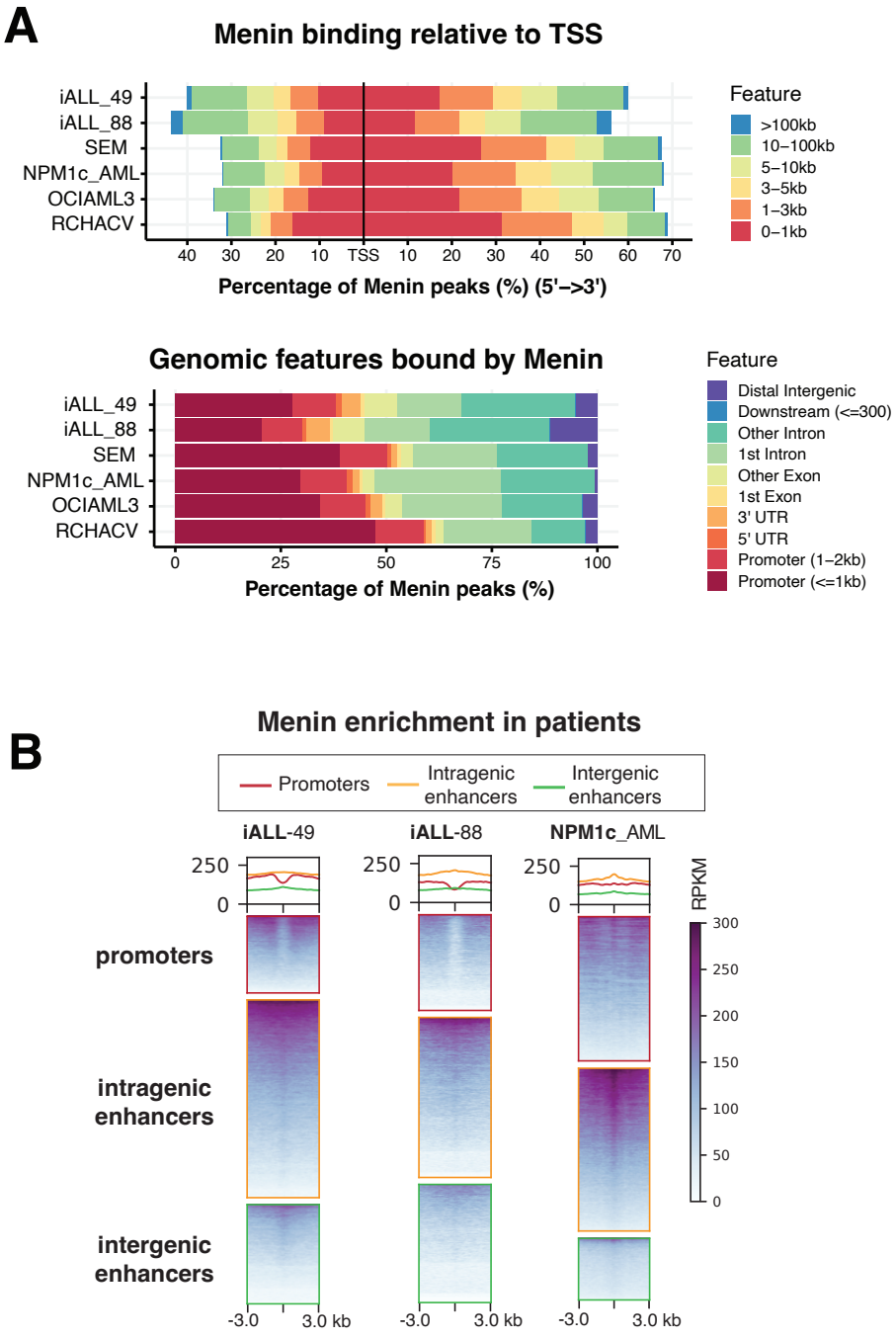


Figure 5.4: *(see next page)*

Figure 5.4 *(previous page)*: Menin binding follows similar distributions in cell line models and patient samples. **A.** Distribution of Menin binding sites relative to TSS (top) and at annotated genomic regions (bottom). **B.** Heatmaps of Menin binding at promoters and enhancers in two infant ALL patients (iALL) and one NPM1c AML patient.

enhancers based on feature annotation and distance to the nearest TSS. The metaplot analysis of the mean Menin signal showed that, in contrast to the cell lines, Menin binding was more enriched at intragenic regions compared to promoters (Figure 5.4A). However, this may be an effect of noisier ChIP-mentation data in patient samples relative to ChIP-seq performed in cell lines (Figure 5.1A), which has a higher signal-to-noise ratio.

To assess the effects of Menin inhibition on Menin-bound enhancers, I compared H3K27ac levels at intragenic and intergenic enhancers stratified by differential gene expression following Menin inhibition for 24 hours. Intergenic enhancers were linked to the gene with the nearest TSS. In SEM cells, H3K27ac was specifically reduced at Menin-bound intragenic enhancers within down-regulated genes, although the effect was relatively subtle (Figure 5.5A). In contrast, up-regulated genes showed, on average, increased H3K27ac at both intragenic and intergenic enhancers, indicating that these enhancers may be promoting gene activation. In OCI-AML3, the average H3K27ac data at non-Menin bound enhancers is noisier due to the low number of genes in the respective categories (*i.e.* a total of 31 up-regulated genes). Despite this, OCI-AML3 cells also exhibited a decrease in H3K27ac at both Menin-bound intragenic and intergenic enhancers associated with down-regulated genes (Figure 5.5B). In both cell lines, H3K27ac levels remained unchanged at genes that did not alter expression. Overall, these data indicate that acetylation changes induced by Menin inhibition at enhancers are linked to transcription

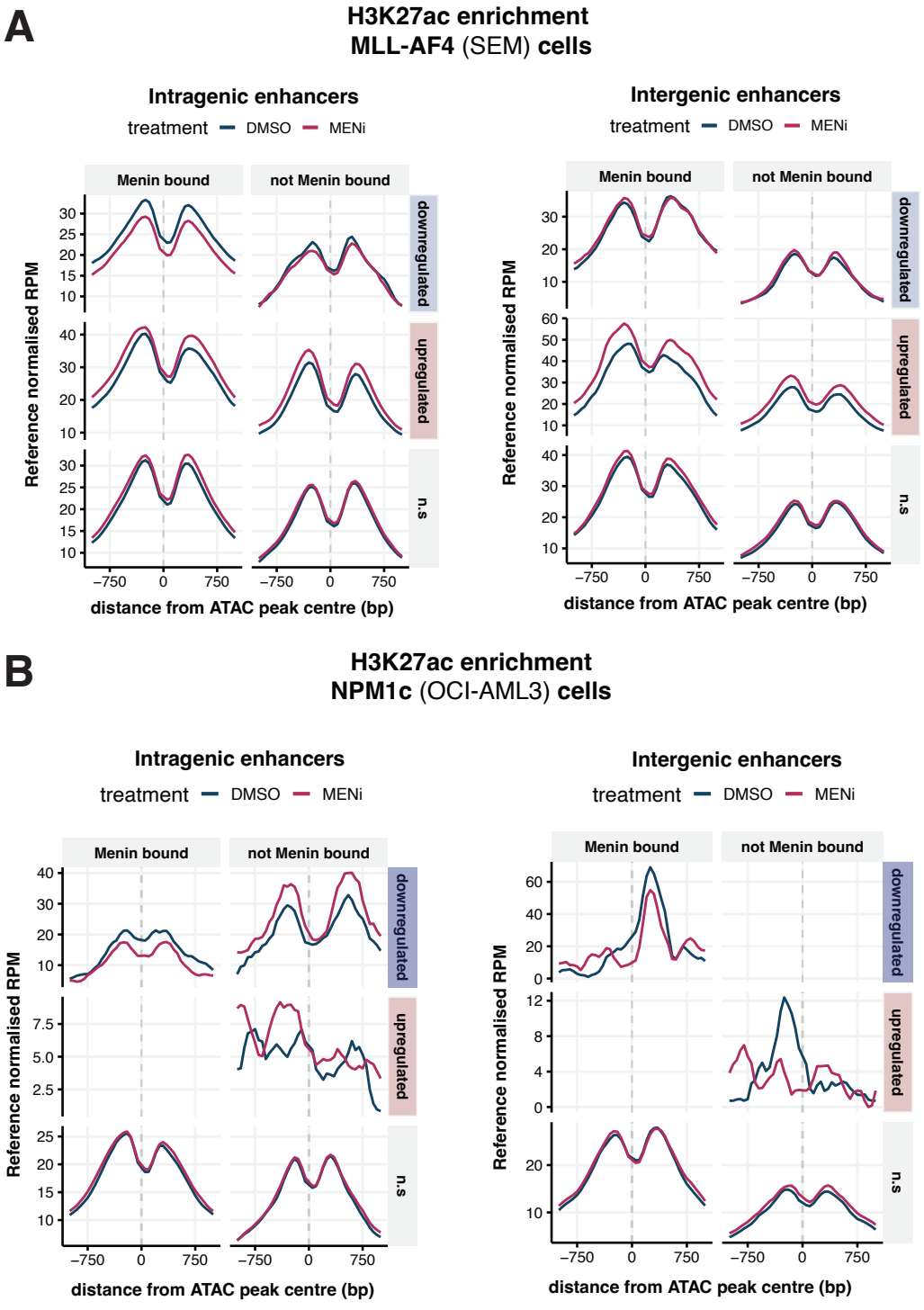


Figure 5.5: *(see next page)*

Figure 5.5 *(previous page)*: Menin inhibition is associated with reduced H3K27ac at intragenic enhancers. Metaplots representing mean H3K27ac at intragenic (left) and intergenic (right) enhancers at differentially expressed genes (vertical annotation) at 24 h of Menin inhibition in **A.** SEM cells and **B.** OCI-AML3 cells. "n.s" indicates non-significant genes from the differential gene expression analysis. Empty panels are due to the absence of genes in this category.

changes.

The effect of Menin inhibition specifically on intragenic enhancers in SEM cells was surprising given that Crump *et al.* (2023) showed disruption of the MLL-AF4 complex affects both intergenic and intragenic enhancers (Crump et al., 2023). To confirm that Menin inhibition primarily affected intragenic enhancers, I analysed MLL-AF4 enrichment at enhancer elements with and without Menin enrichment in SEM cells. Similarly to H3K27ac, MLL-AF4 was specifically reduced at intragenic enhancers and retained at intergenic enhancers, indicating that Menin is not required at intergenic MLL-AF4-bound enhancers (Figure 5.6A).

Further, the effects of Menin inhibition on bi-directional transcription of short enhancer-associated RNAs (eRNA) — another feature of active enhancers — at intergenic Menin-bound enhancers was also very mild, even at down-regulated genes (Figure 5.6). This analysis is not possible for intragenic enhancers due to the overlap with gene transcription, making it impossible to distinguish eRNAs. Together, these data indicate that Menin specifically maintains MLL-AF4 binding and H3K27ac at potential cis-regulatory elements within gene bodies in SEM cells.

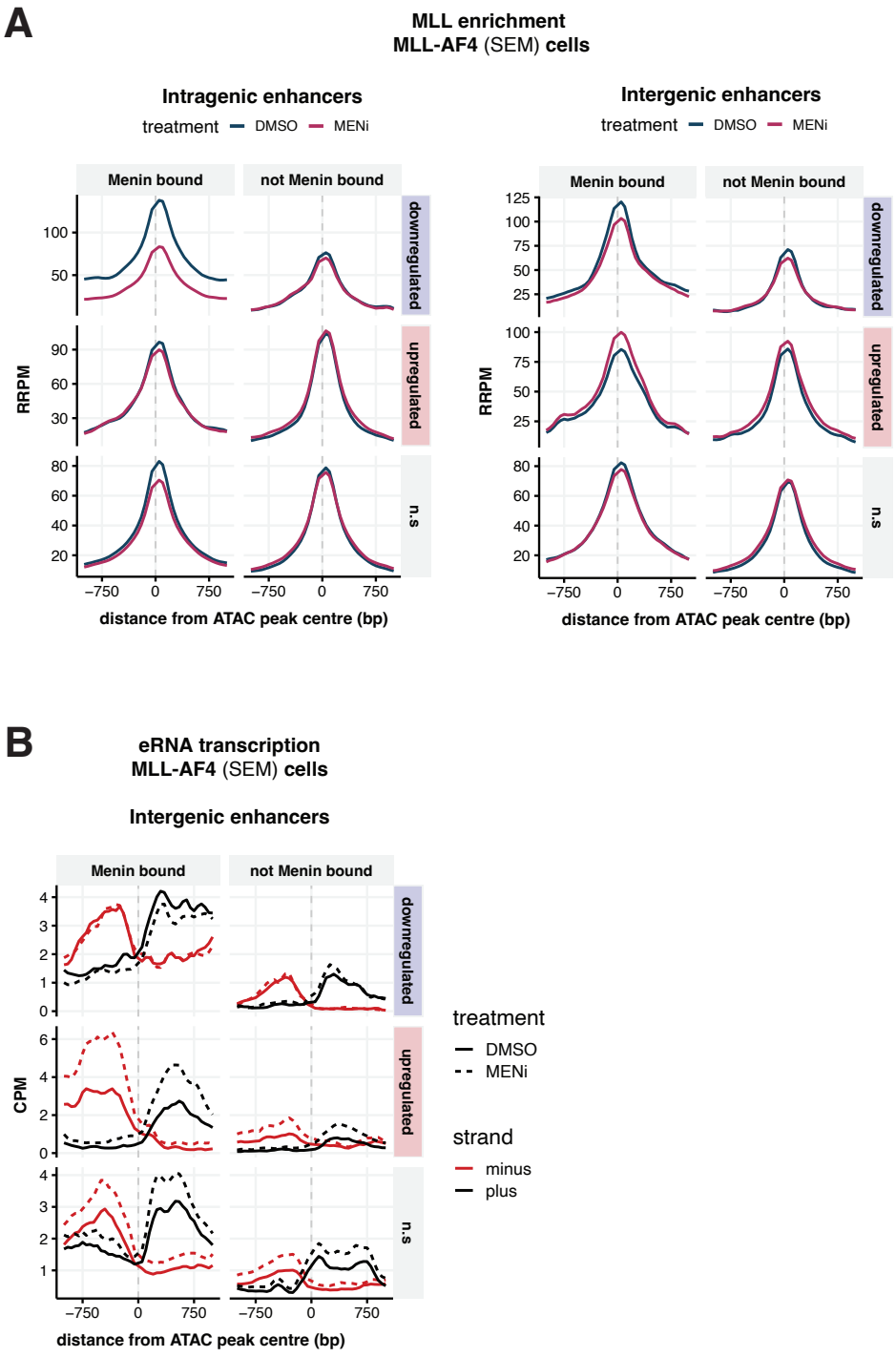


Figure 5.6: *(see next page)*

Figure 5.6 *(previous page)*: Menin inhibition reduces MLL/MLL-AF4 binding at intragenic enhancers but does not affect intergenic enhancer activity in SEM cells.**A.** Metaplots representing mean MLL/MLL-AF4 at intragenic (left) and intergenic (right) enhancers at differentially expressed genes (vertical annotation) following 24 h of Menin inhibition in SEM cells. "n.s" indicates non-significant genes from the differential expression analysis. **B.** Metaplot of mean nascent transcription (average of 3 biological replicates per condition) at intergenic enhancers in SEM cells.

5.2.2 Menin inhibition affects enhancer-promoter contacts in MLL-AF4 cells but not NPM1c cells

The loss of H3K27ac with Menin inhibition suggested that Menin supports the activity of intragenic enhancers in both SEM and OCI-AML3 cells. Next, I aimed to determine if these Menin-enriched intragenic enhancers were in 3D proximity to promoters and whether Menin was also required for the maintenance of enhancer-promoter contacts in both inhibitor-sensitive cells. After treating SEM and OCI-AML3 cells with Menin inhibitor for 24 h, I used the high-resolution 3C technique Micro-Capture-C (MCC) (Hua et al., 2021) to identify enhancer contacts for key gene promoters. The panel of promoter viewpoints included key MLL-AF4 and NPM1c target genes, such as *MEIS1* and *CDK6*, as well as genes which were down-regulated in response to Menin inhibition.

To assess whether our promoter viewpoint panel would likely capture genes with Menin-bound enhancers, I annotated cell-line specific enhancers (defined as overlapping ATAC and H3K27ac signal over 2 kb away from a TSS) to the nearest gene and intersected their coordinates with Menin ChIP-seq data from untreated cells. Genes with nearby Menin-bound enhancers elements were included for both cell lines (Figure 5.7). The full list of probes is provided in

Appendices B.1 and B.2.

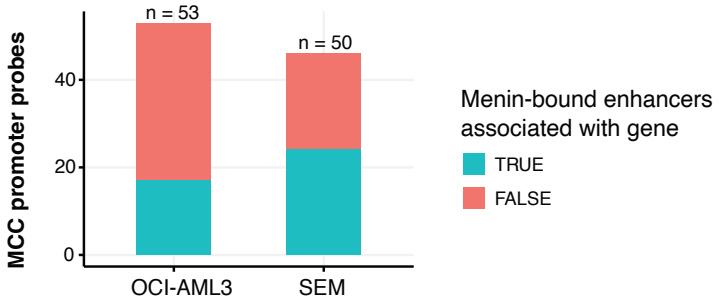


Figure 5.7: Proportion of Menin-bound enhancers near genes sampled with MCC for promoter interactions.

The base-pair resolution of MCC allows for investigating changes in enhancer-promoter contacts at smaller, more defined regions, including at protein binding sites, compared to restriction enzyme-based 3C techniques. To define the enhancer-promoter contact sites in each cell line, peaks were called from the MCC data using LancOtron (Hentges et al., 2022). The MCC peaks were then intersected with ChIP-seq datasets for H3K27ac, Menin, and MLL, and annotated with genomic regions. To enrich for contacts with putative active enhancers, only MCC peaks that overlapped with H3K27ac were retained for analysis. Peaks adjacent to TSS were not excluded, so this list includes both other promoters as well as putative enhancers.

In SEM cells, Menin was enriched at 69% of acetylated promoter contact sites, the majority of which fell within intronic regions or other promoters (regions within 2 kb of a TSS) (Figure 5.8A). Non-Menin bound contact sites in SEM cells were primarily localised to promoters, distal intergenic sites, and a smaller proportion of introns relative to Menin-bound sites. Conversely, in OCI-AML3 cells, Menin enrichment was only detected at 7.5% of promoter contact sites (Figure 5.8B). These Menin-bound contacts were primarily within 2 kb of a

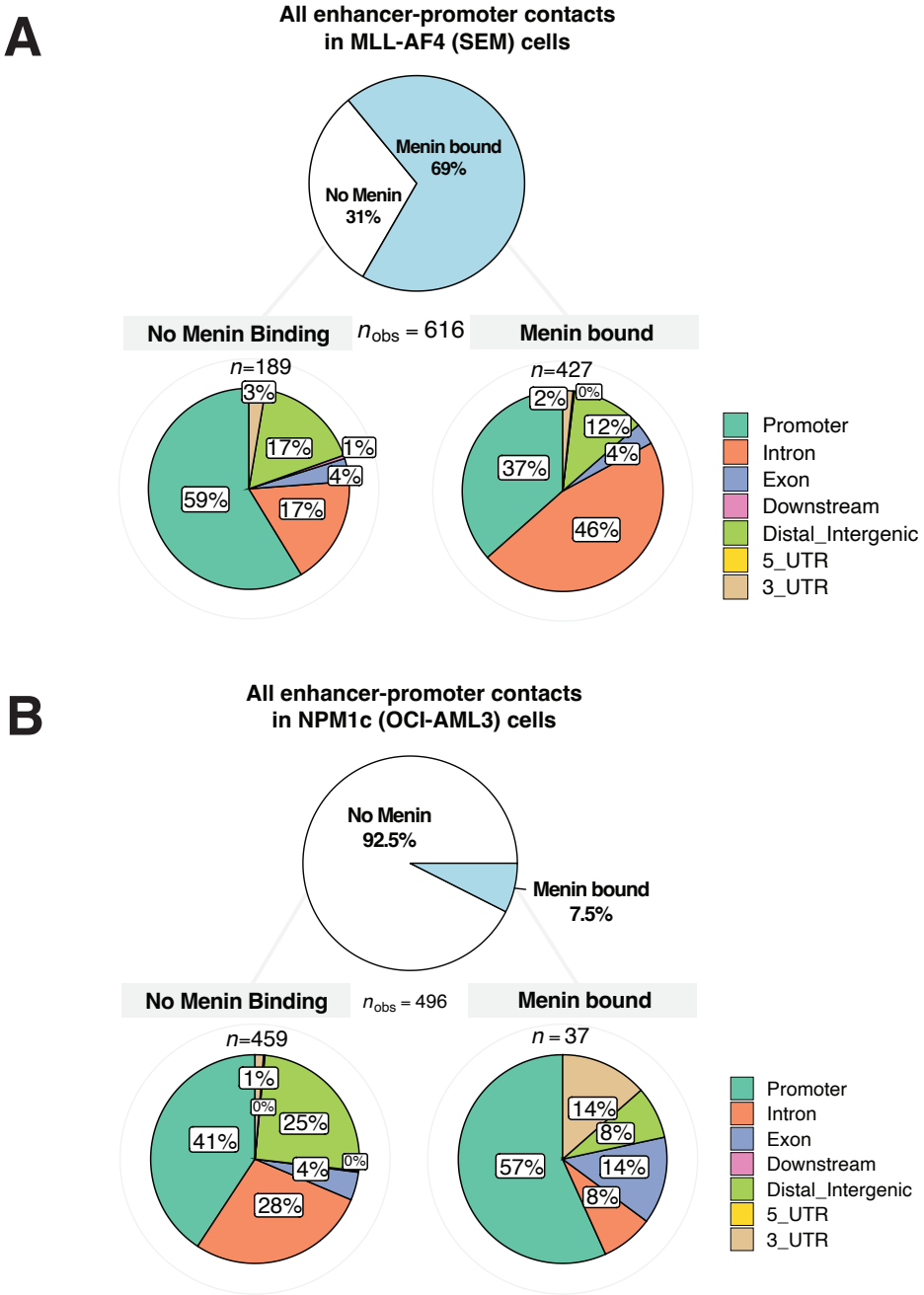


Figure 5.8: Menin is enriched at enhancer-promoter contact sites in MLL-AF4 (SEM) cells but not in NPM1c (OCI-AML3) cells. Annotation of enhancer-promoter contact sites with or without Menin enrichment in **A.** SEM cells and **B.** OCI-AML3 cells.

TSS, whereas, in contrast to SEM cells, the non-Menin bound contact sites formed a higher proportion of intronic contact regions. These data are in agreement with the relatively higher Menin signal at intragenic enhancers defined by ATAC and H3K27ac sites observed in SEM cells compared to OCI-AML3 cells (Figure 5.1A), as well as with the observation that more genes have Menin-bound intragenic enhancers in SEM cells (Figure 5.1C).

The analysis of overlaps between enhancer-promoter contacts and Menin binding relied on peak calling Menin ChIP-seq, which can be prone to missing some regions of low-level enrichment which do not resemble classical peaks. To affirm the conclusion that Menin is more enriched at SEM enhancer-promoter contact sites compared to OCI-AML3 contact sites, I performed a qualitative analysis of Menin, MLL/MLL-AF4, H3K27ac and NPM1c signal at promoter contacts (Figure 5.9). Using k-means clustering, contact sites could be separated into three groups of high, medium, and low Menin enrichment — clusters 1, 2, and 3, respectively — in both SEM and OCI-AML3 cells.

In SEM cells, clusters 1 and 2 (high and medium Menin enrichment) made up a higher proportion of contact sites than the equivalent clusters in OCI-AML3 cells, corroborating the intersections of MCC and Menin peaks (Figure 5.8). The contacts in these clusters also showed higher levels of MLL/MLL-AF4 and H3K27ac, as well as NPM1c in OCI-AML3 cells.

At the contact sites in cluster 1 (high Menin enrichment, dark blue average line), loss of Menin also correlated with a decrease in MLL-AF4 and H3K27ac in SEM cells (Figure 5.9A). Conversely, in OCI-AML3 cells, H3K27ac and NPM1c remained unaffected at the Menin-enriched contact sites despite the loss of Menin with Menin inhibition (Figure 5.9B). This result appears to contradict the previous observation that Menin inhibition correlates with a

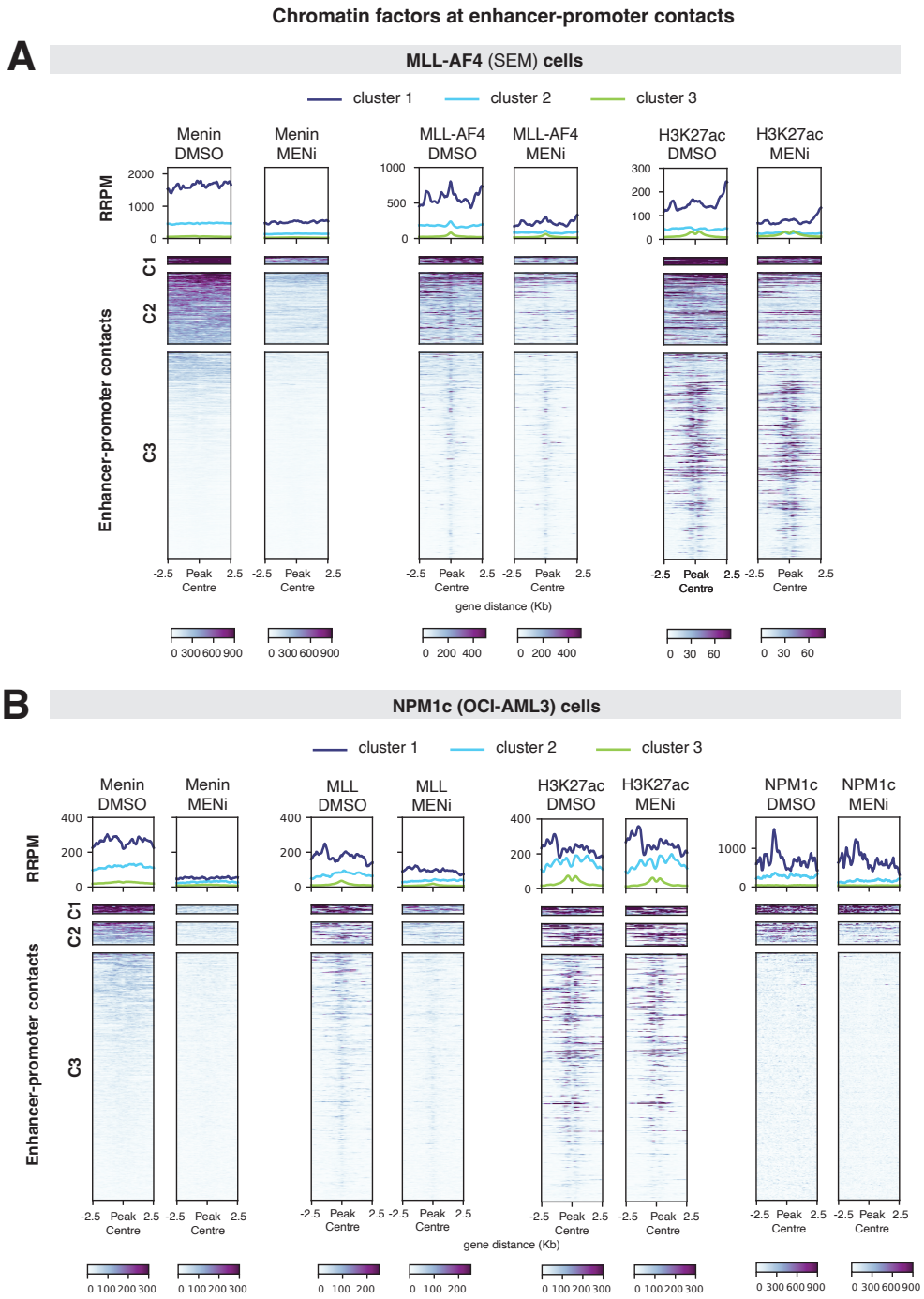


Figure 5.9: *(see next page)*

Figure 5.9 *(previous page)*: Menin is enriched at a subset of enhancer-promoter contact sites. Heatmaps and associated metaplots of the average Menin, MLL, H3K27ac, and NPM1c enrichment at enhancer-promoter contact sites (MCC peaks) in **A.** SEM cells and **B.** OCI-AML3 cells. Clustering was performed using k-means. Signal is represented as reference normalised reads per million (RRPM).

decrease in H3K27ac at intragenic and intergenic enhancers in OCI-AML3 cells (Figure 5.5B). However, this may be a consequence of stratifying contact sites by the levels of Menin enrichment as opposed to by the effect on gene expression, and as a result the average over MCC peaks may obscure individual decreases in H3K27ac at specific down-regulated genes. Furthermore, not all non-promoter H3K27ac peaks correspond to an enhancer-promoter contact site, and it is possible that contact sites are specifically insensitive to Menin loss. The majority of Menin-bound contact sites in OCI-AML3 cells are within 2kb of a TSS (*i.e.* within promoter regions), where H3K27ac may also be less sensitive to Menin loss.

In order to quantify changes in enhancer-promoter interactions upon Menin inhibition systematically across the promoter viewpoints, the mean count of unique ligation junctions was compared between conditions at each acetylated MCC peak. In SEM cells, there were 49 sites with a significant change in interaction (Mann Whitney U test, FDR q-value < 0.05), of which 43 showed decreased interaction (Figure 5.10A, left). Of the sites with decreased promoter contact, 39 overlapped with Menin enrichment. Overall, the mean $\log_2$ fold change in promoter interaction frequency at all Menin-bound enhancers was significantly different (Wilcoxon test ρ-value < 0.01) from the mean $\log_2$ fold change at non-Menin-enriched sites, pointing to a specific Menin inhibitor-driven effect. On the other hand, there were no significant

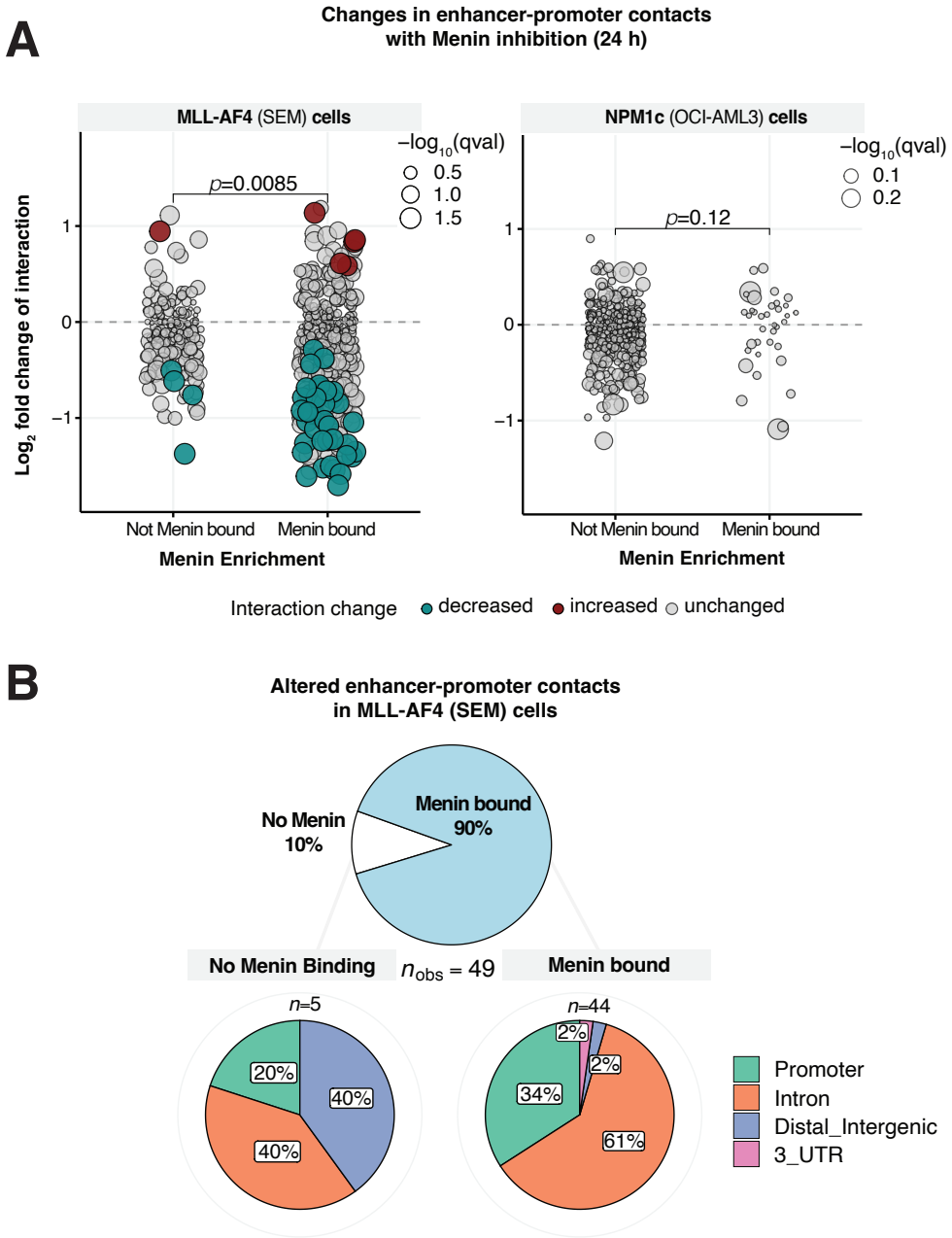


Figure 5.10: *(see next page)*

Figure 5.10 *(previous page)*: Menin inhibition leads to reduced enhancer-promoter contacts in MLL-AF4 cells but not NPM1c cells. **A.** Differential enhancer-promoter contacts in SEM (left) and OCI-AML3 (right) cells treated with Menin inhibitor for 24 hours, categorised by Menin binding. Coloured data points represent differential contact sites with Mann-Whitney U qval $<$ 0.05. The mean $\log_2$ fold change of Menin-bound enhancers is significantly different from the mean $\log_2$ fold change of non-Menin bound enhancers in SEM cells (Wilcoxon test ρ-value = 0.0085). **B.** Annotation of differential enhancer-promoter contact sites with or without Menin enrichment in SEM cells.

changes in the interaction frequency at OCI-AML3 enhancers, including at Menin-bound sites (Figure 5.10A, right), suggesting that Menin is dispensable for enhancer-promoter contacts in these cells.

Consistent with the finding that Menin inhibition primarily affects intragenic enhancers in SEM cells, the majority of Menin-bound contact sites with altered interaction frequency were located in introns (61%) (Figure 5.10B). While 12% of Menin-bound contact sites were at distal intergenic sites (Figure 5.8A), these interactions were not significantly altered with inhibition — in line with the unaffected H3K27ac, MLL/MLL-AF4, and eRNA levels at intergenic sites (Figure 5.5, 5.6).

The *ARID1B* locus provides a useful illustration of the differential effects of Menin inhibition on enhancer activity in MLL-AF4 versus NPM1c cells (Figure 5.11A). In SEM cells, there are two large Menin-bound intragenic enhancers (highlights 1 and 2). To quantify the potential loss of MCC signal upon Menin inhibition, the DMSO condition was subtracted from the inhibitor condition (labelled as "subtraction"). This reveals that the frequency of the interactions at these sites is decreased in SEM cells, along with binding of Menin, MLL-AF4, and H3K27ac, indicating a loss of enhancer activity.

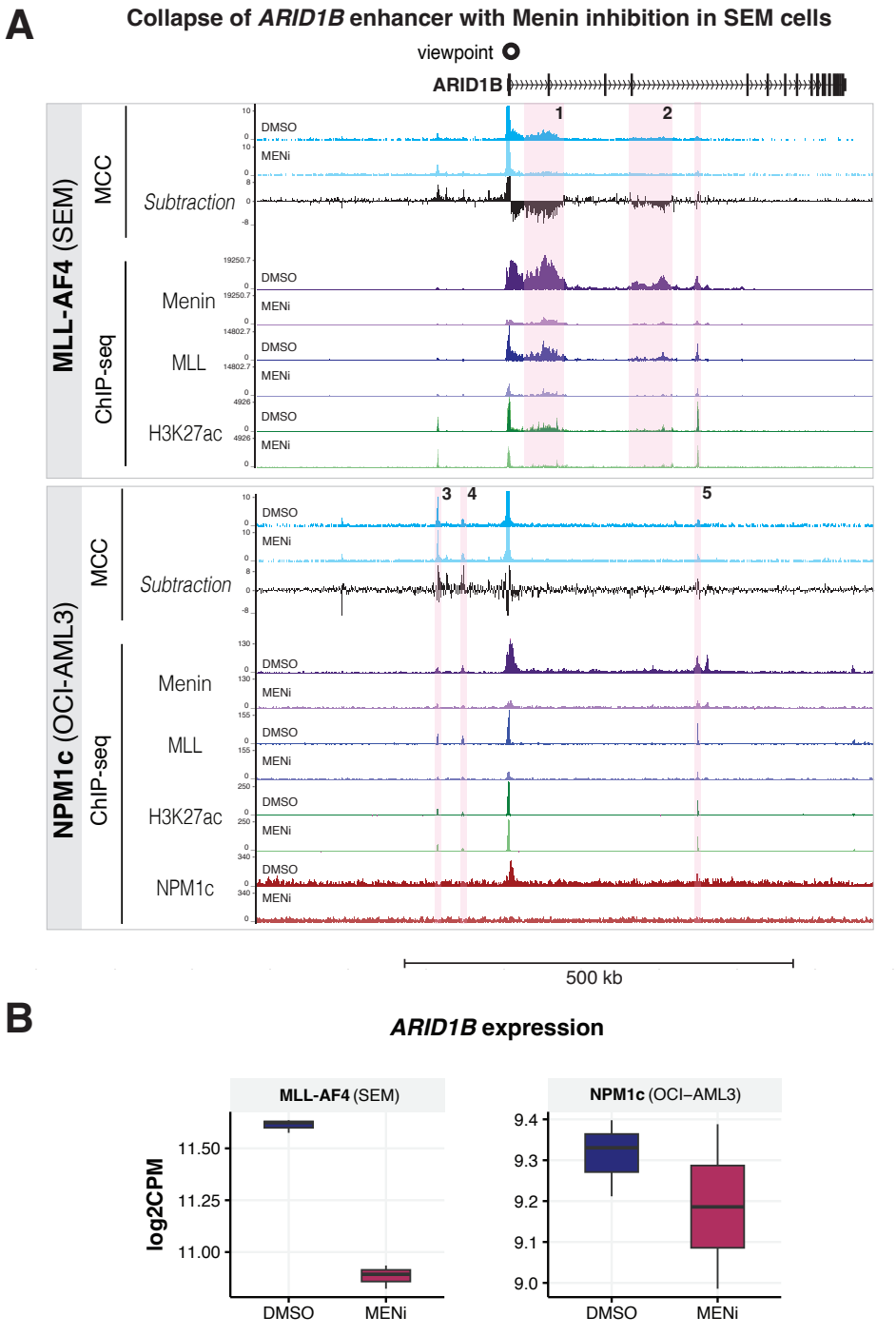


Figure 5.11: *(see next page)*

Figure 5.11 *(previous page)*: Differential enhancers at *ARID1B* highlight the role of Menin in sustaining enhancer activity in SEM cells but not OCI-AML3 cells. **A.** Visualisation of MCC and ChIP-seq signal at the *ARID1B* locus in SEM cells and OCI-AML3 cells. Enhancer-promoter contact sites are highlighted in pink. MENi refers to a 24 h treatment with Menin inhibitor. **B.** *ARID1B* transcription (TT-seq) in SEM and OCI-AML3 cells in DMSO and Menin-inhibitor (24 h) treated cells.

On the other hand, the *ARID1B* locus in OCI-AML3 cells does not contain the same large intragenic enhancers marked by Menin enrichment. Instead, the interaction sites with the OCI-AML3 *ARID1B* promoter both upstream (highlights 3 and 4) and within the fourth intron (highlight 5) have low levels of Menin and are unaffected by the inhibitor treatment. The collapse of the intragenic Menin-bound enhancers in SEM cells correlates with decreased *ARID1B* expression (Figure 5.11B), which was unaffected by Menin inhibition in OCI-AML3 cells.

The *BAHCC1* locus illustrates an example of Menin-bound enhancers in OCI-AML3 cells with relatively higher Menin signal at the contact sites (Figure 5.12). Nonetheless, the interactions between the promoter of *BAHCC1* with these regions, highlighted in pink, do not decrease with Menin inhibition with statistical significance, despite the decrease in Menin binding. This gene, which is commonly over-expressed in leukaemia (Fan et al., 2020), is also not differentially expressed following Menin inhibition ($\log_2$ fold change = 0.179, FDR = 0.80 at 24 h). NPM1c binding is retained at the contact site upstream of the *BAHCC1* promoter (highlight 1), indicating that Menin inhibition is insufficient to disrupt NPM1c at this site, similarly to the promoter. Although it is unclear whether NPM1c itself sustains enhancer-promoter contacts at this target gene, the overall insensitivity to Menin inhibition at the locus suggests there is an alternative pathway for stabilising NPM1c and promoting

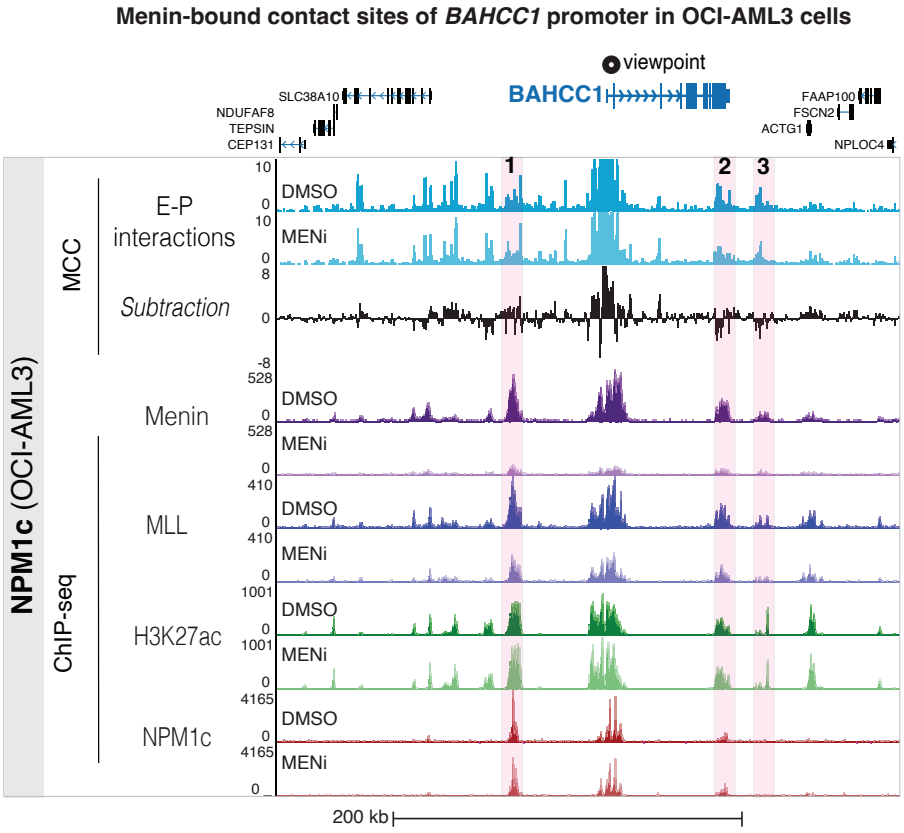


Figure 5.12: Menin-bound enhancers in contact with the *BAHCC1* promoter in OCI-AML3 cells do not decrease in interaction frequency with Menin inhibition. Menin-bound enhancers are highlighted in pink.

enhancer activity.

Given that Menin inhibition led to both decreased H3K27ac and MLL-AF4 at intragenic enhancers in SEM cells (Figure 5.5A), it is difficult to attribute the decrease in interaction frequency to Menin loss specifically, versus to the combination of decreased acetylation and MLL binding. To examine the relationship between these factors, I quantified the enrichment of Menin, H3K27ac and MLL at promoter interaction sites in DMSO- and Menin inhibitor-treated cells. I then compared the change in ChIP-seq signal to the change of the interaction with the promoter to identify the strongest

relationship between these alterations.

Most of the ChIP-seq data points lie below 0, showing that, on average, there was a decrease of H3K27ac and MLL/MLL-AF4 signal along with the expected loss of Menin at all enhancer-promoter interaction sites (Figure 5.13). Fitting a linear model specifically for sites with a significant decrease in interaction (blue data points) revealed that the decrease in interaction frequency was most strongly correlated with changes in MLL enrichment — although this correlation was nonetheless relatively weak ($R^2 = 0.32$).

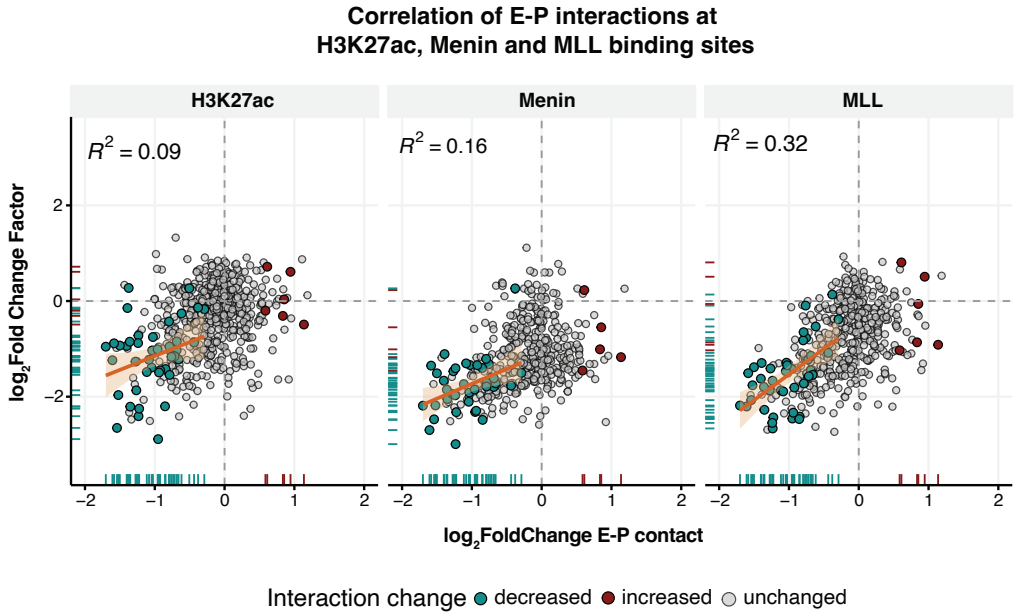


Figure 5.13: Decreased enhancer-promoter interaction frequency correlates with decreased H3K27ac, Menin and MLL-AF4 at contact sites in SEM cells. Scatter plots show the $\log_2$ fold change of the enhancer-promoter interaction and the $\log_2$-fold change of H3K27ac, Menin or MLL enrichment at the interaction site after 24 h of Menin inhibition. Coloured data points are sites with a significant (q-val < 0.05) change in interaction. A predicted line and corresponding R^2 from a linear model fit is shown for sites of decreased interaction.

The analysis of differential MLL peaks with Menin inhibition in SEM cells

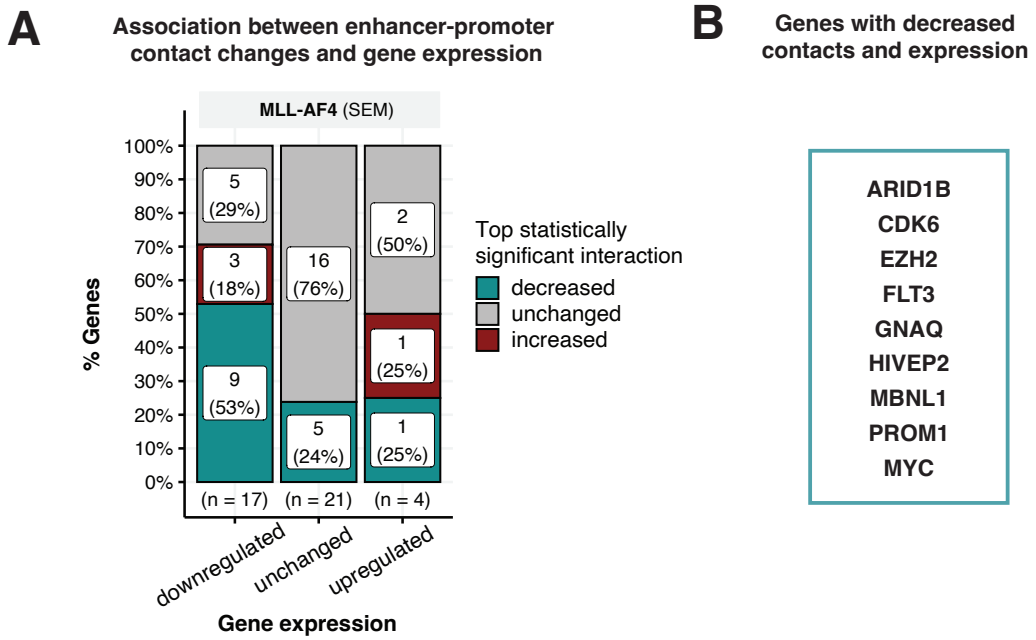


Figure 5.14: Loss of enhancer-promoter interactions is associated with gene down-regulation at specific MLL-AF4 targets. **A.** Proportion of differentially expressed genes from the MCC panel with differential enhancer-promoter contacts in SEM cells. For each gene, the most statistically significant differential promoter interaction is presented. **B.** List of genes with a significantly decreased enhancer-promoter contact and decreased expression after 24 h Menin inhibition.

(Chapter 3) showed that not all loci which lose MLL-AF4 binding exhibit a concordant decrease in gene expression at 24 h. Thus, I next sought to test the extent to which the observed loss of MLL-AF4 and decreased enhancer interactions corresponded to alterations in transcription. After filtering MCC contact sites to those which overlapped with H3K27ac, the remaining data contained 42 genes which had a detectable contact with an active enhancer. I intersected this gene list with the nascent transcription data in order to classify the genes according to differential expression. Nine out of 17 down-regulated genes (53%) had a decreased interaction as the most statistically significant enhancer-promoter contact alteration, whereas 5

genes had a decreased enhancer-promoter contact but no change in expression (Figure 5.14A). Among the genes with a concordant decrease in enhancer contacts and expression were *ARID1B*, *FLT3*, *PROM1*, and *CDK6* (Figure 5.14B) — key oncogenes at which the MLL-AF4 complex has previously been shown to sustain enhancer-promoter contacts (Crump et al., 2023; Godfrey et al., 2019). Thus, similar to how MLL-AF4 redistribution associated with transcription changes at specific loci, this subset of genes appears to be more sensitive to the loss of enhancer-promoter contacts maintained by Menin and the MLL-AF4 complex than other genes with Menin-bound enhancers.

5.3 Discussion

The characterization of active enhancer elements with ATAC-seq, ChIP-seq and Micro-Capture-C in the Menin inhibitor-sensitive SEM and OCI-AML3 cells revealed that a subset of intragenic enhancers in SEM cells are uniquely dependent on Menin enrichment. Disruption of Menin binding with the Menin inhibitor led to decreased H3K27ac, MLL-AF4 binding, and enhancer-promoter contacts at these loci. In contrast, enhancer activity in OCI-AML3 cells was unaffected by Menin inhibition.

One caveat is that this was not a genome-wide analysis of enhancer-promoter contacts and could therefore be affected by my choice of promoter capture viewpoints. However, the genes represented in this analysis were associated with Menin-bound putative enhancers in similar proportion, and also included both NPM1c and MLL-AF4 targets whose expression is sensitive to Menin inhibition. It is therefore unlikely that the choice of probes introduced a significant bias against Menin-bound regulatory elements in OCI-AML3 cells. Unfortunately, genome-wide 3C techniques, such as Promoter Capture Hi-C or HiChIP, are not compatible with this sort of high-resolution, quantitative analysis (Mumbach et al., 2016; Lieberman-Aiden et al., 2009).

Previous work from the Milne lab has explored the role of the MLL-AF4 complex in sustaining both intragenic and intergenic enhancers. Godfrey *et.al.* reported on a subset of enhancer elements marked by the DOT1L-deposited H3K79me2/3 mark, whose activity was disrupted with DOT1L inhibition (Godfrey et al., 2019). Similar to Menin-bound enhancers, in SEM cells the majority of H3**K**79me2/3 **E**nhancer **E**lements (named "KEEs" by Godfrey *et al.*) were in intragenic regions. The *ARID1B* intragenic enhancers

that interact with the promoter and show sensitivity to Menin inhibition are also highly sensitive to DOT1L inhibition. On the other hand, MLL-AF4 knockdown as well as degradation of members of the PAF1 and FACT complexes, which interact with the MLL-AF4 complex, has also been shown to decrease both intragenic and intergenic enhancer activity in SEM cells (Crump et al., 2023). My data suggests that Menin functions more similarly to H3K27me2/3 in stabilizing the MLL-AF4 complex at intragenic enhancers, thereby facilitating interactions with gene promoters.

Intragenic enhancers enriched for Menin may be connected to the MLL-AF4 spreading phenomenon described by Kerry *et al.* (Kerry et al., 2017). Menin was shown to stabilize MLL-AF4, but not wild-type MLL, in the gene bodies of a subset of highly-expressed genes, which are also highly sensitive to DOT1L inhibition. Together, these observations suggest a model in which Menin specifically stabilizes the MLL-AF4 complex, including DOT1L, within gene bodies, thereby facilitating multivalent interactions between transcriptional activators and proximity with the promoter, which together sustain high gene expression. The sensitivity of these regions to the loss of various MLL-AF4 complex components — Menin, PAF1 and SSRP1 (Crump et al., 2023), or the DOT1L-deposited H3K79me2/3 (Godfrey et al., 2019; Kerry et al., 2017) — suggests that the overall high density of proteins is required for enhancer activity and promoter contact, as opposed to one specific single component creating these enhancers. Conversely, loss of any single protein appears to be sufficient to collapse the complex at these enhancers.

In the NPM1-mutant OCI-AML3 cells, the enhancer landscape differs from SEM cells in terms of Menin enrichment. While there was still detectable Menin enrichment at regions of open chromatin and H3K27ac away from TSS (*i.e.* at presumed enhancers), this was lower relative to the Menin enrichment

at promoters. In contrast, in SEM cells Menin levels at intragenic enhancers were similar to promoters. Further, the majority of enhancer-promoter contact sites did not overlap with Menin peaks in OCI-AML3 cells. Although this analysis relied on Menin peak calling, which in its binary nature may exclude some regions with relatively low but genuine enrichment, a more qualitative inspection of the Menin ChIP-seq signal at MCC peaks corroborated this, and showed that these sites do not lose H3K27ac or NPM1c binding upon Menin inhibition.

Menin inhibition in OCI-AML3 cells did not affect enhancer-promoter contacts even at down-regulated genes, indicating that the primary function of Menin in these cells is at promoters, where it stabilizes NPM1c binding. Wang *et al.* (2023) detected NPM1c chromatin binding outside of promoters, although they did not delve into its function at these sites (X. Q. D. Wang et al., 2023). Similarly, my ChIP-mentation data on NPM1c revealed peaks in gene bodies and intergenic regions more than 2 Kb away from an annotated TSS, and NPM1c enrichment was detectable at some enhancer-promoter contact sites. However, whether NPM1c has a functional role in enhancer activity is inconclusive, as NPM1c binding was unperturbed by Menin inhibition.

It remains possible that NPM1c and Menin occupancy at some non-promoter regulatory elements has a function distinct from promoting enhancer-promoter proximity, such as recruitment of Mediator or other co-activators. Menin exhibits a similar "spreading" pattern at some NPM1c target genes in OCI-AML3 cells to that observed in SEM cells (X. Q. D. Wang et al., 2023). For example, Menin is enriched in the gene bodies of *JMJD1C*, *PHF10*, *SATB1*, *SATB2* and *RUNX2*, but promoter contact sites were not altered with statistical significance following Menin inhibition. Nonetheless, Menin enrichment in the 5' of genes is characteristic of down-regulated genes in

OCI-AML3 cells (X. Q. D. Wang et al., 2023) and may therefore be important for transcriptional activation independent of enhancer-promoter proximity.

What drives the specificity of the sensitivity of enhancer-promoter contacts to Menin loss in MLL-AF4 cells remains an open question. My analysis showed that only some Menin-enriched enhancer-promoter contact sites exhibited a decrease in interaction, and of those, just over 50% were linked to a decrease in transcription. The limitations of a small sample size of genes for which there was a detectable enhancer-promoter contact (n = 42, out of 50 probes) made it challenging to identify factors that correlated with a concordant loss of transcription and of enhancer-promoter interactions, which was detected at a total of nine genes. While there was a positive correlation between loss of interactions and a decrease in MLL-AF4 enrichment, a larger sample size of genes would be needed to analyse the relationship between Menin-bound enhancers and transcription of the corresponding gene more thoroughly. For instance, the size of the enhancer, the extent of MLL-AF4 and Menin spreading, levels of baseline transcription, or the binding of additional transcription factors at a subset of Menin-bound enhancers could be associated with the effects of Menin inhibition on enhancer-promoter contacts.

Crump *et al.* (2023) showed that while no transcription factor binding motif was specifically associated with MLL-AF4 enhancers compared to enhancers generally, MLL-AF4 spreading was associated with increased RUNX1 and MAZ enrichment in gene bodies (Crump et al., 2023). In its role as a scaffold protein, Menin may be stabilizing a high density of transcription factors, interacting with the MLL-AF4 complex, to create highly active intragenic enhancers. To test this hypothesis, the Menin interactome data (Chapter 4) could be integrated with motif analysis at enhancer-promoter contact sites to shortlist candidate transcription factors recruited to Menin-enriched enhancers.

Given that these enhancers appear sensitive to the disruption of multiple factors, screening for additional enhancer-associated transcription factors could be an opportunity to identify synergistic vulnerabilities in targeting enhancers in MLL-AF4 leukaemias.

In summary, the unique effects of Menin inhibition on enhancer activity in SEM cells suggest that the stronger transcriptional response observed in SEM cells compared to OCI-AML3 cells is triggered by a depletion of MLL-AF4 at both promoters and enhancers, and a loss of promoter contacts. As a result, SEM cells may be more sensitive to inhibition in the short term because of this combined effect on transcriptional regulation. On the other hand, the milder transcriptional response in OCI-AML3 cells could be attributed to insensitivity of enhancer activity to Menin loss, thereby restricting the inhibitor-responsive gene set to highly sensitive promoters.

6 | Discussion

The aims of my project were to:

- In **Chapter 3**, evaluate the transcriptional consequences of Menin inhibition in sensitive and insensitive cell line models of leukaemia, including NPM1c acute myeloid leukaemia (OCI-AML3), MLL-AF4 (SEM) and E2A-PBX1 (RCH-ACV) acute lymphoblastic leukaemia cells.
- In **Chapter 4**, characterise protein complexes that interact with Menin in NPM1c, MLL-AF4 and E2A-PBX1 cells.
- In **Chapter 5**, test the effect of Menin inhibition on enhancer activity in MLL-AF4 and NPM1c cells.

6.0.1 Novel insights into the role of Menin in transcription activation and enhancer activity

Overall, my results highlight the distinct requirement for Menin in NPM1c and MLL-AF4 cells, which are both dependent on the interaction between Menin and MLL-N to regulate expression of NPM1c or MLL-AF4 target genes.

In Chapter 3, I showed that SEM cells, ALL cells expressing MLL-AF4, exhibit rapid transcriptional dysregulation, which affects thousands of genes, upon Menin inhibition. On the other hand, OCI-AML3 cells, an AML cell line with NPM1c, showed transcription changes in approximately a hundred genes in the short term, and instead altered gene expression more gradually over a longer exposure period to Menin inhibition. E2A-PBX1 cells also exhibited

gene expression changes but showed no effect on viability, confirming that the Menin-MLL interaction is dispensable in this context. Although some Menin target genes were cell type specific, Menin enrichment was generally detected at the promoters of highly expressed genes. While Menin inhibition disrupted both MLL-AF4 and NPM1c binding at some of their target genes, the variable transcriptional responses indicated that Menin has different regulatory roles in the MLL-fusion protein complex compared to the NPM1c complex.

As Menin is a scaffold protein, I reasoned that the distinct effects of Menin loss on transcription regulation are linked to the complexes it interacts with and stabilises. In Chapter 4, I showed that Menin-MLL1/MLL-FP complexes may interact with transcription regulators and transcription factors with some cell-type specificity. By focusing on transcription activators, I found that EP300, as well as proteins in the SEC, FACT, and SAGA complexes were more enriched in the Menin interactome of MLL-AF4 cells compared to NPM1c cells. We had previous evidence that the FACT complex co-operates with MLL-AF4 to regulate enhancers (Crump et al., 2023), and this, in combination with the strong enrichment of EP300 in SEM cells, led me to investigate whether Menin inhibition could disrupt enhancer activity in MLL-AF4 cells or NPM1c cells.

In Chapter 5, I showed that Menin was enriched at intragenic and intergenic enhancers in both MLL-AF4 and NPM1c cells. However, Menin inhibition specifically reduced H3K27ac at intragenic enhancers at down-regulated genes, suggesting that Menin activity at enhancers was closely linked to transcription. Using high-resolution Micro-Capture-C to map enhancer-promoter contacts, I found that Menin inhibition disrupted enhancer-promoter proximity specifically in MLL-AF4 cells and primarily at intragenic enhancers. The decrease in enhancer-promoter proximity was correlated with decreased

gene expression at only a subset of genes, indicating that the requirement for Menin is specific to select enhancer-promoter contact sites.

Together, my data points to a model (Figure 6.1) whereby MLL-AF4 uniquely co-opts Menin to associate more strongly with transcription co-activators and stabilise enhancer-promoter proximity to activate genes, which could account for the greater transcriptional disruption observed with Menin inhibition compared to NPM1c cells. In contrast, NPM1c requires Menin at select gene targets, which could be critical for maintaining the AML cells' differentiation block. This provides new insights into Menin activity outside of stabilising MLL-fusions or NPM1c at promoters and furthers our understanding of the different effects of Menin inhibition in *MLL*r ALL versus NPM1-mutated AML.

I chose to use the Menin inhibitor VTP50469 throughout my experiments, which specifically disrupts the interaction between Menin and MLL, and depletes Menin from chromatin genome wide. Therefore, my results pertain specifically to the functionality of the interaction between MLL and Menin, as the Menin protein itself remains expressed. Whether Menin has additional function outside the MLL-AF4 or NPM1c complexes in the presence of Menin inhibition remains to be clarified. Inducible degradation of Menin, such as through the dTag system (Nabet et al., 2018) or a Menin-specific PROTAC, would be useful to compare against Menin inhibitor in order to test whether the activity of other protein complexes is affected.

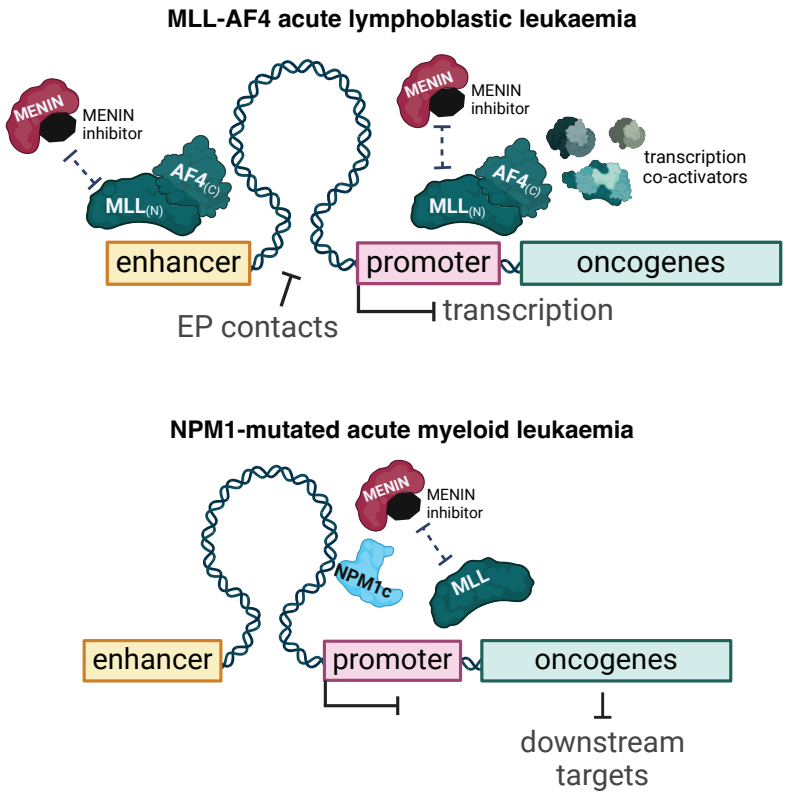


Figure 6.1: Model for Menin activity in MLL-AF4 and NPM1-mutated leukaemia. "EP" refers to "enhancer-promoter."

6.0.2 Transcriptional effects of Menin inhibition beyond *MEIS1*

My findings pertaining to the role of Menin in activating transcription and maintaining enhancer integrity in MLL-AF4 ALL cells add to investigations that have primarily focused on MLL-rearranged AML. *In vitro*, AML and ALL cells with MLL-rearrangements have been demonstrated to respond differently to Menin inhibition: AML cells tend to differentiate, whereas ALL cells undergo apoptosis (Adriaanse et al., 2024). Further mechanistic work comparing the responses to Menin inhibition in *MLL*r ALL and AML, as well as *MLL*r AML and NPM1c AML, is needed to clarify to what extent the interaction between cell lineage and the driver mutation determines the dependency on Menin in transcription.

Additionally, whereas models of Menin activity to date have focused on the decreased expression of *MEIS1*, *HOX* genes, and other direct MLL-FP or NPM1c target genes, I also identified a number of genes which concurrently gain MLL-AF4 or NPM1c binding and increase expression upon Menin inhibition. This observation corroborates recent reports of the redistribution of MLL1 upon Menin inhibition (Sparbier et al., 2023), which merits further investigation into whether the redistribution of MLL-AF4 and NPM1c also activates genes that have a tumour suppressive role. If so, identifying the factors which recruit or stabilise MLL-AF4 and NPM1c at these new binding sites could also point to additional TFs and chromatin proteins which are important for the response to Menin inhibition.

In OCI-AML3 cells, the *ONECUT3* gene showed a dramatic gain in NPM1c and MLL1 binding, H3K27ac enrichment, and increased gene expression upon Menin inhibition. Not much is known about this transcription factor in AML,

which, according to the Human Protein Atlas, is not highly expressed in immune cells (Uhlen et al., 2010). In myelodysplastic syndrome, a condition characterised by myeloid cell expansion and a high risk of progression to AML (Platzbecker, Kubasch, Homer-Bouthiette, & Prebet, 2021), *ONECUT3* over-expression was found by Luo *et al.* to be associated with karyotype abnormalities, whereas ectopic expression of *ONECUT3* in AML cell lines led to mulinucleation (Luo et al., 2024). Further investigating whether increased genomic instability is also a consequence of *ONECUT3* up-regulation in response to Menin inhibition could reveal potential therapeutic combinations.

How NPM1c is recruited and stabilised at target genes has only recently been described (Uckelmann et al., 2023; X. Q. D. Wang et al., 2023). Interestingly, Uckelmann *et al.* (2023) showed that some NPM1c target genes varied between PDX samples derived from four AML patients. Testing to what extent NPM1c target genes vary between NPM1c AML patients, and whether the same set of targets is consistently sensitive to Menin inhibition, could be important to understanding whether NPM1c can also contribute to transcriptional heterogeneity within this AML subtype.

6.0.3 MLL-AF4 confers additional regulatory activity to key interactors, including Menin

The endogenous Menin immunoprecipitations add to a complex body of literature that has described a variety of context-specific Menin interactions and downstream regulatory processes (Matkar et al., 2013). In line with our understanding of the Menin-MLL1 complex as generally enriched at actively transcribed genes, I identified key transcription machinery, including the RNA polymerase II complex, enriched in the Menin interactome in all three cell lines.

The unique activities of Menin in SEM cells, including its association with the FACT complex and its role in stabilising enhancer-promoter contacts, concurs with previous observations from the Milne lab that proteins in the MLL-AF4 complex can have distinct regulatory roles. For example, while DOT1L and PAF1 are typically associated with transcription elongation (Kim et al., 2010; Steger et al., 2008), Godfrey *et al.* (2019) and Crump *et al.* (2023) and showed that these factors can also regulate enhancer activity in MLL-AF4 cells. PAF1 depletion was found to decrease enhancer activity in MLL-AF4 SEM cells but not in the multiple myeloma cell line MM1.S, despite enrichment at enhancers and super-enhancers in both cell lines (Crump et al., 2023). This observation is analogous to how Menin inhibition specifically reduced enhancer-promoter interactions in MLL-AF4 cells but not NPM1c cells. Although NPM1c and Menin also co-localised at a small subset of contact sites in OCI-AML3 sites, H3K27ac and enhancer-promoter interactions were not affected by Menin inhibition. This supports the model that Menin gains additional regulatory activity in the MLL-AF4 complex compared to the wild-type Menin-MLL1.

6.0.4 Open questions about Menin's potential participation in multiple complexes

The focus of my investigation of Menin interactomes in the leukaemia cell lines centred on chromatin complexes and transcription activators, which leaves the additional complexes that were detected to be explored further. There is evidence from different model systems that Menin can regulate other pathways such as DNA repair in addition to transcription activation (Matkar et al., 2013). My protein complex enrichment analysis also showed the enrichment of DNA repair machinery in SEM cells, which fits with previous observations implicating Menin in DNA repair. For example, Menin was shown to interact

with CHK1 and promote homology-directed repair (Gallo, Agnese, Esposito, Galgani, & Avvedimento, 2010). In *MLL*r AML cells, Menin inhibition was found to impair activation (phosphorylation) of Chk1, which was potentiated by co-treatment with HDAC inhibitor (J. Ye et al., 2019). Menin has also been found to interact with FANCD2 (Jin et al., 2003), another protein involved in homology-directed repair (Garcia-Higuera et al., 2001). MEFs lacking Menin accumulate chromosome abnormalities, including breaks and cross-links, in the presence of the DNA crosslinking agent DEB, suggesting that Menin may play a role in their repair (Jin et al., 2003). Investigating whether cells with NPM1c or MLL-FPs are more sensitive to DNA damage when treated with Menin inhibition could point to potential combination therapies.

Interactions with specific TFs, such as RUNX and IKZF proteins, also merit further investigation, as they may reveal therapeutically actionable co-dependencies, as was recently demonstrated by the combination of Menin inhibitors and immunomodulatory drugs (IMiDs) in AML (Aubrey et al., 2022). To systematically interrogate additional Menin dependencies, the NPM1c- or MLL-AF4-specific Menin interactomes and nuclear proteomes could be used to guide the design of targeted CRISPR screen libraries. CRISPR screens investigating Menin inhibitor sensitivity and resistance published to date (Soto-Feliciano et al., 2023; Zhou et al., 2024) have been performed in *MLL*r acute myeloid leukaemia cells, which leaves a significant gap in our knowledge regarding resistance mechanisms or co-dependencies in *MLL*r acute lymphoblastic leukaemias or NPM1c myeloid leukaemias.

6.0.5 Enhancer usage in MLL-AF4 cells merits further investigation in Menin inhibitor resistance

In addition to the redistribution of MLL and NPM1c, evaluating how other chromatin modifying complexes are redistributed could reveal mechanisms that modulate non-genetic adaptations to Menin inhibition. Altered binding patterns of MLL3/4-UTX and of Polycomb Repressive Complex 1 have already been implicated in the response to Menin inhibition in AML cells (Soto-Feliciano et al., 2023; Zhou et al., 2024). Observations from the Revumenib clinical trial also pointed to non-genetic mechanisms of resistance to Menin inhibition, in addition to the mutations in the Menin-drug interface (Perner, Stein, et al., 2023).

My Micro-Capture-C results indicated that Menin inhibition can alter enhancer usage. Although at 24 h of treatment I observed primarily decreased interactions, it would be worth exploring whether long-term exposure to Menin inhibition causes new enhancer-promoter contact sites to emerge. In a conference abstract, the group who characterised the *MEN1* mutations driving resistance to inhibition have described cases where PDX models acquired Menin inhibitor resistance in the absence of mutations and in spite of a successful transcriptional response, including down-regulation of *MEIS1* (Perner, Rahnamoun, et al., 2023). Testing to what extent enhancer re-wiring contributes to this, and the factors which activate new enhancers, could potentially identify pathways that can be targeted to restore sensitivity to Menin inhibition. This has been shown to be a viable strategy in the context of BET inhibition in AML; Bell *et al.* reported that enhancer switching, which had given rise to AML resistant to BET inhibition, could be therapeutically targeted with LSD1 inhibition (Bell et al., 2019).

Investigating enhancer re-wiring in MLL-AF4 cells in response to Menin inhibition could be accomplished by longitudinal sampling of Menin inhibitor-treated patient or PDX material (Perner, Rahnamoun, et al., 2023). However, limiting cell numbers can be a challenge for enhancer profiling with MCC. An alternative model system for evolving resistance to Menin inhibition *in vitro* could be the fetal liver derived model generated by Rice *et al.*, which recapitulates infant MLL-AF4 ALL gene expression (Rice et al., 2021). These xenoblast cells can be expanded in culture, making them a suitable tool for evolving resistance to Menin inhibition, CRISPR screens, and genomic profiling.

Overall, my finding that Menin sustains enhancer-promoter contacts to regulate transcription in MLL-AF4 ALL cells but not NPM1c AML cells indicates that Menin inhibition can have distinct consequences on transcription regulation in different sensitive leukaemias. Future work to analyse how Menin inhibition affects transcriptional plasticity through the interplay of chromatin complexes and enhancer usage in different leukaemias can further our understanding of how this promising targeted therapy can best be used in the clinic.

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A | TT-seq proportions of reads mapped

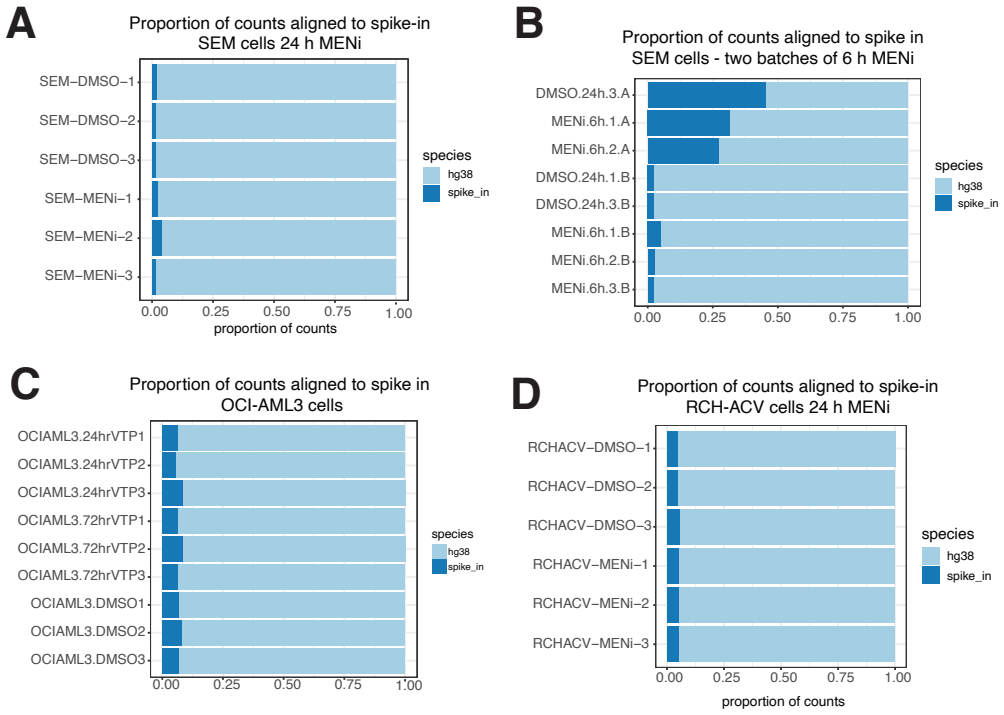


Figure A.1: Proportion of reads mapped to either the human genome build hg38 or to the synthetic spike-in controls in TT-seq experiments. **A.** SEM cells treated with DMSO or MENi for 24 h. **B.** Two experiments in which SEM cells were treated with DMSO or MENi for 6 h. Samples are labelled with "-A" for batch 1 and "-B" for batch 2. **C.** OCI-AML3 cells treated with DMSO or MENi for 24 h or 72 h. **D.** RCH-ACV cells treated with DMSO or MENi for 24 h.

B | Micro-Capture-C Probes

B.0.1 MCC probes for SEM cells

Table B.1: Probes used for MCC in SEM Cells.

Probe list for MCC in SEM cells	
Gene	**Probe sequence**
KRAS	GGCCGGGCGGCCGCAGAGGGCAGAGCTATCGATGC
	GTTCCGCGCTCGATTCTTCTTCAGACGGGCGTACG
	AGAGGGAGCGGCTGAGGGCGGTGTGGGAAGAGGG
	AAGAGGGGGAGGCAGC
NF1	CGGCCCAGAGGAGTTAGATGACGTCACCTCCAGGA
	GGACTCGCTTTTTCATTAATGAAACCGGCCGGCGC
	GGGCGCATGCGCGGCAGGCCGCCTTCCCTCTCGCT
	TCCCCCTCCCCTTTC
TP53	CCAGTCTTGAGCACATGGGAGGGGAAAACCCCAAT
	CCCATCAACCCCTGCGAGGCTCCTGGCACAAAGCT
	GGACAGTCGCCATGACAAGTAAGGGCAAGTAATCC
	GCCTGCCGGAGGAAG
ASXL1	CACGCCGGGAAGTGTGGTCCACGCGCTTCACGCGG
	CCATATTCTTCCGGAGCGGGGGAGGCCAGAGAGGC
	CGTTGGGACTACGACTCCCAGGCGCCCTTTCGGTT
	TGTTTTTGTTCAAAA
IKZF1	CCTGGTCTGAGCCGGCTGGGGGCGGGGAGTGTGGC
	GGAGAAATGGGGAACAATGCGAGTGAGCAACTTCA
	GGAAGTCATTGTGAAAGAAAGCTGGGAAGAGCTCC
	GCGGCCAAGTTAGCA

Probe list cont. B.1

Gene	Probe sequence
PAX5	TGTCCTGTTCTCGCCAACATCACAAGATGTCCCCA
	GACACGCCGCGCCCCCAGCGCGCCGCCCCACACTG
	CCGGCCCGGAGCGAGGAAAGGGTAGGCGCTGCGCG
	GCCGGGCCTGCTCAG
JAK2	CAGCCAGGGTGAGTCACTCCCGGCCGCTTCCCTGG
	AGGGCGGCGGCCACACACACACCCGCCCATCTAGT
	GAGGGCCGGGCCCAGCGCTGGCGCCTAGCTCCAGC
	CGGGCACCAGCGTTT
PTPN11	CGGGGGGCAGCTGCACAGTCTCCGGGATCCCCAGG
	CCTGGAGGGGGGTCTGTGCGCGGCCGGCTGGCTCT
	GCCCCGCGTCCGGTCCCGAGCGGGCCTCCCTCGGG
	CCAGCCCGATGTGAC
RUNX1	GTGCCTGGAAATGAACGTGCTTTTACTGTAAGCCC
	GGCCGGAGGAATTCCATTCCCTCAGCTCGTTTGCA
	TAGGGGCGGCCGGCGGCCAATCACAGGCCTTTCCG
	GTATCAGCCAGGGCG
JUN	TCGCTCTCAGGGGGGCGGGCCCGCCCGTCACCATG
	GAGACTCCACCCTAGAAGATTCTTCTCTGGGCCCG
	CGGAGGCTCACGGGATGAGGTAATGCTCCGCTGCC
	CTCCTACAGTCGGGC
LMO4	ATTATTCCGCCTGAACAATCCGGCCGCCCAGTGGC
	TGAGGGTCGCTGACGTCGGAGGCAGAGCCGGGGAG
	TAGTTGGGATTTTGCTCTGTCAGTAACACATGTGT
	AAGAGCCGCGGAGGG

Probe list cont. B.1	
Gene	**Probe sequence**
FLT3	CCGGAGCCCGGGGTCCCCAGGCCGCGCCGGCCCAG
	CCCTGCGATGCCGCCTGGAGCGGCGCGCCTCGCGC
	TGCAGGTGGCTCTCTTAAGGATGCGCGTCACCGAC
	CGCAAATTCCCTCGG
FOS	TGACGTTTACACTCATTCATAAAACGCTTGTTATAA
	AAGCAGTGGCTGCGGCGCCTCGTACTCCAACCGCA
	TCTGCAGCGAGCATCTGAGAAGCCAAGACTGAGCC
	GGCGGCCGCGGCGC
ZNRF1	AAGGGTTAACCCGGCGGGGGGAGGGGGAGGGGGC
	GCGGGCCGCGCCTGCGCTCTGCCGCCCTCCATTGT
	CTCCACGGCGGCGAGGAGCGCCGGCGAGCGCAGCC
	CGGGACCGAGCGGGGC
BCL11A	GCCCCCCTGAGCTGCAAGTTCAAGTGCGGACGTGA
	CGTCCCTGCGAACTTGAACGTCAGGAGTCTGGATG
	GACAGAGACACACAAAACATGGGCAGGGCGAGCAG
	GAGAGAAGGGGAGGA
MEIS1	ACTTGCTGCAGCCCAATGCATTGTGTAAGACGCGA
	CCTGTTATGGCCACCACTACTTCCGGGTTCTAGCA
	TTCTGGTCGGAATCCACCTCTCCGCCTGTGCAACA
	CACACTTTACACACG
ASAP2	CGGCGCGCGGCGGCCAGGAACGGCTCGGCGGGCCG
	GGGCGCGCGCCGCCTTCCTTCCTGCGGGATCCGCT
	TCCCGGCGGCGCGGCAGCGGCGAGCGCGCCGATTG
	GCGGCCCGGTCCTCC

Probe list cont. B.1	
Gene	**Probe sequence**
EZH2	CTTTTATTTGGCCCCCCCAGCCCAATCAAGCGCCGC
	CCCGATTGGCGGGACGCGAGGCGGGCGCCCGTCCA
	ATCACAGGGCCCGCCGGGCTCGGAGGCGCCGAGCC
	AGCCCCACGCAGAG
CDK6	GCGCGGAGAGGTTGCAGGGGCCCCTCGGGGATGAG
	CGAGCGGCGCGGGACGCAGTGGAACGGGAGGGGG
	CGTGCCGAGCAGCCCAGAGTGTGCCGGGAGCGCGG
	GGGAGGGGAGGCGCCG
MYC	CGAGAAGGGCAGGGCTTCTCAGAGGCTTGGCGGGA
	AAAAGAACGGAGGGAGGGATCGCGCTGAGTATAAA
	AGCCGGTTTTCGGGGCTTTATCTAACTCGCTGTAG
	TAATTCCAGCGAGAG
ARID1B	GTGTGCACCACCACCCCCTGCTCCCCCGTCACGAAC
	TCAACATGGCCCATAACGCGGGCGCCGCGGCCGCC
	GCCGGCACCCACAGCGCCAAGAGCGGCGGCTCCGA
	GGCGGCTCTCAAGG
BCL2	GGCGGGAGCGCGGCGGGCGGGCGGGCAGGCGGCG
	CGGAGGGGCGGGCGCGGGAGGAAGGGGGCGGGAG
	CGGGGCTGTGGTGCCTGTCCTCTTACTTCATTCTC
	TGCACAGCCCGACCGGT
BRD4	GCGCCGGCCCCGGCCCACGTGAGCGCCGCGGCCGA
	GCCCGCCTAGAGGACCGCGGACCGGGACAGCTGCG
	CTCGCGGCCCCCGCGCCAGGCCGGGACTTACCCGG
	GCCGGGCGCGCCGCG

Probe list cont. B.1

Gene	Probe sequence
ERG	TTGTGCTAAGTCTGGGAAATGAAGTCAGCCAATGG
	CAGGAAGAGGTTTCTATTGGTCCTGGCTGTCCAGC
	CCAAAGAAACAGGATATTTGGGAGTGGAGAGATAA
	GAGACCCTGAAAACA
JMJD1C	AGCGCGTCTCCTTCCGCCGGCGCGGGCGGGGCAGC
	AGCCGCGGGAGGGTCGGCGGAACCACCGCAGACGG
	AAAGTAGTGCCCGGCCCTGCGGAGGCGGCAGCGGC
	CACTGGGTCCGTCCA
MSL3	ACGGCCGCGCGGAGCTGAGGGACCGGCCGGGCTCC
	TCGGCCTGCGAGGAGCGGTGCGGCCGGCAGGGGGC
	GCTCACAGGGCGCTACGCCCGGGAGTCGGGGCGGG
	GTCTGGGATGCTTCC
TAPT1	GCCGCCTTCTCCCGGAGCGGCCGCGTCGCCGACGC
	CCGCCATGTTCCGAGCACAACAAACTGTCCCCGCC
	GCCTCCCTCCAGCCTCTGCCTCCGGCCGGCCCCCTC
	CCCGCCTCGCCCCG
HIVEP2	GCGCGGCCCCCTCCCCCCAGGACATTACGCAAAGC
	AGCCTCCCCTCATCTGCATAGATCCGCGGCCAGGG
	TATTCCCCGCATTTCCCCACGCGCCCGACACCCCTC
	CCGCGGTCTCCCCC
MEF2C	CCCACACAGAACCTTCAAAGTCAATCCAATAGCAG
	CCCGAAGATGTCTGGGTGTACGCGTGTGCTCAAGT
	GTCTGTGACTGCGAGCAGAGCGAGCAGAGAGCTGG
	AGACGATGTGTGCGT

Probe list cont. B.1

Gene	Probe sequence
PROM1	CTGCTTCGCCACGTAGCCCTGGGGCGCCCACCAGC
	ACCTGGACAGCATCCATTTCCTGTAGAGGTGCCTG
	CTCAACCCCCTGCACTCCCTTCTTGCAACGCTTGCG
	CCTCTTTTCCCCCC
EXO1	GGAAGCCGCAGGAGGCGGAACCGGGTTGCGGGATA
	CGCGTTGACGTCACATCCTCTGGGCGGGGCCGCAA
	GGAACCCGCGCAAATTGAAAGGTCAGCCTTTCGCG
	CGCTGTGTAGGCAAG
WT1	CAGCTTCCCAAAGCTCAAATAAGAGGGGCCGGCGG
	CGCGGGGAAGAGGAGGAGCCAGGAGGCTCGGCCG
	CTCCATTCACTCAGCAGCCCAAGCCCGGCCAGGCA
	GCGCGCGCTGCTCCTT
SMAD3	CTCCGCCCCGCGTTCGGGGCCTTCCCGACCCTGCA
	CTGCTGCCGTCCGCCCGCCCGGCCGCTCTTCTCTTC
	GCCGTGGGAGCCGCTCCGGGCGCAGGGCCGCGCGC
	CGAGCCCCGCAGGC
CXCR4	TTTCTGGCCGCGGCCGGACTTTTATAAAAACACGC
	TCCGAGCGCGGCGCATGCGCCGCTGGGGCGGGGAG
	GGAGAAGGCGGGGTGGGGGAGAGGGGCAGACGCG
	AGGAAGGAGGGCGCAG
ERG	CTTCCAGAAGTTTTCGTTTCTACGCGAATTCCGAC
	CGCAAAATCCAGCTTACCTGAGCGCCGCTCCTCTT
	CTCTCATGTCCCTCGGGGGCCGTCACGACCGTCCC
	GGAGCGATCTCTAGG

Probe list cont. B.1	
Gene	**Probe sequence**
MBNL1	GGGCGCCGGCGGAAAGTTGAAGAGCGTTTTTCTCG
	CCGCCGCGTGCATTAGGAGCTCGACGAGTCCGCCC
	TGGGCTTCCTGGTGGGGCTGGGCGGGCGGGGGAG
	GGGCCGCGCAGCAGCA
CDC25A	AAAGCGGCTCGCGGGACTCTGCCAGGTCATCGGCT
	CTCAGAAGGGGGCGGAGCCCAGAGCAGAAGAGCGG
	GGATCAAAATGAGAGGGGGAGCAGAGGTCAATGAA
	AACTCCCAGCAGAAC
CD38	TGAGGCAAGGGGTTGGGGGTGGGAAGGGAAACAG
	AGAAAAGGCAAGTGAAACAGAAGGGGAGGTGCAGT
	TTCAGAACCCAGCCAGCCTCTCTCTTGCTGCCTAGC
	CTCCTGCCGGCCTCA
TERT	GCGGAAAGGAAGGGGAGGGGCTGGGAGGGCCCGG
	AGGGGGCTGGGCCGGGGACCCGGGAGGGGTCGGG
	ACGGGGCGGGGTCCGCGCGGAGGAGGCGGAGCTG
	GAAGGTGAAGGGGCAGGA
MCM3	GATTGGCTGAGTTCTCTGAGGTCGGACTAGCGACT
	GTCCAATCAAAAGCTTCTTCTTCTTCGGGTTTTCCG
	CTGCGCGCCCCGCGGTGGCGGGAAACGTACCATCC
	GGGATTCTCTGTTC
CASP2	CGCCCTCGCTCTTTCCCCGCCCTTTGCCCCGCCCCG
	CTCCTTCCCCTCCTTTTGTCTGTCCGCCGAGCACCC
	CACTTCACCCCATTGGACCGCGCGGCCGCCGCTAG
	AGCTCTGCGCCTG

Probe list cont. B.1	
Gene	**Probe sequence**
ABCA2	CTCTGCGCGCCCCGCCGGGCCCCGCAGCCCGCCGC
	GCCGCTGGGCATCGCCCGCGCGGGGGCGGGGCGCT
	CGGGCGTCCGGGACCCGATCCGCGCTGGCTCCGCC
	CCGGCGGCCGCGGCG
GNAQ	AGTGGCCGGGGGCGCGCCCGGGAGGGTAGGGGCG
	AACCGCGGGCGCCGGGGGGCGGGGGCGCCGAAGG
	CAGCTGCCCCGCAGGGCCAAGGATGGTGGGCTGGG
	GGCGCAGAGGCCCGGCG
E2F1	GGGGCGGGGCGCGCGCAGGCTTTGTCCGGATGGTA
	CCAGGCGCGGTGTGACTCCCCGCCCCCAGCCCGGG
	CGTCCCCTGCTGGAGGCGTGGGGACACCGGAACAT
	AGGCCCCGCCCCGCC
GFI1	CAGCCCCCAAAACCACAAGCTTCACTTCCTGAGCG
	TTACTGAGTCGCTCGCATAGCCCTCCCAGACCTGCT
	CCCAACCCCCGTCGGGCGGCCGGGCGCGCTCCTTT
	GCGCAAGTGGCCAG
MEF2D	CCCCGCCCCTCGCTTCCCCAGGGCTGGCCGGGCCA
	GGCCGGGTCGGCCCCCACCCCACCGCCCTCCCAAG
	CTAGGCTGCGGCGCTGGGAGCGGCGGATCCCTCCG
	CCTGCCGGGAGGGAG
IKZF1	CCTGGTCTGAGCCGGCTGGGGGCGGGGAGTGTGGC
	GGAGAAATGGGGAACAATGCGAGTGAGCAACTTCA
	GGAAGTCATTGTGAAAGAAAGCTGGGAAGAGCTCC
	GCGGCCAAGTTAGCA

Probe list cont. B.1	
Gene	**Probe sequence**
PAX5	TGTCCTGTTCTCGCCAACATCACAAGATGTCCCCA
	GACACGCCGCGCCCCCAGCGCGCCGCCCCACACTG
	CCGGCCCGGAGCGAGGAAAGGGTAGGCGCTGCGCG
	GCCGGGCCTGCTCAG
RUNX1	GTGCCTGGAAATGAACGTGCTTTTACTGTAAGCCC
	GGCCGGAGGAATTCCATTCCCTCAGCTCGTTTGCA
	TAGGGGCGGCCGGCGGCCAATCACAGGCCTTTCCG
	GTATCAGCCAGGGCG
KRAS	GGCCGGGCGGCCGCAGAGGGCAGAGCTATCGATGC
	GTTCCGCGCTCGATTCTTCTTCAGACGGGCGTACG
	AGAGGGAGCGGCTGAGGGCGGTGTGGGAAGAGGG
	AAGAGGGGGAGGCAGC
End of Table	

B.0.2 MCC probes for OCI-AML3 cells

Table B.2: Probes used for MCC in OCI-AML3 Cells.

Probe list for MCC in OCI-AML3 cells	
Gene	**Probe sequence**
ARID1B	GGGGACGAGTCCAGTCCGCGTTTTGCGAGTGCGCG
	GGAGTAATGCGAGCGAAGTGGATAATAGTTGCTCG
	AGCTCGCCCGCTGCCTCTCAAGCCATGCTGGGCCC
	GATAGGCTCAGCTAG
ARID2	GTGTGCGTGTGAAATGTGCGGCACATTGCAGACGA
	ACCCTAAGCCCGGCGAGTCGAGTCTATTGTTCCCC
	GCGAGGATCTGCCGGGGCGGTGTGGAGCCAGGCGC
	AGTGCCGCGGCCAGC
ASXL1	CCACCCAGCCCCTTCCCCGAACAGCGACCCGTCCCC
	GTTCCTAGCTGAGGTCGGTGCTAGGCGGAGGACGC
	GAGAGGGAGAGCGGAAGGGGGCCGCCGGGCACGC
	CGGGAAGTGTGGTCC
BAHCC1	ATGATCTGCGCAGAGGAGCTGCGCAGTCGCCGGGC
	TTGAATTAGGCGCCATCGGGCTTGGTAGTAGCCCC
	GCTGCTTCCTGATTGGCAGTTCCCTGGCCCGGCGC
	CAGCCTATTGGGAGG
BCL2	GGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTGT
	GGTGCCTGTCCTCTTACTTCATTCTCTGCACAGCCC
	GACCGGTTTCCTGTGCGTAACGTCACACGGTTCAT
	TCAAAAAAAGAAGAA

Probe list cont. B.2	
Gene	**Probe sequence**
CDK6	CCCCTCGGGGATGAGCGAGCGGCGCGGGACGCAGT
	GGAACGGGAGGGGGCGTGCCGAGCAGCCCAGAGT
	GTGCCGGGAGCGCGGGGGAGGGGAGGCGCCGGGC
	ACGTCAATGTCACGGCT
E2F1	AAGAGCCACGAGCCGCCGAGCGCCGCCACAATTGG
	CTGCCCCGGCCGTGACGGGAACAACGGCGGCGGTT
	CCTATTGGCTTTAACGCGCCGGACCCGGCGGCGAG
	GGGCGGGATGGGGCG
EZH2	GCGGCGGGGTTCGCTGTAAGGGACGCCACTGGCCG
	TGTGGAAGCGCCGCCGCCGTCTCTTTGTTCTTTCG
	CTGAACACACGGCGCCTCTCAGGAAGGCGGTGTGC
	AGGCACGCGGGGACC
FLT3	CGTCACCGACCGCAAATTCCCTCGGACTGGTGCAA
	AGTGGAAGGGGGGAGGAACCCTCTCCCCAGAGGCA
	GGGACCGAGGCGGGAGCGGAGAGGTTGGGCAGAG
	CCGAGCAGCCGGTGCC
IKZF1	GAGTGAGCAACTTCAGGAAGTCATTGTGAAAGAAA
	GCTGGGAAGAGCTCCGCGGCCAAGTTAGCAGGACA
	CTCTAACAAGTGACTGCGCGGCCCGCGCCCGGGGC
	GGTGACTGCGGCAAG
IRF8	AGAAGAGGCCCGGACCCCACTGCCGTGGCTGCCGG
	GGGCGGGAAAGTGATTTCTCGGAAAGCAGAGCACT
	TCGAAGAAGGCGGGCCGCGCGAGCCAAGCTGACGC
	TATTGGTCGGTGTGG

Probe list cont. B.2	
Gene	**Probe sequence**
IRX5	GGAGGAAGAGGGAGAGGGCGAGGCGCGCCTGGCG
	GCCGGGTTGGCTGCGGCCGCCCAGAAGCTCCTGCC
	AGTCCTCCCACCGACTACACCCCGGGAAGGAGGAG
	CTTCGGGCGCGCACAA
ISL2	GATCCTTGTAGGTTTCGGAGGTGGGGGGGCTGCAC
	TCCATTGTTCACTCCGGGCCAATCAGGGTTGGCCC
	ACTTCCTCCCAGCCAATCTCCCTTCACCCCCAGCCT
	CCAACCCAACCCAC
MEF2C	CATGCAGCCACGGCGACCCACACAGAACCTTCAAA
	GTCAATCCAATAGCAGCCCGAAGATGTCTGGGTGT
	ACGCGTGTGCTCAAGTGTCTGTGACTGCGAGCAGA
	GCGAGCAGAGAGCTG
MEF2D	AAGGGACGAAGCGAGGCAAAGTAGGGAGGCGAGA
	CCCAGAGGCCTTTAAAGGGATCGGTCCGCTCCCAC
	GGTGCCCCTCGGAGCACGCCGGCCGCCCGCCCGCC
	CGCCTGGCGCTGCTGC
MEIS1	CTGCCGAGGAGCCTACTTGCTGCAGCCCAATGCAT
	TGTGTAAGACGCGACCTGTTATGGCCACCACTACT
	TCCGGGTTCTAGCATTCTGGTCGGAATCCACCTCT
	CCGCCTGTGCAACAC
MKX	CTCCCAGGACTCCCGCGCGCGGCCACCCGCGCCTC
	TTCTCAAATCACTTACCCCGATTCACTCCAGACTGT
	GGCCGGGGAGGTCACTCCCTGCAGAAGTGTCCCCC
	TCCCCCAACGCCGG

Probe list cont. B.2	
Gene	**Probe sequence**
MYC	GCGGGAAAAAGAACGGAGGGAGGGATCGCGCTGA
	GTATAAAAGCCGGTTTTCGGGGCTTTATCTAACTC
	GCTGTAGTAATTCCAGCGAGAGGCAGAGGGAGCGA
	GCGGGCGGCCGGCTAG
PHF10	TGCACCAGGGCACTTGTAGGACCGCACTAAAAACA
	GCGGAAAGTGAGGAGCCAAGCCTGGGTCCGGGGCG
	GCCCGCCGTACAGCTGGCCTCACGGATTCCACTGC
	CTGCGCCTGCAGATG
PIK3R5	CGTCGGAAGCGCGGCTTTGGCCCCGCAGGAGGGCT
	GGGCGGAGCAGGGGGGCAGGTCTGGGGTAGGGGC
	TTAGGGGAAAGACGAGAAAGAGGAAGCTCCGACGA
	GCTGCTTCCTTCTCTC
RUNX1	AATGAGAGTGCCTGGAAATGAACGTGCTTTTACTG
	TAAGCCCGGCCGGAGGAATTCCATTCCCTCAGCTC
	GTTTGCATAGGGGCGGCCGGCGGCCAATCACAGGC
	CTTTCCGGTATCAGC
RUNX2	GCGGCAATGCCGGCCCCGGGGCTGGACTGCTGAAC
	CCACACCGCTTCACCACCGGACTCCAACTGGGAGG
	GGGCGCCAGCGCACCCAGGACGCGGTGCCCCAAGG
	GACCCCATTCCAAGC
SATB1	GTTACTGTAGTGTGTGTGCAGGCGGAGGAGGAAGT
	CAGGTGAGAGGCCGCGAAGCACCCCCACCCCAACC
	CAGCCTGCGTGGGGTGTATGCTTCCCCACTAGGGG
	CATTTGGGCCATTTT

Probe list cont. B.2	
Gene	**Probe sequence**
SATB2	GAGGAAGGGGAGAGTCGGCGGTCGGAGCGGGGTG
	ACGAGGACCTCATGCACGAGGCGGCGAAGGACCGA
	TGGCAGAGAACCGAGTGCGGCGCGAGGCGGTGGCC
	GCGGCCAGCGGGTGGG
STK11	TGTATCCACCCCTCGGGCGGGGCCAAGCGCCGACC
	AATCGCCGCTCGGGCGCCCGGCCGGGTCCAAACGC
	TCCAATCGTCAGCGGCGGCGGGGCGGGCAGAGGGC
	CGGGGATGGCAGGTT
ZEB2	CTTTTCTGTAGGAGAGAGACGAGAGACCCTGAAAC
	ACGCGCCACCTATCTTTGTGGGGAGGGATAATTGA
	AGCGCCCTGAGCGTGAGCATCATGTGAAAACGTGA
	TCGCCAAGTTTCTCT
UNCX	TCCGGCTCCCCCCGCCCCCAGCCCGTCTTGTTGTGA
	CAGTTCCTGATACTGTTTATTGAGGTGCATGTCAG
	GTATAATTAGCAATCGATATGGAAAACGTTTCCCT
	GCACCCAGCACGCC
PTPN7	GAAGCCAGCTTCCTCCCTCCGCCCCTCCTTGCTGCC
	ACCCACGCACACCCCAGCTGCATTCTGCCCTCCTGT
	GCCTGCTGCCGCTGCCACCAGGGGTCGGCTGCCTC
	CCGCCTGTGCCCT
ETS2	CGGCTCCCAGGGCGCACACTCGCGCGCACGTGGGG
	CCGAGGCCCTGCTCCCGGGGCCTCAGGGCCAGCCG
	GCGAGGGACCCAGCCGAGTGACAGCAGGAGGCGGA
	GGGAAGGTTGGGCCG

Probe list cont. B.2	
Gene	**Probe sequence**
NRAS	CCCAGGCGCCTGAGTCCCCGCCCCGGCCACGTGGG
	CCTCCGAACCACGAGTCATGCGGCAGGCCGCACCC
	AGACCCGCCCCTCCCACACGGGACGTTTCAATAAT
	GAAAGCGCTAGGTGC
SPI1	CTCCCTTGACATTGCAGGGCCAGCACAAGTTCCTG
	ATTTTATCGAAGGGCCTGCCGCTGGGAGATAGTCC
	CCTTGGGGTGACATCACCGCCCCAACCCGTTTGCA
	TAAATCTCTTGCGCT
DOCK2	AGCGGGGAGCCCCGGCGAGGGAGGAGAGGGCGGG
	CCCTGAGAGGAGTTTGAAAAGAGGAAGGAAGTGGG
	GCCCTGCGGCGCCCAGCCACCCCCTGACGGCTTCC
	CCACGGGAGGACGCGA
CBFB	TGGCCGCGGGCGAGCAGGGGGAAAAGGGCCCTGAT
	GCGGAAGGAGCCCCGGGGCGCCACAGCCAGGCCGC
	TCCGCCAGCGGGCGGGTGGCGCATGCGCGGCGTGG
	GCCGCGGAGTTGTGA
FZD1	GTCCCTGAGCCTCCAGTTCTGCGTCGGTTTCAAGG
	CTCCTCCCTCCTGGTGAAAGACAGACTACGGGGCG
	CCTGGAAACCGGTCCGAGGGCGCGCGAGGCAGAGG
	AGAGGGAGCGAGTTG
JMJD1C	AACACTCCTTTGGACTCCCAGATTCGCAGCCTTGT
	GCTGCAGCGCCACACAAGAAAACTGAAACAAAACC
	CAACGCGGCCGTCGAAGACCCCGAGGCAGCCCAGC
	CGCCGCCACCGCGCC

Probe list cont. B.2	
Gene	**Probe sequence**
KRAS	CCGGGCCGGCGGGGGAGGAGCGGGGGCCGGGCCG
	GCGGAGGAAGGGGTGGCTGGGGCGGTCTAGGGTG
	GCGAGCCGGGCCGGCTGGAGAGCGGGTCTGGGCG
	GCGCCTTGGCGGGAGGAG
XPO1	TCGGAGGAGGCATTTAGACCAGGCGCCTTAAGTTC
	ACCGCCCCTTTCTCTATGCAGCATGGCCTCGGCGT
	GATCAGAGGAGGTCCGGGCTGAGCCAGTACTGAAT
	GCTTCCTCTACTACA
IGF2BP2	CGGCCGGCCCGGCCCGCCCGGGGGCAGAGGCGGAG
	GCGGGGACTCGGCGCGGCTGCCTCCTCGGGGCAGA
	GTCCCGGGCCGGGCGGCGGCGCGCGGACAAAGCGC
	TCTCTCTGCCTCCCT
PTBP1	CCGCCTGACTCGCCACGTACCCACTCTCAAGATGG
	CGTCTCCCGCAAAGCCACGCGCCAGCTGACTGGCT
	CAGACGCCGTCACGTGATCCCATTTTCTCCCCCTCC
	CCCGACTACACAAG
SMC4	GTACCTCCCCGTCACCATAGTGACTTCTAAGAGTT
	ACGCTCCCTTCCTCTTCCCGCGATACTTCTACGCGG
	CACGTCATCCGAGTGGGCCCGAGACCCCGCCTCTC
	GGCGAGCCCGCCCT
PTMA	CGGCAAACTCCGGCCTTGAATGACAGTGCGATCGG
	CGACTGCGCAGCGCGGGACGCGCCGGGGCACTGCC
	GCTTTAAGCACGCTTGTCCATTGTTCGGAATCGAG
	CCAATGAGCGAGCGC

Probe list cont. B.2

Gene	Probe sequence
SOX4	CCGGGGGAGGGGTTTGGCATGAGGAAGCGTTGGTT
	ACAGCAGCTGATTGGCTGCAGCCAAGACTGTGAAA
	GGATAAAGAGGCGCGAGGCGGAATTGGGGTCTGCT
	CTAAGCTGCAGCAAG
CDKN1B	CTTAATTTTAATTTCTCCGAGGCCAGCCAGAGCAG
	GTTTGTTGGCAGCAGTACCCCTCCAGCAGTCACGC
	GACCAGCCAATCTCCCGGCGGCGCTCGGGGAGGCG
	GCGCGCTCGGGAACG
LHX2	GCCTGGGGCGCCGAGCGGCCAATGGCACGGCGGCA
	GGACGTGATGTCAGGCGCGGCTGTAGAAAAGGCGC
	GGAGGCTTGCGCTGGCGCGGACTGCAGAGCCGGGG
	CTGGGCTAGGCGCGC
SMAD3	GAGCGAGCGGCGAGCCGGGAGGAGGAGGGTGGCG
	GGGCGGTGAGGCCGCAGAGGCGGAGGGATCTGCG
	CATCAAAGCTAGCGAGGCGAGCGAAGTTTGGCCGG
	GGGTTGGACTTTCCTTC
CXCR4	GCCCAGGGGACCCTGCTGTTTGCGGGTGGTCGGTA
	GTGAGTCCGGAGCCGGACAGGACCTCCCAGAGGCA
	TTTCCTAAGTTTGAGGGAAGCGGGATGCGCCTGAA
	GACAGGTGGGAAGCG
TERT	GGCAGCGGGGAGCGCGCGGCATCGCGGGGGTGGC
	CGGGGCCAGGGCTTCCCACGTGCGCAGCAGGACGC
	AGCGCTGCCTGAAACTCGCGCCGCGAGGAGAGGGC
	GGGGCCGCGGAAAGGA

Probe list cont. B.2	
Gene	**Probe sequence**
GNAQ	AGTGGCCGGGGGCGCGCCCGGGAGGGTAGGGGCG
	AACCGCGGGCGCCGGGGGGCGGGGGCGCCGAAGG
	CAGCTGCCCCGCAGGGCCAAGGATGGTGGGCTGGG
	GGCGCAGAGGCCCGGCG
TAPT1	GCCGCCTTCTCCCGGAGCGGCCGCGTCGCCGACGC
	CCGCCATGTTCCGAGCACAACAAACTGTCCCCGCC
	GCCTCCCTCCAGCCTCTGCCTCCGGCCGGCCCCCTC
	CCCGCCTCGCCCCG
DNMT3A	CGGTGTCAAGTGCCTCCCCCCGCAGCCCGCGGAGC
	CCCCGGCGGCCTTCGCCCTGCAGTTCTCCCGACCTC
	CGCCAGAGCCCACTGCGCTCTGCCTGCCTCAGCAC
	TGGGGCTGGGGACA
IDH1	CAATTCCCGTGGGATGGGCGGAGTCAGCGAGAGGG
	GCGTGGCCACAGTCAAAGATGGAGAATCCATGGTG
	CCACTTTCCCGGTGATCCCGGTGGGGGAAGGGGTT
	GCCGCCCGGCAGAGA
IDH2	CCCGGCGGCCAGGCCCGGCTGGTGCTTCTCTGGGC
	GCCGCTGAAAGCCCAGCTCCGCAGGAGCTACAGGC
	GTGAGACCCACCGGGACCCGCGAGCGGGATCAGGG
	CTGGGGGATTAGCAG
CEPBPA	GTTAGAGTTCTCCCGGCATGGCGAGCCTCGGCGGC
	CTCCAGCCTGCGCGGGGCGTCGCCGCCGCCCACCC
	GGAGACCCTGCTCGCCCGCGCCCGCGCACCTCCGG
	GTCGCGAATGGCCCG
End of Table	