Chapter 11
Cellular and Molecular Basis of *KMT2A/MLL* Leukaemias: From Transformation Mechanisms to Novel Therapeutic Strategies

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Contents

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Abstract The Mixed Lineage Leukaemia (KMT2A/MLL) gene encoding a H3K4me3 histone methyltransferase is a frequent target of chromosomal aberrations resulting in various forms of aggressive leukaemia with poor prognosis. Treatment of MLL leukaemia presents a major challenge, thus novel and more effective therapies are urgently needed to improve patients' outlook. While internal deletion and amplification of MLL have been reported, MLL mutations mostly manifest either as chromosomal translocations resulting in the generation of fusion proteins, in which the C-terminus of MLL is replaced by 1 of more than 70 identified fusion partners, or as partial internal tandem duplications (PTD). Some of the most frequent MLL fusion partners exist in multiple complexes associated with histone methyltransferase, DOT1L or positive elongation factor b (P-TEFb). Aberrant recruitment of these complexes by MLL fusions among other mechanisms such as dimerization of MLL fusions or recruitment of other histone modifying enzymes resulting in aberrant transcription of downstream targets such as *HOX* genes has been identified as critical steps in MLL fusion mediated transformation. Among them are various key components of epigenetic machinery including DOT1L, PRMT1 and BRD4, which emerge as promising therapeutic targets. On the other hand, recent studies also identified other essential pathways and molecules such as beta-catenin, ITGB3/SYK, polycomb proteins that are not necessarily under the direct control of the MLL fusions. While development of small molecule inhibitors against most of these emerging targets is still in very early stages, the latest development of DOT1L inhibitors currently in a phase I clinical trial on MLL leukaemia demonstrates the promise of translating our knowledge into novel treatments to improve the outcome for *MLL* leukaemia patients.

Keywords Acute leukaemia • KMT2A/MLL leukaemia stem cells • Cell of origin • Epigenetics • Targeted therapy

11.1 Introduction

Acute leukaemia is characterized by rapid expansion of immature white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. Clinical classification based on lineage characteristics of the leukaemic

blasts can broadly divide acute leukaemia into acute myeloid leukaemia (AML), acute lymphoid leukaemia (ALL) and acute biphenotypic leukaemia (ABL), which features markers of both myeloid and lymphoid cells. While ALL is the most common cancer in infants and children, ABL uniformly present at all ages whereas the incidence of AML increases with age. Recurring chromosomal aberrations tend to associate with particular subtype of acute leukaemia, e.g., t(15;17) with *PML-RARA*, t(8;21) with *RUNX1-RUNX1T1*/*AML1-ETO* found only in AML; whereas t(1;19) encoding *TCF3*/*E2A-PBX1* only occurs in ALL. Interestingly, 11q23 chromosomal aberrations involving the *KMT2A*/*MLL* (*mixed lineage leukaemia*) gene, which generally confer very poor prognosis, are found promiscuously in ALL [1], AML [2] and ABL [3]. *MLL* rearrangement can be found in up to 80 % in infant leukaemia, 3–10 % in ALL and AML, and up to 18 % in ABL. The *MLL* gene located at 11q23 is the human homolog of *drosophila trithorax* (*trx*), and encodes a SET-domain histone methyltransferase (HMT) that tri-methylates histone 3 lysine 4 (H3K4me3) positively associated with transcription. 430 kDa MLL protein also contains N-terminal DNA binding domains (AT-hooks and CXXC domain) as well as central PHD fingers and a transactivation domain. MLL protein is proteolytically cleaved between PHD fingers and the transactivation domain into two fragments (MLL-N and MLL-C), which specifically associate via consensus interaction motifs to regulate gene expression for normal development, including haematopoiesis. In contrast, its mutations in the haematopoietic system lead to acute leukaemia. In this book chapter, we will review the recent advance in understanding the cellular and molecular basis of MLL leukaemia. We will discuss existing *MLL* leukaemia models, the potential cell of origin of MLL leukaemia, compare the molecular functions of wild-type MLL with oncogenic MLL fusions, highlight critical pathways/molecules in MLL leukaemia, and finally describe current therapies and potential development of novel targeted therapies for MLL leukaemia.

11.2 11q23 Abnormalities in Acute Leukaemia

Four recurrent 11q23 chromosomal abnormalities have been identified in acute leukaemia, namely chromosomal translocations, partial tandem duplication (PTD), amplification and internal deletion (Fig. 11.1).

While *MLL*-PTD, amplification and deletion can be found in AML, *MLL* translocations remain the most recurrent 11q23 abnormality in all different acute leukaemia subtypes. The treatments of leukaemia with 11q23 aberrations remain a major clinical challenge. Although cure rates of up to 80 % can be achieved for non-11q23 childhood ALL, the outlook for patients with 11q23 abnormalities is far worse [4]. Similarly, the presence of 11q23 translocations generally confers poor prognosis in AML [2] and in ABL [3].

Fig. 11.1 Schematic overview of wild-type KMT2A/MLL protein and the aberrant KMT2A/MLL proteins resulting from different 11q23 chromosomal aberrations. The incidence of the individual 11q23 aberration amongst all 11q23 re-arrangements in infant/childhood and adult ALL and AML are indicated (Data modified from [7]). All 'rare' MLL translocation partners are presented together in 'all others'. Functional protein domains of MLL are indicated. AT-hooks and CXXC, DNA binding domains; FRYN/FRYC, consensus interaction motifs; TAD, transactivation domain; SET domain, mediates H3K4me1,2,3 methylation

11.3 *MLL* Chromosomal Translocation Fusions

Pre-natal backtracking of concordant leukaemia in identical monozygotic twins provided evidence that 11q23 translocations can occur in utero [5], and the very short latency (<1 year) to develop full blown leukaemia in infants suggest few (if any) additional mutations are required. This may differ from the aetiology of leukaemia harbouring 11q23 translocations found in adults where the cellular origin of the disease can be very different. On the other hand, *MLL* translocations not only occur in de novo acute leukaemia, but are also frequently observed in therapy related AML (t-AML). Strikingly, compared to de novo acute leukaemia where 11q23 translocations occur in similar frequencies in AML and ALL, the vast majority (>90 %) of 11q23 translocation therapy related leukaemia is t-AML, that arise after topoisomerase II treatment of an unrelated primary neoplasm [6].

All *MLL* translocations occur within the same 8.3 kb breakpoint cluster region between the CXXC DNA-binding domain and the central PHD fingers. As a result, all MLL fusions retain the MLL N-terminus with its DNA binding motifs, but the C-terminal SET domain is replaced with the fusion partner that may possess other transcriptional effector or homo-dimerization domains. Although more than 70 different MLL fusion partners have been identified and can be broadly divided into nuclear and cytoplasmic proteins, AFF1/AF4, MLLT3/AF9, MLLT10/AF10, ELL, MLLT1/ENL and MLLT4/AF6 account for more than 85 % of cases [7]. With the exception of AF6, they are all nuclear proteins. Strikingly, the most frequent nuclear fusion partners biochemically interact with each other [8], and many of them were subsequently found co-existing in protein complexes. The first description of such a complex was named ENL associated proteins (EAP) [9, 10] (Fig. 11.2), which included amongst other components also known MLL fusion partners ENL, AF9, AF4, AFF3/LAF4, AFF4/AF5Q31 as well as the H3K79 histone methyltransferase

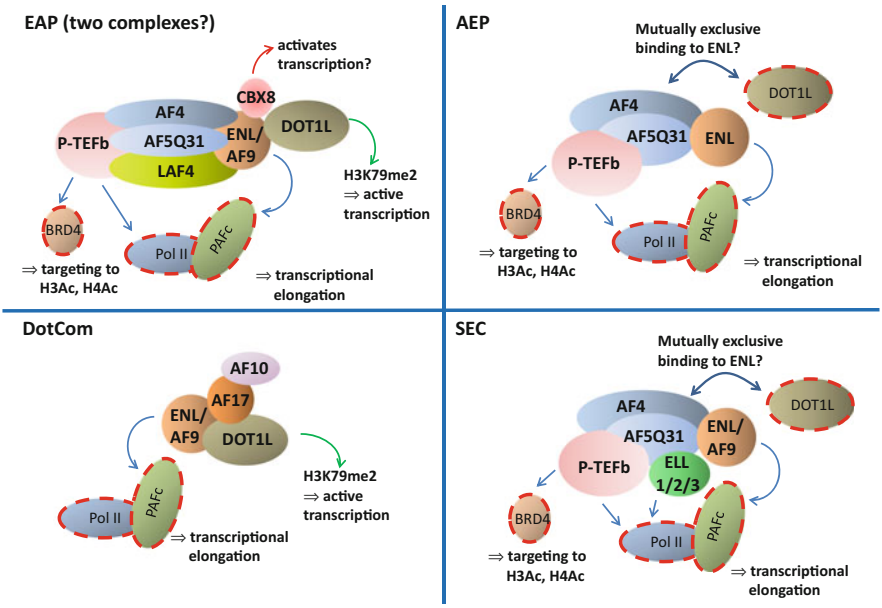

Fig. 11.2 Schematic overview of the complexes associated with the most common KMT2A/MLL fusion partners. *EAP* ENL associated proteins, *AEP* AF4 family/ENL family/P-TEFb, *DotCom* Dot1L complex, *SEC* super elongation complex. Proteins not originally identified in these complexes but shown to interact with components of these complexes, indicated with *blue arrows*, are marked with a *dotted red outline. Green arrows* indicate H3K79me2 chromatin modification mediated by Dot1L, whereas *red arrow* indicates potential positive transcriptional effects. BRD4 recognizes and binds to acetylated histones and interacts with P-TEF-b. PAF1 promotes transcriptional elongation of RNA polymerase II (Pol II) and interacts with ENL/AF9. Pol II itself interacts with P-TEFb and ELL/ELL2/ELL3. A mutually exclusive binding of ENL to either AF4 or DOT1L was suggested [12]

DOT1L and the positive transcription elongation factor b (P-TEFb) that plays an essential role in regulation of the transcription by RNA polymerase II.

However, more recent biochemical and functional studies have identified three slightly different complexes, namely the super elongation complex (SEC) [11], AF4 family/ENL family/P-TEFb (AEP) complex [12] and DotCom [13]. While AEP and SEC largely overlap and contain P-TEFb as well as known MLL fusion partners AF4, AF5Q31 and ENL (note that SEC also contained ELL1/ELL2/ELL3 and AF9), they do not have DOT1L. On the other hand, DotCom lacks P-TEFb but contains DOT1L plus MLLT3/AF9, AF10, MLLT6/AF17 and ENL. These results suggest the presence of at least two different complexes (one with and one without DOT1L) associated with the most common nuclear MLL fusion partners (Fig. 11.2) that may link transcriptional elongation with histone methylation leading to deregulated target gene expression, such as *HOX* genes. Interestingly, while the incidence of the different MLL fusion partners in ALL as well as AML changes with the age of the patients (Fig. 11.1), certain MLL fusions are almost exclusively associated with AML (MLL-ELL) or ALL (MLL-AF4) whereas others can be found in both (MLL-AF9, MLL-ENL). Therefore, some MLL fusions may in part determine the lineage specificity, although other factors including cell of origin and microenvironments likely also have important roles in controlling the MLL leukaemia phenotypes. On the other hand, given that transcriptional complexes associated with an individual MLL fusion are likely similar in AML and ALL, these findings suggest a lack of functional relationship between transcriptional complexes and the leukaemia lineage.

11.4 MLL-Partial Tandem Duplication (MLL-PTD)

MLL-PTD occurs within the same breakpoint cluster region observed in *MLL* translocations, but results in an internal duplication of N-terminal sequences flanking the DNA binding domains AT hooks and CXXC. Importantly, this re-arrangement of MLL does not affect C-terminal sequences of MLL. Therefore MLL-PTD possesses a functional H3K4me3 SET domain and has been reported with strong transcriptional activity [14]. MLL-PTD predominantly occur in AML (~5 %) and is enriched in AML with trisomy 11 [2], although very rare cases in ALL have been reported [7]. While MLL-PTD enhances self-renewal of haematopoietic progenitor cells, it does not induce AML in mouse model, suggesting additional mutations are required for full-blown leukaemia [15–17]. Similar to *MLL* translocations, the presence of *MLL*-PTD in AML confers a poor prognosis with markedly reduced remission duration [18].

11.5 MLL Amplification and Internal Deletion

In contrast to *MLL* translocation and *MLL*-PTD, *MLL* amplification and internal deletion are much less frequent 11q23 aberrations. *MLL* amplification is mainly the result of intrachromosomal (HSR, homogenous staining regions) or extrachromosomal (dmin, double minute chromosome) amplifications, and may confer poor prognosis [19]. *MLL* amplification is predominately found in AML (up to 1 %), but a case report of its existence in ALL has also been described [20]. On the other hand, only three ALL patients have been reported with internal deletion in one *MLL* allele, in which exon 11 together with parts of intron 10 and 11 (new nomenclature) were lost [19]. Since *MLL* translocations represent the most frequent 11q23 abnormalities in acute leukaemia and *MLL*-PTD/amplification has been recently reviewed [19], we will focus our discussion on *MLL* translocations in the rest of the chapter.

11.6 Modelling *MLL* Leukaemia

11.6.1 AML Models of MLL *Fusions*

In the past two decades, a number of *MLL* leukaemia models have been reported that recapitulate several aspects of the human disease. A breakthrough in modelling *MLL* fusions came around 4–5 years after the identification [21] and cloning of *MLL* gene at 11q23 [22, 23] when Rabbitts lab [24] created the first *MLL* fusion mouse model. Using knock-in technologies in ES cells, the cDNA of the fusion partner AF9 was inserted just after the exon 8 (old nomenclature) of the *MLL* gene resulting in an MLL-fusion gene under the expression of the endogenous *MLL* promoter. Chimeric MLL-AF9 knock-in mice developed AML with a latency of 4–11 month. The second seminal *MLL* leukaemia model was established by the Cleary lab using retroviral transduction to transfer MLL-ENL into murine primary haematopoietic progenitor cells. MLL-ENL expressing murine primary haematopoietic cells induced myeloid leukaemia when transplanted into mice with a shorter latency of 2–5 months [25]. This system has also been used to define and describe the disease progression from pre-LSC (pre-leukaemic stem cell) to LSC [2, 26]. In order to mimic and recreate the chromosomal translocations found in human acute leukaemia patients rather than just the expression of the dominant der(11) transcripts, the Rabbitts lab further pioneered in the generation of the translocator mice [27]. These mice harboured loxp sites within the Mll and Af9 genes at specific introns, which correspond to the breakpoint regions found in MLL-AF9 acute leukaemia patients. Exposure to *Cre*-recombinase resulted in the interchromosomal recombination of Mll and Af9, thus creating both derivative transcripts and resulting in AML [28]. A similar approach was also used in Mll-Enl translocator mice that rapidly developed myeloid leukaemia after *Cre*-recombination [29] with latencies similar to that

observed in the retroviral transduction model. The use of a conditional *MLL* fusion by employing either the mutant oestrogen receptor/tamoxifen [30] or Tet off/doxycycline system [31] together with retroviral transduction of murine primary cells has further demonstrated the biological relevance of MLL fusions and their downstream targets such as *HOX*/*MEIS* genes in MLL leukaemia. Modified retroviral transduction approaches using MLL fusions and human lineage negative or CD34+ primary haematopoietic cells isolated from cord blood led to the first humanized in vivo leukaemia model [32, 33]. Together, these landmark studies demonstrated the feasibility of modelling AML as a result of 11q23 translocations/fusions, and these models have been widely used to gain mechanistic insights into the underlying disease mechanisms. However, modelling the ALL phenotype observed in 11q23 patients proved to be more challenging.

11.6.2 ALL Models of MLL Fusions

The first ALL model of MLL leukaemia was reported in a murine retroviral transduction approach using *MLL-GAS7*, which was capable of transforming HSCs leading to multiple lineage leukaemia including ALL, AML and ABL [34]. *Mll-Enl* translocator mice developed T-ALL among other haematological malignancies when *Cre* expression was restricted to the t-cell compartment using a *Lck-Cre* [28]. On the other hand, immuno-compromised NOD/SCID mice transplanted with *MLL-ENL* or *MLL-AF9* transduced human primary cells developed ALL, or ALL/AML respectively [33]. Interestingly, the phenotype of leukaemia seems to depend largely on the microenvironment of the recipient mice as *MLL-AF9* transduced human CD34+ cells have shown different disease outcomes, ALL vs AML, depending on the recipient mouse strain [32]. Modelling the ALL disease phenotype of *MLL-AF4*, the most frequent *MLL* fusion associated with ALL has also been challenging. Early unsuccessful attempts to model *MLL-AF4* in murine primary cells using a retroviral approach were succeeded by two mouse models (knock-in and inverter), in which *MLL-AF4* expression led to B-cell lymphomas with long latency and low penetrance [35–37]. A much improved conditional *MLL-AF4* model was reported to have about half of the knock-in mice develop B-ALL [38]. However, the observed ALL phenotype was still different from that observed in patients and the other half of the animals developed AML, suggesting that the right cellular target might have been missed in these studies [39]. Of note, another *MLL-AF4* murine retroviral transduction model has been reported, in which *MLL-AF4* as well as the reciprocal translocation product *AF4-MLL* causes B-ALL with a long latency and low penetrance [40]. Surprisingly, this study also showed that *AF4-MLL* alone is sufficient to cause leukaemia, suggesting a critical role for the reciprocal product in the pathogenesis of *MLL-AF4* leukaemia. However, it should be noted that the

reciprocal MLL fusions, including AF4-MLL cannot be detected in all patients harbouring *MLL* translocations [7, 41, 42].

11.6.3 ABL Generated by MLL Fusion

While ABL comprises only 2–5 % of all acute leukaemia cases [3], bi-phenotypic leukaemia expressing MLL fusions have been frequently described in different model systems. The first ABL models were reported using retroviral transduction approach on murine cells with *MLL-ENL* [43] or *MLL-GAS7* [34]. In both cases, *MLL fusion* transformed cells expressed both myeloid and lymphoid markers, and induced ABL in mice with a relative short latency. ABL was also observed in three *MLL-AF4* mice [38] and one *AF4-MLL* mouse [40], respectively, Interestingly, ABL was recurrently observed in the retroviral transduction approach with human CD34+ primary haematopoietic cells isolated from cord blood [32]. These results provide further experimental evidence for the specific association of *MLL* fusions with lineage infidelity/promiscuity.

11.7 Cell of Origin for MLL Leukaemia

In order to define potential origins of MLL LSCs, phenotypically and functionally defined haematopoietic populations (such as HSC, haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte erythroid progenitor) were purified from mouse bone marrow for retroviral transduction and transformation assays. While MLL-ENL failed to transform MEP, it induced phenotypically identical myeloid leukaemia when expressed in HSC, CMP and GMP populations [44]. In contrast, MLL-GAS7 was reported to transform HSC, CMP and GMP but with different leukaemia phenotypes. MLL-GAS7 expression in HSCs could produce multi-lineage leukaemia whereas its expression in CMP and GMP led to exclusive myeloid transformation [34]. These results together with subsequent global expression analyses on MLL LSC-enriched populations [45, 46] revealed that MLL fusion can induce a self-renewal programme in otherwise short-lived myeloid progenitor cells in AML, whereas HSCs may be the cell of origin for multi-lineage leukaemia. On the other hand, an important insight about the potential origin of LSC was obtained in a study examining the transformation abilities of HSCs, CMPs and GMPs expressing MLL-AF9 driven by endogenous *MLL* promoter in *MLL-AF9* knock-in mice. While *MLL-AF9* expressing HSCs led to AML, GMPs expressing

MLL-AF9 driven by the endogenous *MLL* promoter, which is in contrast to those driven by MSCV retroviral promoter [45], failed to induce leukaemia, highlighting the importance of the expression level in determining the transformation ability of MLL fusions in different cellular targets [47]. Although it is feasible to compare murine retroviral and knock-in models, it is more challenging to assess the impact on MLL fusion expression levels in the human system. Recent technological advances in genome editing technologies utilizing custom made zinc finger nucleases (ZFNs) [48] or transcriptional activator-like effector nucleases (TALENs) [49] that recognize any given DNA sequence with high specificity can facilitate the creation of novel MLL leukaemia models in human primary cells where the MLL fusion is expressed at physiological levels. Such a model system could also provide unique insights into the long sought-after infant ALL leukaemia with an extremely brief latency. Given that MLL fusions arise in utero, it would be important to assess the impact of physiologically expressed MLL fusion in early, embryonic haematopoiesis if hES or iPS cell are employed.

11.8 Transcription Regulation by MLL and MLL Fusions During Normal and Disease Development

11.8.1 Wild-Type MLL and Its Transcriptional Complex

It had been realized very early on that the *MLL* gene shares significant homology with the drosophila trithorax (*trx*) gene [22]. Trx is the founding member of the trithorax-group (Trx-G) proteins, which antagonize the polycomb group (Pc-G) proteins to maintain cellular memory/identity by epigenetically maintaining information about gene expression of key developmental master regulators such as hox genes [50]. Consistently, *MLL* knockout mice were embryonic lethal and showed homeotic transformations with abnormal *Hox* gene expression [51], indicating MLL as the functional homolog of trx with critical roles in cellular memory and the transcriptional regulation. Important cues to the molecular function of MLL came from the discovery that MLL is the mammalian homolog to yeast set1 [52], which was later shown to possess histone methyltransferase activity specific for H3K4 [53]. Subsequently, this enzymatic activity was also confirmed in MLL [54] and Trx [55]. The H3K4me3 mark is generally associated with active transcription (active mark) although it can also be present together with K3K27me3 (a repressive mark) at bivalent genes poised for transcription. The exact role of the H3K4me3 SET domain of MLL in normal development is still unclear since mice expressing a

mutant MLL without the SET domain (MLLΔSET) are viable and fertile, although they displayed homeotic transformations and reduced hox expression [56].

Purification of the yeast set1 (COMPASS), mammalian MLL (mammalian MLL COMPASS-like) and *Drosophila* trx (*Drosophila* trithorax COMPASS-like) complexes revealed that many core components are indeed common between these complexes [57], further endorsing a conserved function of trx/set1/MLL in transcriptional regulation of target genes across different species. The core components of the drosophila trithorax COMPASS-like and mammalian MLL COMPASS-like complex (Fig. 11.3a) include WDR5, RBBP5, DPY30, ASH2L, which are similar to yeast COMPASS core components Cps30, Cps50, Cps25, Cps60 respectively. In contrast to the yeast COMPASS complex, *Drosophila* and mammalian COMPASS-like complexes contain MEN1 [57, 58], which binds to trx/MLL N-terminal sequences. There are also other critical cofactors not originally co-purified but later shown to interact with components of the mammalian COMPASS-like complex. These include (1) polymerase associated factor complex, PAFc, which is thought to play a

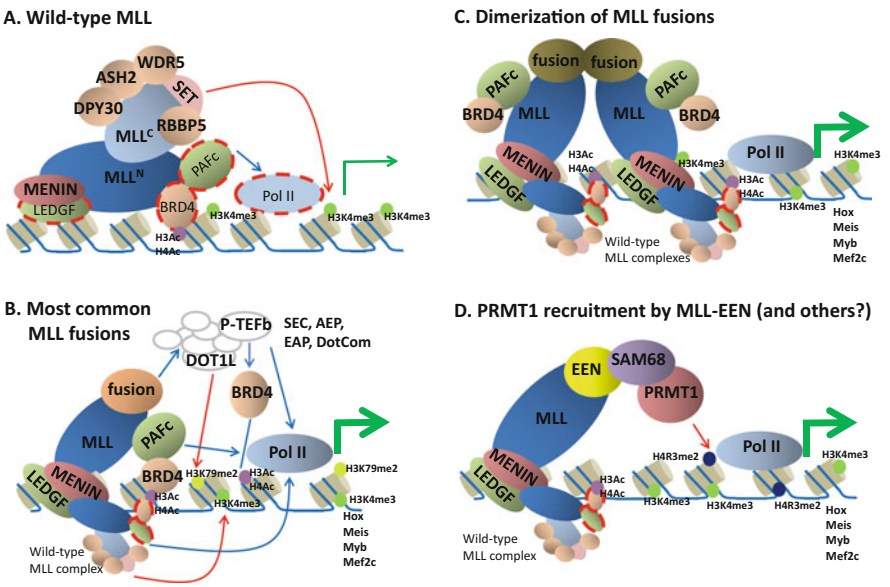

Fig.11.3 Schematic overview of the wild-type KMT2A/MLL complex and the different oncogenic transcriptional complexes formed by KMT2A/MLL fusions. (**a**) **Wild-type MLL complex**. Proteins not originally identified but shown to interact with components of the MLL COMPASS-like complex are marked with a red outline. PAF1 binds directly to MLL N-terminal sequences and interacts with BRD4. MEN1 interacts with LEDGF. RNA polymerase II is present at *MLL* target genes. (**b–d**) **Proposed different oncogenic transcriptional MLL fusion complexes**. *Green arrow* indicates transcription. *Blue arrows* indicate protein interactions. *Red arrows* indicate enzymatic histone methyltransferase activity. For clarity, no arrows have been depicted in the two panels on the right (**c** and **d**) except for PRMT1 enzymatic activity. Note that both wild-type MLL as well as MLL fusion proteins have been reported to co-localize at target gene promoters

role in targeting MLL complex to target loci [59]; (2) acetylated histone binding proteins of the BET family such as BRD4 potentially via interaction with PAFc [60]; and (3) the chromatin associated protein PSIP1/LEDGF via its interaction with MEN1 [61].

11.8.2 MLL Fusion Complexes

Over the past few years, it has become clear that multiple common MLL fusion partners co-exist in various endogenous complexes (EAP, AEP, SEC, DotCom) that are aberrantly recruited to MLL target genes resulting in abnormal transcriptional regulation of target genes such as *HOX* genes. These complexes often contain the H3K79me2 histone methyltransferase DOT1L and/or the positive elongation factor P-TEFb that is critical for the transition of transcriptional initiation to elongation by phosphorylating of the CTD of RNA polymerase II. Moreover MLL fusion complexes also interact with mammalian MLL COMPASS-like associated proteins such as MEN1, LEDGF and PAFc. Hence it is believed that these MLL fusions may transform haematopoietic cells by aberrant recruitment of histone modifying enzymes (e.g., DOT1L) and elongation factors (e.g., PAFc, P-TEFb) (Fig. 11.3b). For the less common MLL fusions, several alternative transformation mechanisms (Fig. 11.3c, d) have been described including (1) dimerization of MLL fusions observed in MLL-EPS15/AF1p and MLL-GAS7 [62], MLL-GPHN [63] and synthetic MLL-FKBP [14]; (2) recruitment of different histone modifying enzymes such as the protein-arginine methyltransferase, PRMT1 in MLL-SH3GL1/EEN [64]; or (3) indirect recruitment of AEP in case of MLL-MLLT4/AF6 [12], all resulting in de-regulated gene expression of critical targets such as *HOX* genes.

11.8.3 Crosstalk Between Wild-Type MLL and MLL Fusion Complexes

Interestingly, the N-terminus of MLL is preserved in all MLL fusions (Fig. 11.1), yet it is still unclear how wild-type and MLL fusion complexes are recruited to specific downstream targets. Proteins that interact within the N-terminus of MLL such as MEN1 and LEDGF are present in wild-type MLL as well as MLL fusion complexes suggesting putative binding to the same target genes. Indeed, up to 80 % of MLL-ENL target genes overlap with wild-type MLL targets [65]. On the other hand, MLL fusion target genes (n = 223) represent only a small minority (~7 %) of wild-type MLL targets (n = 2595), suggesting a highly selective binding exhibited by MLL-fusions to these loci. In contrast to wild-type MLL, all MLL fusions lack the C-terminal H3K4me3 SET domain. Yet, MLL fusion target genes are enriched not only with the H3K79me2 but also with the H3K4me3 chromatin mark [66, 67]

suggesting a possible crosstalk of MLL fusions and H3K4me3 HMTs. Although there are six different mammalian HMTs (MLL, KMT2B/MLL4, KMT2C/MLL3, KMT2D/MLL2, SETD1A and SETD1B) that can all contribute to the H3K4me3 mark, it is generally believed that MLL fusions may interact with wild-type MLL probably through proteins associated with both complexes. Supporting this hypothesis, experimental evidence for roles of PAFc and MEN1 in mediating this crosstalk has been reported. Wild-type MLL/PAFc complex is required for the efficient recruitment of MLL fusion to the same loci [68] as MLL fusion could not bind to *Hoxa9* in the absence of wild-type MLL or PAFc recruitment in MEFs. On the other hand, it was shown that knockout of *Men1* resulted in not only loss of H3K79me2 but also H3K4me3 at MLL fusion target genes in KMT2A/MLL fusion transduced cells [69], suggesting that enzymatic activities from wild-type MLL as well as MLL fusion complexes are acting in concert simultaneously on the *Hoxa9* gene locus. Consistently, wild-type MLL and MLL fusion complexes are recruited to *Hoxa9* in a Men1 dependent manner, and a conditional deletion of wild-type MLL inhibited MLL fusion mediated leukaemogenesis. While this suggests a critical role of wild-type MLL in *MLL* fusion mediated transformation, it should be noted that the conditional *MLL* mouse used in the Thiel et al. study suffered from a much more severe haematopoietic phenotype [70] than what has been reported for another conditional *MLL* mouse model [71]. It is not clear if these cells are generally compromised for any transformation. Interestingly, it was very recently reported that *MLL-AF9* AML is initiated and propagated normally in MLLΔSET haematopoietic cells [72] suggesting that the wild-type MLL H3K4me3 activity is dispensable for MLL fusion transformation. Furthermore, the leukaemic cell line ML2 [73], which was derived from an AML patient with *MLL-AF6*, lacks wild-type MLL. Further studies including comparison of both conditional mouse models and their impact on MLL fusion mediated transformation as well as the role of H3M4me3 and other histone modifications in MLL fusion mediated transformation will further advance our understanding of this fascinating crosstalk.

11.9 Pathways and Downstream Targets Critical in MLL Leukaemia

While characterizing the oncogenic MLL fusion transcriptional complexes in *MLL* leukaemia revealed several target genes and transcriptional programmes critical for self-renewal of *MLL* LSC [45, 46], recent data suggest that also other pathways and molecules which are neither under the direct control nor recruited to MLL fusion complexes play important roles in MLL leukaemia. Furthermore, it has been known for many years that several mutations such as *FLT3* and RAS genes recurrently found in AML and ALL patients also co-exist in MLL leukaemia patients, albeit their functional contribution, requirement and therapeutic value is much less clear. FLT3 has been reported to cooperate with some MLL fusions (e.g. MLL-ENL,

MLL-SEPT6) [74] and is expressed in high levels in *MLL* leukaemia patients [75, 76], but patient's responses in early FLT3 monotherapy clinical trials were rather limited both in depth and duration which severely dented the promise of FLT3 inhibition in acute leukaemia treatment [77]. Consistently, it was more recently shown that MLL fusions were able to induce leukaemia in *Flt3* knockout cells with expected latency and phenotype as by MLL fusion transformed wild type cells [78]. Moreover, co-expression of Flt3 or its constitutively activated mutant (Flt3-TKD) did not cooperate with MLL-AF4 in the transformation of human primary stem/progenitor cells [79] adding further doubt to the exact functional role and therapeutic value of Flt3 in *MLL* leukaemia.

11.9.1 MLL Downstream Targets

Identification of the oncogenic transcriptional MLL fusion complexes revealed that MLL fusions act as transcriptional activators and enhance gene expression of downstream targets. Among them, *Hox*, *Meis1*, *Pbx3*, *Myb* and *Mef2c* [30, 45, 80] represent the best characterized MLL downstream targets which are all part of a wider transcriptional programme critical for self-renewal of MLL LSCs. Knockdown or knockout of *Meis1*/*Pbx3* [81] or *Myb* [80] resulted in impaired MLL fusion cell growth and colony formation, whereas conditional deletion of *Mef2c* [82] did not impair induction or maintenance of MLL fusion mediated leukaemia but affected homing and invasiveness of MLL leukaemic cells in vivo. The functional role of *Hox* genes in MLL leukaemia remains controversial. While *HOXA9* has been shown to be required for human MLL leukaemia cell lines [83], which is in line with findings that *MLL-ENL* cannot transform *Hoxa9*−/− or *Hoxa7*−/− cells [84], it is also reported that both *MLL-AF9* and *MLL-GAS7* mediated transformations are independent of *Hoxa9* [85, 86]. Interestingly, only one microRNA, miR-495, is expressed at very low levels in *MLL* leukaemia compared to non-*MLL* leukaemia and it has been shown to target *Meis1* and *Pbx3* transcripts [87]. Indeed over-expression of miR-495 prolonged the latency of *MLL* leukaemia in vivo, providing a potential avenue to target the expression of certain MLL fusion downstream target genes.

More recently, MLL fusions have been reported to activate expression of the DNA demethylase *TET1* gene (ten eleven translocation 1) [88], and *miR-9* [89]. Moreover it has been shown that MLL fusions maintain the expression of *MECOM*/*EVI1* [90] in LSK (Lineage$^-$, Sca1$^+$, Kit$^+$) cells. However, expression of MLL fusion did not lead to an up-regulation of *MECOM*/*EVI1* in GMPs that have only basal expression levels of *MECOM*/*EVI1*. Common to these target genes is their requirement for MLL fusion mediated leukaemia as knockdown/depletion of *Tet1* and *Mecom*/*Evi1* has been reported to impair cell growth and leukaemia induction, whereas overexpression of *miR-9* promotes MLL-AF9 leukaemia in vivo.

11.9.2 Critical Pathways/Molecules Not Directly Regulated by MLL Fusions

11.9.2.1 Canonical WNT/beta-catenin

The evolutionarily conserved WNT/CTNNB1 pathway has been implicated in the pathogenesis of AML [91], and is required for the development of *MLL* LSCs [26, 92]. MLL fusions fail to induce leukaemia in the absence of CTNNB1 and its activation confers drug resistance to *MLL* LSCs [26]. Interestingly, deletion of *Ctnnb1* in haematopoietic cells has little/no effect on the function of adult haematopoiesis [93, 94], suggesting a therapeutic window. Consistently, pharmacological inhibition of Ctnnb1 by Indomethacin showed efficacy in inhibiting *MLL* leukaemia in vivo [95], although these experiments were done in serially transplanted animals probably due to the drug toxicity. It is noted that CTNNB1 also plays important roles in other haematological malignancies including CML-blast crisis [96–98], suggesting its broad therapeutic application.

11.9.2.2 ITGB3

In vivo shRNA screen carried out in a *MLL* mouse model identified integrin beta 3 (Itgb3) signalling amongst others as essential for MLL-AF9 mediated leukaemia [99]. Loss of Itgb3 signalling reversed the transcriptional programmes established by *MLL-AF9* such as self-renewal and led to up-regulation of differentiation programmes. Itgb3 dimerizes with Itgav on the cell surface, and transmits extra-cellular signals via a cascade of protein kinases such as Syk, Src and Ptk2b, guanine nucleotide exchange factors such as Vav1, Vav2, Vav3, and GTPases such as Rho and Cdc42. In the same study, Syk was identified and validated as a critical mediator of Itgb3 signalling in *MLL* leukaemia. Although the functional relationship between Itgb3/Syk and MLL fusion proteins in leukaemia are still largely unknown [100], the identification of tractable signalling molecules and the availability of Syk inhibitors that have already been shown effective in various diseases including B cell malignancies [101] add to our expanding repertoire of promising targets for *MLL* leukaemia.

11.9.2.3 Polycomb Group Proteins

Classically, trithorax and polycomb group proteins have been viewed as antagonistically acting proteins for regulating gene expression and cellular memory during normal development. However, increasing evidence suggests that polycomb proteins such as Bmi1 also play an important role in *MLL* leukaemia, which cannot be simply explained by their antagonistic functions [102, 103]. In addition, the polycomb protein CBX8, which has been shown to interact with the MLL fusion

partners ENL and AF9 [104], is critical for MLL-AF9 mediated transcriptional activation and transformation [105]. While it was suggested that CBX8 is critical for *MLL* leukaemia because of its recruitment of KAT5/TIP60 to the MLL fusion complex [105], another study provided evidence that the interaction between ENL and CBX8 allows MLL-ENL to inhibit the repressive function of polycomb group proteins on MLL fusion downstream target genes such as *HOX* [106]. Interestingly, two more polycomb group proteins (EZH2 and EED) genes have been reported to play key roles in *MLL* leukaemia [107, 108]. While the functional requirement of EZH2 in MLL leukaemia is less well defined, ablation of Eed impaired MLL-AF9 leukaemia although it is not clear if Eed is generally required for cell survival.

11.9.2.4 RUNX1

The heterodimeric core binding factors composed of RUNX1/AML1 and CBFB subunits are critical for definitive and adult normal haematopoiesis. Interestingly, RUNX1 and CBFB are also the most frequently translocated genes in acute leukaemia resulting in *RUNX1-RUNX1T1*/*AML1-ETO*/t(8;21) or *CBFB-MYH11*/inv(16) fusion genes. It has been recently shown that RUNX1 recruits wild-type MLL to activate RUNX1 target genes such as *SPI1*/*PU.1*, and the MLL-RUNX1 interaction prevents RUNX1 proteasome degradation [109]. Despite the RUNX1 interaction domain being located in the C-terminal portion of MLL, the N-terminus seemed required for enhancing RUNX1 protein levels, suggesting a differential effect of MLL fusion on RUNX1. Indeed a role for RUNX1 in MLL fusion mediated transformation was subsequently suggested since suppression of RUNX1 inhibited the growth of various MLL fusion transformed cells [110, 111]. However, it was very recently reported that MLL fusion down-regulates *RUNX1*/*CBFB* expression [112]. In contrast to the previous reports, down-regulation of *RUNX1*/*CBFB* expression levels accelerated MLL-AF9 leukaemia, whereas overexpression of *RUNX1* impaired *MLL-AF9* leukaemia. Therefore, further studies are needed to define the role and exact contribution of RUNX1 in *MLL* leukaemia.

11.9.2.5 Other Emerging Molecules

Involvement of myeloid specific transcription factors (such as CEBPA [113] and SPI1/PU.1 [114]), general transcription factor (NFKB [115]) and ubiquitin ligase (RNF20 [116]), which are all not under the direct transcriptional control of MLL fusion, further highlight a widespread crosstalk of other signalling pathways in *MLL* leukaemia. Genetic ablation/knockdown of *NFKB*, *Rnf20*, *Spi1*/*Pu.1*, or *Cebpa* impaired leukaemia growth. Interestingly, CEBPA is only important for the development of MLL fusion LSCs but not for their maintenance. While transcription factors are poor therapeutic targets to date, signalling cascades upstream of these

transcription factors may represent more promising targets. Inhibition of the IKK complex, a major upstream regulator of NFKB signalling suppresses cell growth and colony formation of murine *MLL* fusion transformed cells, suggesting that therapeutic targeting of this pathway is possible in *MLL* leukaemia. Moreover, a recently identified TNFa/NFKB autocrine positive feedback loop critical for the establishment of both MLL and non-MLL LSCs further highlights the role of NFKB, and the possibility of targeting TNFa for leukaemia therapy [117].

11.10 Current Therapies and Development of Novel Targeted Therapies

Currently, AML patients with 11q23 aberrations receive standard induction cytotoxic therapy "3 + 7" of daunorubicin and cytarabine, and they represent one of the worst prognostic subgroups [2]. Treatment of ALL patients, including those harbouring 11q23 abnormalities usually comprise a remission-induction phase, consolidation phase and continuation therapy [4]. While allogeneic HSCT is a key element in adult treatment, it confers no survival advantage in infant ALL with 11q23 aberrations and may only have limited benefits in a small subset of 11q23 patients, which possess additional poor prognostic factors [118, 119]. Strikingly, the drugs currently in use for these ALL and AML treatment regimens were developed in the 1950s–1970s. Although their dosage and schedule have been optimized resulting in higher survival rates accompanied by less general cytotoxicity in the majority of ALL cases and some AML cases, patients with 11q23 aberrations in general have had little to no benefits from these improvements, highlighting the need for novel drugs and therapies especially for this poor prognostic subgroup. The recent advances in identifying critical molecules, which are mainly part of the oncogenic MLL fusion complex and essential for *MLL* leukaemia, have provided novel targets for effective treatment, some of which are already being developed for early phases of clinical trials (Fig. 11.4).

11.10.1 Targets with Enzymatic Activities

11.10.1.1 DOT1L

DOT1L has been identified in several MLL fusion partner complexes (Fig. 11.2) and is essential for MLL fusion mediated leukaemia as demonstrated by conditional knockout of *Dot1l* for in vitro transformation [120] and in vivo leukaemogenesis [66, 122]. Noticeably, loss of Dot1l impaired haematopoiesis in all three different conditional knockout models, albeit with different severity possibly due to the use

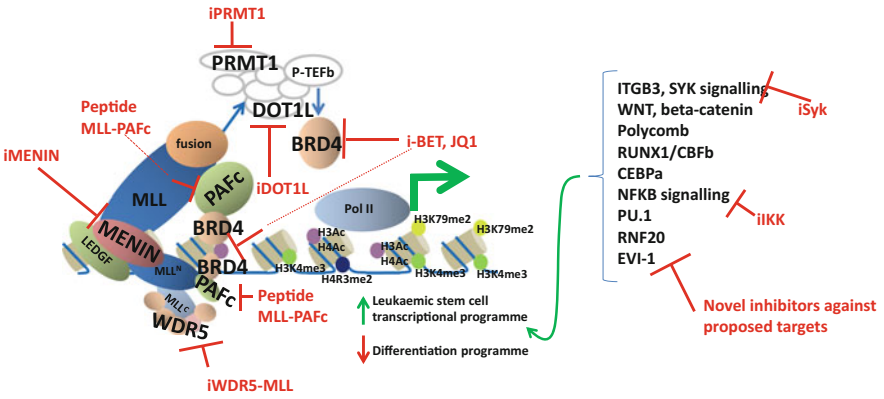

Fig. 11.4 Novel players in KMT2A/MLL leukaemia and development of targeted therapies. Schematic generic MLL fusion complex together with wild-type MLL complex at target gene locus driving distinct gene expression profiles. Highlighted in larger font size are individual molecules that have been shown important for MLL fusion-mediated transformation and have been experimentally targeted by small molecule inhibitors. On the right are emerging molecules and pathways listed with reported importance in MLL fusion-mediated transformation influencing MLL fusion protein transcriptional programmes. Small molecule inhibitors with already reported efficacy are indicated

of slightly different gene targeting strategies, Cre recombinases or/and incomplete Cre-mediated *Dot1l* deletion. Whereas one study found that loss of Dot1l had limited effects on haematopoietic progenitors [66], the others reported an indispensable function of Dot1l in the maintenance of adult haematopoiesis as the numbers and function of haematopoietic stem and progenitor cells were greatly compromised in the absence of Dot1l [120, 121]. Despite this controversy, the essential role of Dot1l in MLL leukaemia and its unique HMT enzymatic activity associated with non-SET domain makes Dot1l an attractive target. In fact, the first DOT1L inhibitor EPZ004777, a competitive analog of the co-factor S-adenosylmethionine (SAM), has been developed and shown to selectively kill *MLL* rearranged cells in vitro with reported IC50 levels 5–100× higher in non-*MLL* cells [123]. While EPZ004777 was tolerated in mice, it had quite poor in vivo pharmacokinetics, which only resulted in a very modest survival benefit in treated animals compared to the controls. The structural data on EPZ004777 binding to DOT1L revealed remodelling of the catalytic site of DOT1L, and identified positions of the compound that can be optimized to improve its pharmacokinetics and potency [124]. Indeed SGC0946, a brominated analogue of EPZ00477, has been reported to improve molecular effects on H3K79me2 level as well as selectively killing of *MLL* leukaemia cells. Another molecule evolved from EPZ004477 with improved potency is EPZ-5676 that has recently entered into phase I clinical trial [125]. Although future studies are needed to clarify their in vivo pharmacokinetic and efficacy on *MLL* leukaemia in the clinics, these studies demonstrate the promise of translating basic research results into potential patient's benefits.

11.10.1.2 PRMT1

Protein arginine methyltransferase 1 (PRMT1) with H4R3me2 specificity has been identified to be critical for MLL-EEN mediated transformation [64] as knockdown of PRMT1 resulted in impaired MLL-EEN in vitro transformation. Moreover, direct fusion of PRMT1 but not a catalytic dead mutant to the truncated MLL resulted in oncogenic transformation of primary murine haematopoietic stem/progenitor cells. While the role of *Prmt1* in other leukaemia remains to be determined, a small molecule inhibitor targeting PRMT1 has shown promising in vivo efficacy in a *MLL* fusion leukaemia model (N.C., B.B.Z. and C.W.E.S. unpublished data). Although EEN is a rare translocation partner of MLL, it is evident that transformation mediated by other MLL and non-MLL fusions may also depend on Prmt1 (N.C., B.B.Z. and C.W.E.S. unpublished data). Future studies using Prmt1 conditional knockout mouse and additional pharmacological PRMT1 inhibitors will be critical to clearly define the role of PRMT1 in *MLL* leukaemia and as a therapy target.

11.10.1.3 KDM1A/LSD1

Although LSD1 (KDM1A) mono- and di-demethylase does not directly associate with either wild-type MLL or MLL fusion complexes, shRNA mediated knockdown and pharmacological inhibition of LSD1 impaired in vitro growth and in vivo leukaemogenesis of cells transformed by various MLL fusions [126]. While inhibition of LSD1 induced differentiation of *MLL-AF9* transformed cells and spared normal bone marrow cells in vitro, its in vivo efficacy and the specificity (if any) towards MLL fusions remains unclear. Interestingly, another study reported that knockdown or pharmacological inhibition of LSD1 potentiated all-trans retinoic acid (ATRA) induced differentiation of non-*MLL* AML cells and reduced the engraftment ability of human primary AML samples in recipient mice [127]. These results suggest that inhibition of LSD1 alone or in combination with ATRA may have therapeutic value in treatment of other AMLs including those with 11q23 aberrations.

11.10.2 Targeting the Protein-Protein Interactions

11.10.2.1 BRD4

BRD4 is a member of the bromodomain containing proteins of the BET family that binds to acetylated histone, and may be recruited to MLL fusion complexes via its interaction with P-TEFb or PAF1. Its functional requirement in *MLL* leukaemia has recently been demonstrated in a shRNA screen [128]. Although BRD4 itself does not possess an enzymatic activity, small molecule inhibitors JQ1 [129] and i-BET [130] have been reported to competitively interfere with the binding of the

bromodomain to acetylated histones. In murine *MLL* fusion leukaemia models, treatment with JQ1 [128] or i-BET [60] has been very encouraging as it increased the survival of the treated animals compared to controls. Gene expression analysis showed that JQ1/i-BET treatment resulted in down-regulation of the LSC signature and reduced expression of *BCL2* and *MYC*. Transcriptional programmes masterminded by MYC have important roles in a variety of LSCs [131], suggesting that BRD4 inhibitors may have a broader efficacy and effects also in non *MLL* leukaemia. Indeed two recent reports highlighted the efficacy of i-BET in non-*MLL* AML [132] and in *JAK2*^{V617F} driven MPN [133]. They also observed down-regulation of *BCL2* and *MYC* upon treatment, suggesting that BET proteins control transcription of key targets such as MYC and BCL2, independently of the presence of MLL fusions proteins. Nevertheless, these studies provide a strong rationale to use BET inhibition as a novel experimental treatment for AML with and without 11q23 aberrations.

11.10.2.2 MEN1/MENIN

Another molecule associated with wild-type MLL as well as MLL fusion complexes is MEN1, which bridges MLL with the chromatin factor LEDGF. Interestingly, while MEN1 was originally identified as the product of the tumour suppressor gene *MEN1*, whose loss of function mutation causes sporadic neoplasm of various endocrine organs [134], it is critical for *MLL* fusion mediated transformation [61]. In its dual role as tumour suppressor and tumour promoting protein, structural analysis of free Men1 as well as Men1-MLL or Men1-JunD complexes revealed both MLL and JunD bind Men1 in the same pocket. However while Men1-JunD binding results in suppressing JunD-mediated transcription, Men1-MLL binding promotes MLL transcriptional activity [135]. Consequentially, MI-2, a small molecule inhibitor disrupting the Men1-MLL interaction was developed and showed in vitro efficacy in *MLL* fusion expressing cells [136]. Although the vivo efficacy has yet to be demonstrated, in vitro treatments of *MLL* fusion cells with MI-2 led to differentiation and down-regulation of MLL target genes with little effects on non-*MLL* transformed cells. Based on the high-resolution crystal structure of the Men1-MI-2 complex, a second generation inhibitor MI-2-2 has been developed and shows 7–9× higher affinity to Men1 compared to MI-2. Crucially, MI-2-2 displayed further enhanced cellular activities and more potent inhibition of MLL fusion harbouring human cell lines [137] although its in vivo efficacy has not been reported. It is noted that targeting the Men1-MLL interaction could potentially also amongst others inhibit the Men1-JunD interaction resulting in the conversion of proliferation suppressing JunD to proliferation promoting JunD with adverse consequences. Although targeting the Men1-MLL interaction may have therapeutic value in *MLL* leukaemia, Men1 is also a tissue specific tumour suppressor and small molecules targeting Men1-MLL or Men1 in general may have undesirable consequences in other tissues, which may result in the development of endocrine tumours. Future in vivo studies undoubtedly will shed lights onto these important issues.

11.10.2.3 WDR5

WDR5 is a component of the mammalian COMPASS-like MLL complex, which is not only absolutely required for its integrity but also for optimal MLL H3K4me3 activity [138]. Recently, a small molecule inhibitor MM-401 targeting the MLL1-WDR5 interaction has been reported and shows in vitro efficacy towards different MLL fusion proteins in primary cell line models and in patient samples [139]. Interestingly, while WDR5 is also found in other mammalian COMPASS and COMPASS-like complexes associated with different H3K4me3 methyltransferases (e.g., SETD1A, SETD1B, MLL, MLL2, MLL3, MLL4), MM-401 specifically inhibits the MLL1 COMPASS-like complex histone methyltransferase activity. Although its in vivo efficacy has yet to be demonstrated, the in vitro inhibition data provide further evidence that *MLL* fusion mediated transformation may be dependent on the presence of functional wild-type MLL COMPASS-like complex.

11.10.2.4 PAF1/PAFc

Polymerase associated factor complex (PAF1/PAFc) is recruited via MLL N-terminal sequences including its CXXC domain to both wild-type MLL and MLL fusion proteins. It has been reported that knockdown of CDC73 or CTR9, critical components of PAFc, inhibits MLL fusion transformation in vitro and that the interaction with PAF1 is necessary for MLL fusion transformation [59]. The use of a genetic model to delete *Cdc73* confirmed the initial findings, however it also affected non-*MLL* transformed cells raising questions about the specificity towards the MLL fusion [140]. Nevertheless, a competitive peptide that disrupts the binding of MLL fusion with PAF1 suppressed MLL fusion activity and did not adversely affect normal haematopoiesis [140]. These results suggest disruption of PAF1/MLL may also be a potential therapeutic avenue although its in vivo efficacy and potential translation into small molecule inhibitors need further investigations.

11.11 Concluding Remarks and Perspective

In the past decade, our understanding of the mechanisms mediating MLL fusion transformation of normal haematopoietic cells into leukaemic stem cells has dramatically expanded. Novel insights into the underlying molecular mechanisms and their crosstalk with other pathways/molecules have helped to identify a number of new therapeutic targets. Although development of small molecule inhibitors targeting these molecules with critical functions in *MLL* leukaemia is still in a very early stage, the successful application of DOT1L inhibitor, EPZ-5676 into phase I clinical trial for *MLL* leukaemia demonstrates the promise of translating our knowledge into novel treatments. Therefore continuous efforts in characterizing the molecular and

cellular basis of *MLL* leukaemia is expected to provide further important biological insights and more effective novel treatments for *MLL* leukaemia.

11.12 Appendix

During the production of this book chapter the first in vivo studies of MENIN inhibition in solid tumors and leukemia had been published [141, 142]. Using MI-2-2, a structure-based design combined with medical chemistry resulted in the development of MI-463 and MI-503 which improved survival of MLL leukaemic mice in vivo. Conversely, MI-463 or MI-503 had little impact on normal haematopoietic development. Although long-term experiments interrogating the effect of MI-463 and MI-503 on the MENIN-JunD interaction have not been performed, this work demonstrates the feasibility of targeting MENIN in MLL leukaemia.

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