Trithorax and polycomb cooperation in MLL fusion acute leukemia

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Genetic alterations of the *mixed lineage leukemia 1 gene* (*MLL1*, here referred to as *MLL*) located on the long arm of chromosome 11 (11q23) are found in pediatric (particularly infant) and about 5-10% of adult *de novo* and therapy-related acute lymphoblastic leukemias (ALL) or acute myeloblastic leukemia (AML); these types of leukemia are often characterized by early relapse.[1,2] Over 50 different balanced chromosomal translocations have been characterized that result in the expression of chimeric proteins in which the amino-terminal portion of MLL is fused to the carboxy-terminal portion of the partner. The translocations t(9;11), t(11;19) and t(4;11) leading respectively to MLL-AF9, MLL-ENL and MLL-AF4 fusions are the most prevalent. MLL-AF9 is frequently associated with AML with monocytic characteristics, MLL-ENL can be found in AML or ALL, and MLL-AF4 is almost exclusively found in B-cell ALL. Several experimental studies have shown that MLL fusions are potent hematopoietic oncogenes *in vitro* and in different mouse models.[1,2]

MLL is the homolog to the Set1 histone3 lysine4 (H3K4) methyltransferase in yeast acting in a large complex of proteins associated with Set1, also known as COMPASS. Similarly, MLL functions as H3K4 methyltransferase in a COMPASS-like complex.[3] Before integration into the complex, MLL is cleaved by taspase1 into an N-terminal fragment containing domains with transcriptional co-activator activity [plant homeodomain fingers (PHD), DNA binding motifs (AT-hooks, CXXC-zinc finger)] and the C-terminal fragment containing the SET methyltransferase domain. Chromatin binding and transcriptional activity of the MLL-complex need interaction with the lens epithelium-derived growth factor (LEDGF) and menin.

MLL fusions lose the PHD fingers and the SET domain but retain the AT-hooks, CXXC domain and the interaction with LEDGF and menin. Interestingly, previous genetic studies have proposed that a wild-type copy of MLL is required for the transforming activity of the MLL-fusions, however, it remains unresolved how the wild-type complex and the fusion cooperatively regulate gene expression.[4] Like normal MLL, the fusions also form large multi-protein complexes. Despite ongoing controversies about the dynamics and the complex composition, work from several groups suggested that the most prevalent MLL fusion partners, AF9 and ENL, on the one hand interact with AFF1, AFF4, the positive transcriptional elongation factor b (pTEFb) and associated co-factors such as Brd4, but on the other hand also form the bridge to other proteins including the histone3 lysine79 methylase DOT1L. Importantly, there is increasing evidence that the transforming activity of the most prevalent MLL-fusions is dependent on the activity of DOT1L and on the interaction with LEDGF and menin.[1-3]

Menin is the product of the *MEN1* gene on the long arm of chromosome 11, which is mutated in patients with multiple endocrine neoplasia (MEN). As for MLL, conditional MEN1 ablation in the hematopoietic system significantly impaired the self-renewal capacity of hematopoietic stem cells.[5-7] Several studies have demonstrated that menin stably associates with the N-terminus of MLL and is essential for initiation and maintenance of leukemogenic transformation by MLL fusions.[8,9]

The central role of the menin/MLL interaction for transformation by MLL-fusions suggested early on that these protein-protein interactions might offer the possibility for novel therapeutic strategies. Structural studies revealed that replacement of the phenyl-ring of phenylalanine in the hydrophobic pocket of menin with an imidazole or hydroxyphenyl ring abolished the interaction suggesting the possibility for selective blocking of the interaction.[10] Indeed a small molecule was identified that binds to menin with low nanomolar affinity and disrupts the interaction between menin and MLL and impairs the transformed state of the MLL fusion immortalized cells associated with down-regulation of HOXA9 expression.[11,12]

MLL is the mammalian homolog and the archetype of the trithorax group (TrxG) of proteins that regulate developmental programs in an antagonistic manner with the polycomb group (PcG) proteins.[13] Generally, TrxG proteins activate Hox genes while the PcG proteins seem to repress Hox gene expression. Two distinct multi-protein polycomb repressive complexes (PRC) have been defined in mammalian cells of which PRC2 seems to modify histone marks that are interpreted by proteins of the PRC1 complex, although both complexes can also function independently. Enhancer of zeste homologue 2 (EZH2) is a PRC2 protein with histone methyltransferase activity on histone 3 at lysine 27 (H3K27). These repressive marks set by PRC2 are stabilized by components of the PRC1 complexes containing RNF1/2, chromobox proteins (CBX4/8), BMI1 and others. Like MLL and menin, PcG proteins, too, are critical functional regulators of hematopoietic stem and progenitor cells: whereas loss of function of PRC2 components enhances hematopoietic stem cell/progenitor activity, loss of PRC1 activity impairs hematopoietic stem cell/progenitor function.[14,15]

A first link between MLL leukemia and PcG proteins emerged from the observation that MLL-AF9 expressing leukemic stem cells overcome senescence through the interplay between BMI1 and HOXA9.[16] Genetic studies revealed that CBX8 interacts with MLL-AF9 and is required for transcriptional activation and leukemogenesis.[17] Four recent reports, including the study by Thiel *et al.* presented in this issue of Haematologica, have addressed the role of PRC2 complex proteins in MLL-AF9-induced AML.[18-21]

Thiel and colleagues found that the PRC2 protein EZH2 collaborates with the MLL/MLL-AF9/menin complex to block differentiation of MLL-AF9-driven leukemic cells through a functional interaction with CCAAT/enhancer binding protein α (C/EBPα) (Figure 1). Using a conditional menin knockout mouse ($Men1^{fl/fl}$;Cre-ER), they demonstrated that acute depletion of menin induced differentiation of MLL-AF9 immortalized cells *in vitro* and reduction of MLL-AF9$^+$ leukemia initiating cells *in vivo*. They also extended their previous observations that the differentiation block of the leukemic cells and disease propagation are dependent on wild-type MLL.[4] Comparative gene expression profiling of primary MLL-AF9$^+$ cells from control and *Men1*-ablated mice revealed a significant overlap of potentially menin co-regulated genes with previously reported target genes of C/EBPα. However, depletion of menin did not result in changes of C/EBPα protein levels or expression of the potentially leuke-

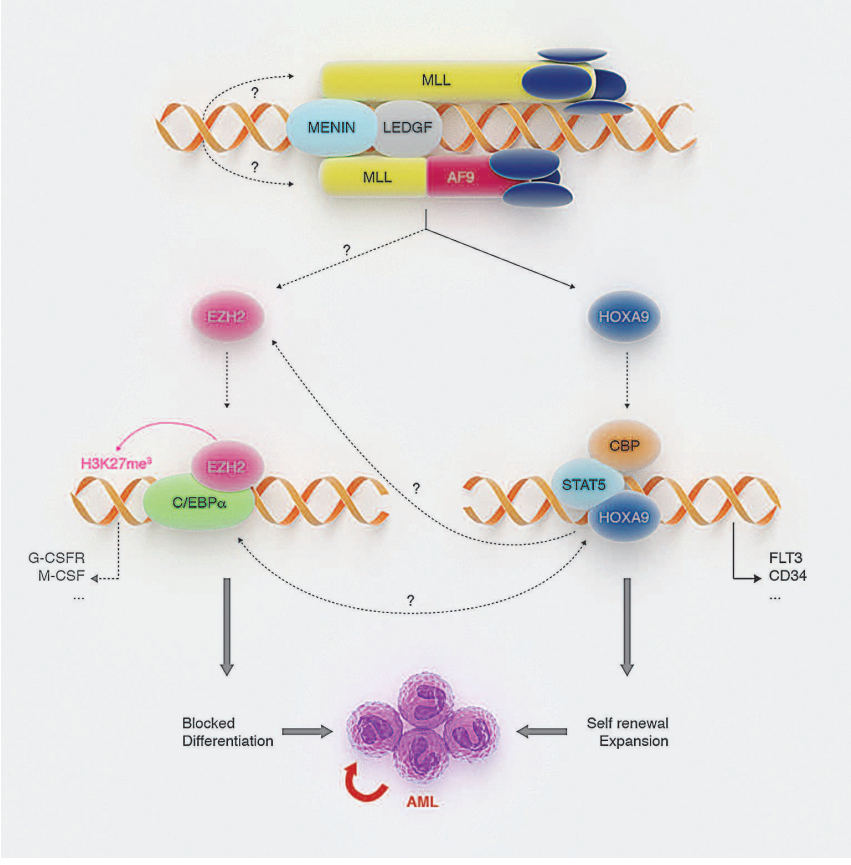

Figure 1. Proposed cooperation between polycomb and trithorax in *MLL*-fusion gene driven AML. Transformation by MLL-AF9 is dependent on wild-type MLL and the interaction with MENIN and LEDGF. Aberrant expression of HOXA9 mediates self-renewal and expansion of the cells as part of a large multi-protein transcriptional complex, whereas differentiation is impaired by functional inactivation of C/EBPα by the EZH2 polycomb protein.

mogenic C/EBPα p30 isoform, nor did it abrogate binding of C/EBPα to the promoter of a target gene such as the monocyte colony-stimulating factor receptor (M-CSFR). Interestingly, menin depletion in MLL-AF9-transformed cells also resulted in decreased expression of the PRC2 member EZH2 independently of the MLL targets HOXA9 or MEIS1. Importantly, chromatin-immunoprecipitation (ChIP) experiments showed enrichment for menin, AF9 and wild-type MLL at the EZH2 promoter in a menin-dependent fashion suggesting that menin/MLL/MLL-AF9 blocks cellular differentiation through EZH2-mediated repression of C/EBPα. Transactivation studies in cell lines demonstrated that EZH2 repressed C/EBPα–mediated activation in a dose-dependent manner. Interestingly, immunoprecipication and ChIP experiments in MLL-AF9^{+} human THP1 cells suggested a physical interaction between EZH2 and C/EBPα and binding at promoters of C/EBPα target genes. Most importantly, EZH2 knockdown in THP1 cells resulted in a dose-dependent increase of C/EBPα targets and signs of cellular differentiation without affecting the expression of HOXA9.

The work by Thiel and colleagues provides several interesting findings. First, it suggests that the blocked differentiation program of hematopoietic progenitors by MLL-AF9/menin is mediated by the PcG protein EZH2. Although regulation of EZH2 by MLL fusions is not yet fully understood, Tanaka and colleagues found that ablation of EZH2 impaired growth and induced differentiation of MLL-AF9-expressing cells.[19] However, Neff and colleagues have shown that EZH2 seems not to be essential for induction of MLL-AF9-induced disease in mice. In contrast, ablation of another PRC2 component, EED, completely abrogated PRC2 function and was incompatible with leukemic growth.[20] Others have used shRNA-mediated knockdown to show that reduced expression of the PRC2 components EED or SUZ12 induced differentiation of hematopoietic cells transformed by MLL-AF9 and NRAS^{G12D}.[21] These studies all suggest that the PRC2 complex cooperates functionally with MLL-AF9 in leukemogenesis. However, beyond MLL, the role of the EZH2 protein in hematopoietic malignancies seems to be rather complex. Previous overexpression experiments suggested that increased levels of EZH2 prevented hematopoietic stem cells from exhaustion and induced a myeloproliferative disorder without impairing myeloid differentiation.[22,23] In addition, EZH2 loss-of-function mutations have been recurrently found in myelodysplastic syndromes characterized by uncoordinated differentiation of myeloid stem and progenitor cells.[24] Additional studies are certainly needed for a better understanding of the functional interplay of EZH2 and other PRC2 proteins with oncoproteins driving leukemia.

The work by Thiel *et al.* also suggests that the menin/MLL/MLL-AF9 complex blocks myeloid differentiation through EZH2-mediated repression of C/EBPα independently of the classical MLL downstream targets HOXA9 and MEIS1 (Figure 1). Previous work showed that knockdown of HOXA9 resulted in differentiation of human MLL-AF9^{+} and MLL-AF4^{+} leukemic cells associated with increased expression of myeloid differentiation markers including M-CSFR1, a known C/EBPα target.[25] This observation raises the question of whether HOXA9 itself is able to regulate EZH2 or C/EBPα. Thiel *et al.* showed that knockdown of EZH2 did not change HOXA9 expression but resulted in differentia-

tion of MLL-AF9+ AML cells. In contrast, work in human NK/T-cell lines suggested that modulation of EZH2 by either knockdown or inhibitor resulted in up-regulation of HOXA9.[26] Additionally, genome wide screening for HOXA9 binding sites in murine cells transformed by overexpression of HOXA9 and MEIS1 suggested that blocked differentiation by MLL-fusions could be the consequence of aberrant HOXA9 expression regulating targets in so-called "enhanceosomes" through co-recruitment of multiple myeloid transcription factors including PU.1, RUNX1 and C/EBPα.[27] It will be important to determine whether the findings by Thiel *et al.* are limited to MLL-AF9-expressing AML cells and to determine the role of EZH2 or other PRC2 family members in ALL blasts driven by other MLL-fusions.

Finally, the observations by Thiel *et al.* and three others studies strengthen the hypothesis that in contrast to normal development, Trx and PcG proteins such as EZH2 can cooperate in leukemic transformation by MLL-fusion genes. Aberrant expression and recurrent gain of function mutations in various human tumors characterize EZH2 as a potential therapeutic cancer target. Structural modeling recently allowed several groups to establish small molecules that selectively block EZH2 methyltransferase activity and impaired proliferation and survival of human cancer cells.[28-30] Two studies presented at the 2012 meeting of the American Hematology Society demonstrated proof of principle that blocking EZH2 by small molecules depleted leukemia-initiating cells in MLL-fusion driven acute leukemia.[31,32]

Considered as a whole, the work by Thiel *et al.* presented in this issue of Haematologica provides another piece of the puzzle, helping the understanding of the molecular mechanisms of MLL-fusion-driven leukemia, which is essential to develop novel targeted therapies for these aggressive leukemias.

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