The role of mutations in epigenetic regulators in myeloid malignancies

Summary: Over the past decade, genomic studies have identified a number of novel and recurrent somatic mutations that affect epigenetic patterning in patients with myeloid malignancies, including myeloproliferative neoplasms, myelodysplastic syndrome, and acute myeloid leukemia. Many of these mutations occur in genes with established roles in the regulation and maintenance of DNA methylation and/or chromatin modifications in hematopoietic stem/progenitor cells. Subsequent genetic and functional studies have revealed that these mutations affect epigenetic patterning in myeloid diseases. In this review, we discuss historical and recent studies implicating epigenetic modifiers in the development and evolution of the various myeloid malignancies and discuss how this knowledge has and will lead to future clinical and biologic insights.

Keywords: chromatin regulation, myelopoiesis, genomics

Introduction

The observation that epigenetic landscapes are consistently deregulated in hematologic cancers has added a new layer of complexity to our understanding of leukemia pathogenesis. In addition, recent candidate-gene and next-generation sequencing studies have identified novel recurrent somatic mutations in patients with myeloid diseases, including myeloproliferative neoplasms (MPNs), myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML) (1, 2). A high percentage of patients with these diseases present with mutations in epigenetic regulators (3). These events comprise what is now accepted as a third novel class of leukemia disease alleles (4). Numerous genome-wide and candidate-gene studies have identified somatic alterations in several genes encoding proteins regulating DNA methylation and posttranslational histone modifications. These data collectively suggest that somatic alterations that compromise the activity of these epigenetic regulators are a common event in the pathogenesis of myeloid malignancies and contribute to hematopoietic transformation.
Epigenetic modifications are the consequence of enzymatic action of proteins that either place or remove particular ‘marks’. Abnormal functionality of an epigenetic modifier protein often disrupts normal epigenetic patterning, which contributes to the aberrant gene expression patterns displayed in myeloid diseases. In this review, we summarize the how somatic alterations in epigenetic regulators promote malignant myeloid transformation, the clinical and therapeutic implications of this knowledge, and discuss outstanding questions and challenges facing future investigations.

**Beyond the two-hit hypothesis for hematopoietic transformation**

Initial studies supported the existence of two discrete classes of leukemia disease alleles that promote hematopoietic transformation: class I alleles, which activate signaling pathway effectors (such as the JAK/STAT, PI3K/Akt, and RAS/MAPK pathways), and class II alleles, which alter the expression of transcriptional targets during myelopoiesis (5). Class I events confer a clonal growth advantage to hematopoietic cells through constitutive activation of signaling pathways that normally mediate homeostatic hematopoietic growth. Class I mutations, such as the BCR-ABL fusion protein in chronic myeloid leukemia and the JAK2 (V617F) mutation in Philadelphia chromosome-negative MPNs, have discrete pathogenic roles form the class II mutations, which lead to a MDS phenotype characterized by impaired myeloid differentiation. However, recent work has identified and characterized new classes of mutations in epigenetic modifiers. This knowledge has advanced our understanding of leukemic disease alleles beyond this conventional binary. These mutations do not explicitly affect either proliferation or differentiation of hematopoietic cells, and have unique roles in both normal hematopoiesis and leukemogenesis.

**Epigenetic regulation of the genome and normal hematopoiesis**

Epigenetics refers to heritable gene expression changes that are independent of DNA sequence changes. Epigenetic modifications can be either transient or physiologically stable, and play essential roles during embryonic development. Epigenetic alterations modify chromatin architecture in a heritable fashion, thus leading to transmissible gene expression patterns. Following embryogenesis, epigenetic changes persist throughout an organism’s lifespan and mediate genetic programming and cellular differentiation of adult tissues (6). Studies of mouse hematopoietic development, in particular, revealed that specific target genes are dynamically regulated during the processes of lineage-specification and differentiation (7). The ‘epigenome’ collectively consists of direct DNA modifications (such as DNA cytosine methylation and hydroxymethylation), postranslational histone tail modifications (8), and even non-coding RNAs that regulate gene expression. Somatic mutations affecting each of these components of the epigenome are detected at significant frequencies across the spectrum of myeloid diseases.

**Mutant epigenetic modifiers in myeloid malignancies**

Large-scale genome sequencing efforts have identified multiple recurrent somatic mutations and rearrangements in genes responsible for epigenetic regulation. Such events are well-understood in the context of AML, particularly translocations involving mixed lineage leukemia (MLL) which are associated with poor patient prognosis (9–11). Since MLL rearrangements and similar translocation events are only observed in AML and not MPN/MDSs, it was initially believed that mutations affecting epigenetic patterning were specific to de novo AML or to AML arising from an antecedent MPN (12). More recently, somatic mutations in epigenetic regulators were reported in MPN patients as well as patients with MDS/MPN overlap syndromes. These data, in collaboration with subsequent functional studies, imply that epigenetic deregulation is a critical event in the pathobiology of all myeloid malignancies.

**DNA methylation regulates gene expression during normal and malignant myelopoiesis**

DNA methylation is one of the key epigenetic signals regulating gene expression in eukaryotic cells. The observation of an early lethal effect of DNA methyltransferase (DNMT)-null mice revealed the critical role of methylation patterns in embryonic patterning, X-chromosome inactivation, and genomic imprinting (13). Gene expression control arises through methylation of cytosine residues in CpG sites—regions where a cytosine and guanine reside are adjacent (4, 13). Mammals (including humans) have a global methylation pattern, consisting of methylated genomic, intergenic, and transposon sequences (8). Genomic regions with a high density of CpG sites are commonly referred to as ‘CpG Islands’ and are associated with the promoter regions of 50% of human genes (13). Methylation at promoter sites is a mechanism of gene repression, as it is associated with
reduced gene expression and corepressor complex recruitment.

Consistent with the coordinated gene expression changes observed during hematopoiesis, researchers have observed regular DNA methylation patterns during lineage specification and differentiation (14). As expected, promoters of genes required for hematopoietic cell fate specification are frequently de-methylated upon progenitor cell differentiation (15). Conversely, genes responsible for maintaining a stem/progenitor fate, such as meis homeobox 1 (MEIS1) and homeobox A9 (HOX9) become increasingly methylated during differentiation (14, 16). Detailed genomic studies of the methylation landscapes of MPN and AML revealed core sets of genes that are differentially methylated between transformed cells and normal hematopoietic cells (17, 18). These data underscore the importance of abnormal (and potentially malignant) DNA methylation changes, and epigenetic dysregulation in general, in the pathobiology of myeloid malignancies.

**DNMT3A**

DNA methyltransferase 3A (DNMT3A) is a member of the mammalian family of methyltransferases that enzymatically adds a methyl group to cytosine nucleotides in CpG islands. DNMT3A is one of two de novo methyltransferases that can methylate both unmethylated and hemimethylated cytosines. Somatic DNMT3A mutations were first identified by Ley et al. in adult AML patient samples following whole genome sequencing of a cytogenetically normal AML patient sample (19). They identified DNMT3A mutations in 22% of adult AML cases, and that these cases were associated with an increased risk of relapse in a single-institution patient cohort. A follow-up study of 489 AML samples revealed that DNMT3A mutations conferred poor prognosis and decreased overall survival (20). These patients were all heterozygous for DNMT3A mutations. Despite being scattered throughout the open reading frame, the majority of somatic mutations cluster in the methyltransferase domain. Mutations also result in either a premature truncated protein (through nonsense or frameshift events), or are missense events that occur most frequently at the R882 amino acid residue. R882 mutations occur in nearly 60% (37 of 62 samples) of DNMT3A-mutant samples, which targets the methyltransferase domain and decreases both DNMT3A’s catalytic activity and DNA-binding affinity (21, 22) as documented in in vitro studies.

The vast majority of DNMT3A-mutant patients retain heterozygosity (19), suggesting that DNMT3A contributes to myeloid transformation as a haploinsufficient tumor suppressor. However, since DNMT3A functions as an oligomer, dominant-negative inhibition of the wildtype enzyme by mutant DNMT3A cannot be excluded. Recent work has confirmed a dominant-negative effect of R882 mutant DNMT3A over wildtype (23). However, this effect only appears when the wildtype and R882-mutant proteins are co-expressed in vivo (24). In this setting, methyltransferase activity is reduced to 20% of wildtype DNMT3A, and this decrease is attributed to reduced tetramerization of wildtype proteins caused by the R882H mutant form.

DNMT3A is the only known DNMT family member mutated in myeloid malignancies, which suggests that DNMT3A may have a unique role in normal hematopoiesis among members of the DNMT gene family. However, partially overlapping functions between DNMT family members, such as DNMT1 and DNMT3b, cannot be formally excluded. Initial investigations using mouse models revealed no overt effects on hematopoiesis unless Dnmt3a and Dnmt3b were codeleted (25). Extensive in vivo efforts aimed to follow-up on this by studying the effect of hematopoiesis-specific conditional Dnmt3a loss on self-renewal during serial transplantation. Challen et al. (26) found that Dnmt3a loss induced a progressive expansion of the long-term hematopoietic stem cell (LT-HSC) pool without altering HSC proliferation or differentiation. Careful analysis of the HSC methylome in this model determined that different loci became hypo- and hypermethylated upon Dnmt3a loss. Notably, a core set of genes crucial for HSC self-renewal and malignancy-promoting factors were both hypomethylated with increased expression levels. It is not yet known if this altered epigenetic pattern also occurs in human DNMT3A-mutant AML.

The picture becomes increasingly more complex, as the different mouse models studied to date vary widely in both their methodologies and findings. Transgenic overexpression of the R882H mutation causes a progressive MDS-like phenotype characterized by anemia, dysplastic features, and a block in erythroid differentiation (27). Mice retrovirally overexpressing DNMT3A(R882H) in a bone marrow transplant (BMT) model developed chronic myelomonocytic leukemia (CMML) at 1 year post transplantation (28). In this BMT model, overexpression of DNMT3A(R882H) also significantly altered gene expression between mutant and wildtype/vector controls at 6 months and 1 year post transplantation, although no substantial change in global
methylation was detected. Several genes involved in HSC 'stemness' and leukemic progression were notably upregulated.

DNMT3A mutations are most common in AML. DNMT3A-mutant AML has been linked to anthracycline resistance and poor prognosis in some studies (3, 29). DNMT3A-mutant leukemia patients present with higher peripheral white blood cell counts and frequently also contain concurrent mutations in nucleophosmin (NPM1), FMS-related tyrosine kinase 3 (FLT3), and IDH1 mutations (19). While a global decrease in DNA methylation is expected with reduced DNMT3A activity, no significant difference in global 5-methylcytosine (5-mc) levels is detectable in DNMT3A-mutant leukemia samples by liquid chromatography-mass spectrometry (LC-MS) (19). Epigenetic studies using MeDIP-chip revealed decreased methylation at 182 specific genomic loci (19). Surprisingly, methylation changes were not associated with changes in gene expression. Efforts to determine if changes in DNA methylation patterns and levels are impacted by the presence of additional mutations are currently underway. A recent re-analysis of 85 DNMT3A (R882H)-mutant AML patient samples found that hypomethylation occurs independently of NPM1 mutations at localized CpGs (24).

While far more common in AML patients, DNMT3A mutations also occur in classical MPN cases (7–15%), as well as in 8% of MDS cases (20, 30, 31). The prognostic relevance and clinical features of DNMT3A mutations in chronic malignancies remain to be determined, and only suggestive information about the timing of DNMT3A mutations during disease evolution and progression exists. DNMT3A mutations are present in nearly all cells obtained from MDS patient samples, irrespective of blast count (32). This observation complements the MDS-like phenotype that arises when DNMT3A(R882H) is overexpressed in a murine BMT setting (28), and has also supported the notion that DNMT3A mutations are an early event in the development of MDS. One study compared MDS/MPN samples with corresponding DNMT3A-mutant secondary AMLs and identified the same DNMT3A variant in the pre-existing chronic malignancy as observed in the secondary leukemia in all ten cases (33). This strongly suggests that DNMT3A mutations might be an early clonal event during leukemic evolution. A recent elegant study provided additional support for this concept. Shlush et al. (34) found that DNMT3A mutations occur before the acquisition of NPM1c and FLT3-ITD mutations in AML, and that DNMT3A mutations reside in preleukemic HSCs that later expand to form the dominant AML clone. DNMT3A-mutant HSCs were also found to be resistant to chemotherapy, and were detectable during remission. Collectively, this suggests that in DNMT3A-mutant myeloid malignancies, the DNMT3A mutation is likely acquired by HSCs at an early preleukemic stage and there is a requirement for the acquisition of additional mutations to develop overt MPN, MDS, or AML.

Taken together, these studies establish that DNMT3A mutations contribute to a whole spectrum of myeloid malignancies. The precise mechanisms through which DNMT3A contributes to normal and malignant hematopoiesis remain elusive and poorly delineated. The relative importance of genetic and epigenetic context in DNMT3A-mutant malignancies also remains to be determined. None of the Dnmt3A mouse models described to date has developed AML. Future work using conditional mutant knockin models in combination with other leukemia disease alleles, and analysis of patient sequence data should shed light onto the roles of DNMT3A in leukemogenesis and MPN/MDS evolution.

TET2 mutations and DNA hydroxymethylation

In addition to compromised methyltransferase activity, cytosine demethylation (the removal of repressive methyl groups on DNA) is also impaired in myeloid diseases.

Cytosine demethylation was originally believed to occur through a passive mechanism. However, work on the TET family of proteins (including TET1, TET2, and TET3) demonstrated that DNA demethylation can occur by an active mechanism (35). TET1 was first identified and cloned as an MLL fusion partner in t(10,11)(q22;q23) AML patients (36). The three TET proteins are iron [Fe(II)] and α-ketoglutarate (α-KG)-dependent dioxygenase enzymes that hydroxylate methylated cytosine residues. The net result of this reaction is the conversion of 5-mC (5-methylcytosine) to 5-hmC (5-hydroxymethylcytosine), which is a key intermediate in the DNA demethylation process. Subsequent steps convert 5-hmC into 5-formylcytosine (5-fmC) and then 5-carboxycytosine (5-caC). 5-caC is recognized and ‘repaired’ (replaced with a non-methylated cytosine residue) by thymine DNA glycosylase (TDG)-mediated base excision repair during the final step of cytosine demethylation (37). In addition to serving as a key intermediate of DNA demethylation, an emerging body of work suggests that 5-hmC might regulate cell-specific gene expression (38, 39).

TET2 mutations rank among the most frequent events detected in myeloid diseases. Up to 50% of CMMML patients harbor mutant TET2. Somatic deletions and loss-of-function
TET2 mutations were first identified in MPN and MDS cases based on mapping of loss-of-heterozygosity and microdeletions within a minimal region of chromosome 4q24 (40, 41). About 10–20% of MPN/MDS cases and 7–23% of AML cases carry TET2 mutations. Mutations occur through exons 3–12 of TET2, and include deletions, loss-of-heterozygosity, and missense, nonsense, and frameshift events which all impair wildtype TET2 function (40, 42–44). While clinical correlative studies have produced varied results, TET2 mutations in the largest set studied to date was associated with poorer prognosis in patients with cytogenetically normal AML (3). As with DNMT3A, the prognostic relevance of TET2 mutations in MPN and MDS patients has not been clearly determined (45, 46).

There are abundant data suggesting that TET2 mutations are an early and potentially disease-initiating event in a subset of MPN cases. They can also be detected in MPN patients whose disease has transformed to secondary AML. In some of those cases, analysis of paired MPN/AML samples demonstrated that TET2 could be acquired as a late event at the time of overt leukemic transformation. In a study of MDS and CMML patients, TET2 mutations were detected in the HSC-containing CD34+ cell population and some patients had more than one TET2 mutation (47). TET2 mutations in early stem cells might confer an advantage during clonal evolution. Most TET2 mutations are heterozygous in leukemia, and expression of the wildtype allele is retained in patients with monoallelic mutations. Biallelic inactivation of TET2 is relatively rare and occurs in less than 10% of leukemia patients (40). This information implies that TET2 acts as a haploinsufficient tumor suppressor in most patients.

In vitro studies indicate that short hairpin RNA (shRNA)-mediated knockdown of TET2 impairs hematopoietic differentiation (48). Several murine models of Tet2 loss have confirmed this observation. In each model described to date, Tet2 loss disrupts hematopoietic differentiation, increases HSC self-renewal, and causes progressive expansion of the stem/progenitor cell-rich Lineage− Sca1+ Kit+ (LSK) compartment. Conditional loss of Tet2 in the hematopoietic compartment gives rise to myeloid leukemia closely resembling human CMML after just 3 months (49). In one Tet2-knockout mouse model, mice developed an AML with erythroid progenitor expansion. There are significant differences in the phenotypes observed in the various models, which may be attributable to the different approaches used to generate these alleles and the genetic background. But there are important common features as well. Importantly, in each model published to date, Tet2-deficient hematopoietic cells give rise to chronic myeloid expansion, which has a variable and long rate of latency progression to acute leukemia. Thus, secondary events likely are required to produce specific myeloid disease phenotypes.

Loss of TET2 enzymatic activity is predicted to result in an increase DNA cytosine methylation and a reduction in 5-hmC levels. Thus, it is not surprising that 5-hmC levels are globally reduced in patients with TET2-mutant myeloid malignancies (48). Reports documenting a corresponding increase in 5-mC levels are not, however, as consistent. Genome-wide DNA methylation studies in MDS and AML have reported both hypermethylated and hypomethylated profiles associated with TET2-mutant disease (48, 50, 51). Use of both HELP (HpaII tiny fragment enrichment by ligation-mediated PCR)-based methylation profiling and LC-MS to detect global methylation and 5-hmC levels in AML patient samples revealed both lower 5-hmC levels and a hypermethylation phenotype in TET2-mutant AML (48). However, a concurrent report that used the Illumina Infinium 27k methylation array to profile the methylome of TET2-mutant MDS and other chronic myeloid malignancies suggested that global DNA methylation was not altered in bone marrow samples from patients with TET2 mutations (48). Patients with relatively low 5-hmC levels also showed hypomethylation at differentially methylated CpG sites compared to controls. It is unclear if this difference is due to variations in the genetic context (presence of additional mutations), disease context (MDS versus AML), or in the platforms and cell populations used by the different groups.

While the specific role of epigenetic changes caused by TET2 mutations is uncertain, TET2-mediated hydroxymethylation likely plays pleiotropic roles in myeloid leukemogenesis. Observations in mouse models, along with the detection of TET2 mutations in a wide range of myeloid disorders in human patients, suggests that TET2 loss induces leukemogenesis by increasing self-renewal capacity, which increases the propensity for the acquisition of secondary genetic events. TET2-mutant AML cases appear to have a unique gene expression signature, characterized by deregulation of genes involved in stem cell self-renewal, cell cycle control, and cytokine/growth factor signaling (45).

IDH1/2 mutations
In addition to being compromised by loss-of-function mutations, the catalytic activity of TET2 can be inhibited by the oncometabolite 2-hydroxyglutarate (2-HG) (52). In normal cells, the metabolic enzymes isocitrate dehydroge-
nase 1 and 2 (IDH1/2) convert isocitrate to α-KG in an NADP⁺-dependent fashion during an essential step of the Krebs cycle. Mutations in IDH1 were initially identified in 12% of 149 glioblastoma samples analyzed by whole-exome sequencing (53). Since then, IDH1 mutations have also been detected in chondrosarcomas, cholangiocarcinomas, colorectal cancer, thyroid cancer, and AML (54). Follow-up candidate-gene sequencing efforts later found IDH2 mutations in glioma and in AML (55, 56). IDH1 and IDH2 mutations are also present in 2–5% of MPN patients, and 3% of MDS patients (54), and are associated with shortened survival and a decreased time to leukemic transformation (57). The mechanism through which IDH mutations promote leukemic evolution remains to be determined.

α-KG is required for the production of 5-hmC by TET2 and the other TET proteins (37). In AML and other myeloid diseases, missense mutations at the conserved R132 residue in IDH1 and either R172 or R140 in IDH2 cause a change in substrate specificity that triggers further catalysis of α-KG to 2-HG by the mutant IDH1/2 enzymes (52). As a result of this neomorphic enzymatic activity, 2-HG accumulates in the cell (56, 58). Levels of 2-HG are also elevated and detectable in the serum of AML patients, suggesting its potential use as a biomarker of IDH-mutant disease (56). 2-HG build-up in IDH1/2-mutant cells results in TET2 inhibition and a corresponding increase in DNA methylation at promoter regions across the genome. During hematopoiesis, this results in impaired differentiation and causes an increase in stem/progenitor cells that phenocopies TET2 loss (18, 51).

IDH1/2 and TET2 mutations in patients tend to be mutually exclusive (3, 51), and share unique DNA methylation and gene expression patterns (59). Conditional knock-in of the Idh1(R132H) mutation in the mouse hematopoietic compartment causes 2-HG production and expands the hematopoietic progenitor pool. Differentiation is also impaired, as mice also exhibit anemia, splenomegaly, and intramedullary hematopoiesis (60). DNA methylation profiling of progenitor cells from the hematopoietic compartment of Idh1 knock-in mice confirmed the genome-wide trend towards hypermethylation seen in IDH1-mutant patients (60). This functional, epigenetic, and genetic data taken together suggest that TET2 and IDH mutations comprise a mutational complementation group in myeloid diseases.

In addition to inhibiting TET2 dioxygenase activity, 2-HG also inhibits other α-KG-dependent proteins, including members of the Jumonji-C (JMJC) domain histone demethylase family (61). Not only is DNA methylation altered in IDH1/2-mutant cells; 2-HG levels also increase levels of histone lysine di- and trimethylation marks due to impaired histone demethylation (62). Differences in the mutational spectrum and clinical correlates of TET2 and IDH-mutant myeloid diseases might be explained by this expanded range of epigenetic writers whose function is compromised by IDH1/2 mutations, whereas TET2 mutations only affect TET2 activity. Other enzymes are likely inhibited by the increase in 2-HG levels seen in IDH-mutant tumors. However, the role these targets play in IDH-mediated transformation is less well established.

Somatic mutations affecting histone modifications

MLL translocations

MLL genes are the mammalian homologs of the Drosophila trio-thorax genes, and encode a family of histone H3 lysine 4 (H3K4) histone methyltransferase proteins. MLL1 is rearranged by chromosomal translocation in 5–10% of AML cases and up to 80% of pediatric leukemia cases (63, 64), and MLL-rearranged leukemias tend to be highly aggressive. All MLL translocation events result in the fusion of the 5’ end of MLL with the 3’ end of one of many fusion partners. The resulting MLL fusion proteins retain aberrant expression of hematopoietic stem cell genes normally regulated by MLL1, such as HOXA9 and MEIS1, which drives leukemogenesis. MLL-rearranged leukemias are a classic example of altered epigenetic patterning promoting myeloid transformation.

EZH2 and genetic loss of polycomb repressive complex 2 (PRC2) activity

Polycomb group (PcG) proteins are transcriptional repressors that target genes essential for regulating cell differentiation and maintenance of cell identity in different cellular contexts. There are two known and distinct complexes of PcG proteins, PRC1 and PRC2, which serve an important and conserved function throughout vertebrate development. PcG complexes initiate and maintain transcriptional silencing by placing and maintaining specific posttranslational histone modifications. The mammalian PRC1 complex is comprised of two core members (RING1A and RING1B) and one of the following: BMI1, MEL18, or NSPC1 (65). In contrast, the mammalian PRC2 complex consists of four core members: EZH1 or EZH2, embryonic ectoderm development (EED), suppressor of zeste 12 homolog, and RBAP48 (65). Alterations of EZH2 are the most common instances of PcG anomalies in human diseases. EZH2 is the catalytic component of PRC2 and is an H3K27 methyltransferase. H3K27methyl marks are an epigenetic modification strongly
associated with transcriptional repression. EZH2 and its homolog EZH1 are enzymatically similar, and the functional redundancy between EZH1 and EZH2 in controlling PRC2 activity likely differs on the basis of cellular context. While mutations in EZH1 or altered expression levels have not been found to date in human malignancies, overexpression of wildtype EZH2 is commonly seen in numerous epithelial diseases (66, 67). Increased EZH2 expression is caused, in part, by the loss of microRNA-guided transcriptional repression (68). Somatic heterozygous activating mutations in EZH2 at the Y641 reside were recently identified in germinal diffuse large B-cell lymphomas (69), and these mutations were shown to increase dimethylation and trimethylation of H3K27 in biochemical studies (70, 71).

In stark contrast, loss-of-function EZH2 mutations are observed in myeloid malignancies and have been shown to impart a adverse patient outcome in MDS and primary myelofibrosis (PMF). Chromosome 7q deletions, which frequently include EZH2 loss, are included in established clinical cytogenetic prognostic schemas for MDS and AML patients (72). More recent mutation profiling of a large MDS patient cohort reported that EZH2 mutations were associated with worse overall survival, independent of established prognostic markers (46). EZH2 mutations are similarly associated with worsened overall survival in PMF (73). EZH2 mutations were not associated with an increased risk of leukemic transformation in MDS, which suggests a differential role for EZH2/PRC2 in chronic and acute myeloid diseases. No EZH2 mutations have been reported in AML patient samples to date, which further support this hypothesis.

While the clinical implications of EZH2 mutations are fairly well-defined, our biological understanding of EZH2’s role in oncogenesis is incomplete. While gain-of-function and overexpression of EZH2 frequently occurs in solid tumors and lymphoid malignancies, EZH2 activity is commonly attenuated in myeloid malignancies. Several mechanisms lead to this net reduction. Deletion of one EZH2 allele occurs in most cases of −7 and some cases of −7q myelodysplastic disorders, while inactivating mutations occur in AML, MDS, MPN, and CMML (74–76). In EZH2-wildtype and diploid chromosome seven patients, defective EZH2 mRNA splicing also occurs due to mutations in splicing factors U2AF1 and SRSF2 (76). Thus, haploinsufficiency, loss-of-function mutations, or aberrant posttranscriptional regulation can all cause the deficit in EZH2 expression and activity seen in myeloid disease.

Conditional deletion of Ezh2 in the hematopoietic system in vivo causes an MDS/MPN-like phenotype (77). In a cohort of 119 AML, MDS, and CMML patients, it was shown that TET2 mutations co-occurred with EZH2 mutations in 6.7% of the patients analyzed (77). Interestingly, loss of both Tet2 and Ezh2 in a double knock-out mouse model caused a more rapid progression to an MDS/MPN/CMML-like syndrome or MDS-like disease than loss of either gene alone. These findings suggest a cooperative effect between these two frequently comutated epigenetic modifiers, although additional investigation is needed to uncover the missing functional link between EZH2/TET2 loss and disease progression.

All together, the literature so far suggests that PRC2 alterations have a haploinsufficient tumor suppressive role in the pathogenesis of myeloid malignancies. While mutations in additional PRC2 components are not mutually exclusive with EZH2 mutations and are rare, they do occur at a significant frequency: 1.4% of MDS/MPN overlap disorder or PMF have mutations in SUZ12, and approximately 1% have deletions or mutations in EED (78, 79). Deletion of the core non-enzymatic components of PRC2 in vivo can cause myeloid transformation and increase HSC self-renewal capacity. Eed−/− mice develop a severe and lethal myelo- and lymphoproliferative disease (80). However, the fact that EZH2 activity is both gained and lost in various disease settings implies that it acts as both an oncogene and tumor suppressor. This dual role of EZH2 can even be observed in the same cellular compartment; Ectopic in vivo overexpression of EZH2 in the hematopoietic compartment using a conditional knockin model also causes myeloid transformation (81). These observations underscore the essential role of adequate epigenetic regulation in maintaining normal tissue development and function. Further work is needed to advance our understanding of PRC2 activity in myeloid leukemogenesis.

ASXL1 mutations

Additional sex combs like 1 (ASXL1) is frequently mutated in myeloid malignancies (82). ASXL1, along with BRCA1-associated protein 1 (BAP1), is part of the Polycomb repressive deubiquitylase complex (PR-DUB), which deubiquitylates H2AK119 marks (83) leading to gene repression. Mutations in ASXL1 occur in 10–30% of all myeloid diseases, and are most common on MDS, PMF, and MDS/MPN overlap syndromes (46, 82, 84). The majority of mutations result in frameshift events in the PHD, which is presumed essential for methylated lysine binding (85, 86). A large proportion of these mutations result in reduced ASXL1 expression (85), which strengthens support that these events are loss-of-function. Recent studies have shown that ASXL1 interacts with the
PRC2 complex, and that ASXL1 loss reduces both global and site-specific H3K27me3 (85).

Conditional deletion of Asxl1 in the hematopoietic compartment causes myelodysplasia, and mice develop progressive anemia and leukopenia (87). Asxl1 loss, surprisingly, does not significantly change H2AK119 ubiquitylation levels in myeloid cells; thus, the relationship between H2AK119 ubiquitylation marks and myeloid neoplasia remains unclear (82). Loss of Asxl1 expression during myeloid pathogenesis results in a reduction in repressive H3K27 trimethylation marks at specific target loci in the genome (85), presumably through inhibition of PRC2 recruitment at these sites and consequent increased expression of PRC2 target genes, such as members of the HoxA cluster. Moreover, shRNA-mediated knockdown of ASXL1 in vivo resulted in accelerated disease latency and increased disease burden in a mouse model of hematopoietic NucG12D expression (85). Interestingly, the combined loss of Asxl1 and Tet2 results in a more penetrant, shortened latency disease, similar to concomitant Asxl1 and Ehh2 loss (87). Interestingly, Tet2 loss reversed the reduction in HSC self-renewal seen upon Asxl1 loss. This combined effect on stem cell function and shortening disease progression suggests an interdependence of these two frequently comutated epigenetic modifiers in myelodysplasia.

ASXL1 mutations were originally reported in MDS, and subsequently found in MDS/MPN overlap syndromes and AML (88). Mutations in ASXL1 are consistently associated with worsened overall survival in MDS (89) and in MPN (90), independent of other prognostic factors such as age, cytopenias, and cytogenetics. ASXL1 mutations are more enriched in secondary AMLs arising from a preceding hematopoietic disorder than in de novo AML (91). While mutational studies of ASXL1 existing in the literature are impaired by the inclusion of non-somatic variants (92), ASXL1 mutations are detected at diagnosis and remain constant throughout disease progression in MDS and MPN (91). This evidence suggests that ASXL1 mutations are early events in myeloid pathogenesis that might precede JAK2 and TET2 mutations (85, 91). ASXL1 mutations are presumably associated with worse outcome in normal karyotype AML (3) and, interestingly, seem to occur in an age-dependent manner (93).

Cohesin complex alterations

One of the most intriguing findings in very recent years was the observation of recurrent somatic mutations in genes encoding members of the cohesin complex. The cohesin complex is an evolutionarily conserved multimeric ring-shaped complex of four proteins, STAG21, SMC1A, SMC3, and RAD21, and is responsible for maintaining sister chromatid cohesion after DNA replication until separation during mitosis (94). Cohesin complex mutations so far have been reported in a variety of malignancies, including: AML, MDS, glioblastoma multiforme, Ewing’s sarcoma, colorectal carcinoma, and bladder carcinoma (95–98). A recent report surveying a cohort of 1060 patients with all myeloid diseases detected somatic cohesin mutations in 12% of patients, while decreased expression was also observed in an additional 15% (99). Mutations in cohesin components are all mutually exclusive, and are predicted to be loss-of-function events. Coincident with this, samples expressing low cohesin levels had similar expression signatures as those seen in cohesin-mutated samples (99). Since cohesin plays a crucial role in maintaining genome integrity, it was initially hypothesized that cohesin mutations would lead to chromosomal instability (100). However, cohesin mutations in AML are not associated with aneuploidy or complex cytogenetic profiles (101). This suggests that cohesin mutations transform hematopoietic cells via an alternative mechanism, presumably by perturbing cohesion-independent cohesin complex functions.

Cohesin mutations might contribute to hematopoietic transformation, like other epigenetic regulator mutations, by promoting aberrant gene expression. Cohesin has established roles in regulating both gene expression and DNA-loop formation. Cohesin was also recently shown to localize at locus-control regions with broad H3K27-acetylation peaks, known as super-enhancers, which regulate the expression of cell identity genes and pluripotency/self-renewal factors in malignant and embryonic stem cells (102). These observations collectively suggest that loss-of-function alterations in cohesin complex members could promote transformation and leukemogenesis by altering transcriptional control of differentiation or proliferation. The vast majority of mutations are heterozygous events (101), which implies haplessufficient tumor suppressor activity. Studies of cohesin dynamics upon BRD4 inhibition with JQ1 led to a twofold decrease in cohesin occupancy at super-enhancer sites and decreased oncogene expression (102, 103). This collectively suggests that reducing cohesin gene dose alters cell identity by regulating the transcription of key cell identity genes.

While there is still extensive investigation required to evaluate these promising hypotheses, recent work has focused on delineating the clinical impact of cohesin mutations. Thota
et al. (99) recently published observations from a large and heterogeneous cohort of 1060 patients with MPN, MDS, MDS/MPN overlap syndromes, primary AML, and secondary AML to elucidate the effects of these mutations on disease phenotype, therapeutic response, genetic background, and clinical outcome. Clonal hierarchy analysis of cohesin-mutant samples revealed that cohesin mutations rarely occurred in the founding clone and likely occurs later during the process of hematologic transformation (99). This observation suggests that aberrant cohesin function could promote expansion of the malignant clone over time. Cohesin-mutant MDS patients also had a higher response rate to azacitidine (99). Future work will be needed to explore the impact of cohesin mutations on patient prognosis, as well as validate patterns of mutational co-occurrence.

Alterations affecting posttranscriptional control of gene expression

One of the most exciting discoveries in recent years is the discovery of somatic mutations that affect genes involved in posttranscriptional regulation of gene expression. These include mutations in genes encoding spliceosome complex members, and most recently, the discovery of somatic mutations in the endoplasmic reticulum (ER) chaperone calreticulin (CALR). Alterations in these genes, while still not entirely understood, could promote myeloid transformation by directly or indirectly influencing the posttranslational expression of differentiation and/or proliferation factors.

Spliceosome mutations in myeloid diseases

Mutations in splicing machinery genes were first reported in MDS (104). Recurrent and mutually exclusive mutations in genes encoding spliceosome components (such as SF3B1, SRSF2, U2AF1, ZRSR2, etc.) occur in all myeloid malignancies but are most common in MDS. It is unclear if splice variants arising from abnormal spliceosome activity are retained during cell division and thus represent true epigenetic information. However, the splicing machinery is intimately coupled to transcription, and increasing evidence suggests that the spliceosome and transcriptional machinery coordinate regulation of target gene expression (105). Chromatin state can regulate mRNA splicing in two different ways: First, the rate of transcriptional elongation by RNA Pol II is dependent on the degree of chromatin compaction and influences alternative splicing. For instance, gene body methylation stimulates splicing during transcriptional elongation. Second, specific chromatin modifications can also interact with the spliceosome machinery. Aberrant splicing events can also contribute to epigenetic deregulation by affecting expression levels or functionality of epigenetic regulators. For example: splice isoforms of DNMT3B increase methylation profile variability (106, 107). Unraveling the role of these events in myeloid disease will be a challenging and interesting avenue of future work. More work is needed to understand the consequences of these mutations on spliceosome function in vivo, and how they promote myeloid transformation either alone or in concert with other disease alleles.

Calreticulin mutations in classical MPN

The most recent set of novel mutations associated with myeloid diseases were observed in the calreticulin (CALR) gene. Two groups recently identified CALR mutations in JAK2-wildtype MPN patients, including 25–27% of patients with essential thrombocytopenia (ET) and 14–35% of PMF, specifically (108, 109). CALR mutations, surprisingly, are mutually exclusive with JAK2 and MPL mutations and occur in up to 84% of JAK2-wildtype MPN cases. No CALR mutations have been reported in AML and MDS as of this time. In retrospective analyses, CALR-mutant ET and PMF cases have improved overall survival and decreased risk of thrombosis, compared to JAK2-mutant cases (109). The relevance of CALR mutations to MPN pathogenesis and therapeutic outcome remains to be investigated.

CALR is an ER chaperone protein that mediates posttranslational transport and modification of membrane proteins. Reported CALR mutations occur only in exon 9, and all of them result in a +1 basepair frameshift. This frameshift creates an alternate C-terminus lacking the KDEL ER retention signal. Initial studies showed that mutant CALR traffics normally. However, in one study, overexpression of mutant CALR in BaF3 cells, a mouse pro-B cell line, led to cytokine-independent growth and increased levels of phosphorylated STAT5 (108). Integrated gene expression profiling has also revealed that a JAK2 activation signature is present in all MPN patients, including in CALR-mutant cases (110). This suggests that CALR mutations contribute to MPN pathogenesis by activation of JAK/STAT signaling through a completely unclear mechanism. On-going work will confirm these hypotheses and help uncover the precise pathophysiologic role of CALR mutations in MPN development, and reveal how CALR regulates JAK/STAT signaling. While CALR mutations do not affect the epigenome in the traditional sense, by altering DNA/histone modifications and chromatin architecture, they could alter gene expression.

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patterns at the posttranscriptional level in a heritable fashion.

**Therapeutically altering the leukemia epigenome**

Given the prominent role of epigenetic dysregulation in the initiation and evolution of leukemia and other hematologic disorders, drugs that target and modulate epigenetic marks and their readers/writers are being developed and used in the clinic.

**DNMT inhibitors**

The clearest example of epigenetically targeted therapy in the treatment of myeloid diseases is the use of DNA methyltransferase inhibitors (DNMTi). DNMTi treatment is now used in the place of conventional chemotherapy regimens in MDS and in AML patients who are not candidates for dose-intensive chemotherapy. The two clinically utilized agents are the nucleoside analogs azacytidine (AZA) and 5-aza-2-deoxycytidine [decitabine (DAC)]. They inhibit DNA methyltransferase activity in S phase, when they become incorporated into DNA and block additional DNA methylation by sequestering DNMTs and promoting their degradation (111, 112). AZA and DAC also induce DNA damage in cells (113). However, potential epigenetic repatterning is only one cellular consequence of DNMTi treatment. AZA is also incorporated into RNA, where it can also affect RNA processing and translation. How these compounds alter the epigenome and gene expression profile of a cell to ultimately achieve clinical efficacy is still not understood.

While AZA and DAC are emerging as common front-line therapy for MDS and AML, they do not show robust efficacy on the majority of patients, which does not appear to be due to their inefficacy at altering methylation levels. One study comparing DNA methylation levels in MDS, AML, and CMML patients at baseline and 15 days after combination therapy with AZA and a histone deacetylase inhibitor (HDACi) revealed that promoter regions became globally hypomethylated, and the effect persisted for 2 weeks after cessation of treatment (114). However, while combined AZA/HDACi treatment can induce robust demethylation across the genome, only 30–50% of patients displayed a clinical response (115). No study to date has also been able to identify gene expression changes which accurately predict or correlate with AZA or DAC treatment, despite the ability of these compounds to induce global methylation changes in vitro and in vivo (116, 117). This disparity between epigenetic repatterning and response demonstrates that DNA demethylation alone is insufficient to treat myeloid malignancy. Continued work is aimed at understanding how DNMT inhibitors function, and at identifying biomarkers of baseline response.

**HDAC inhibitors**

As histone modifiers are frequently mutated in myeloid diseases, various drugs affecting abnormal histone modifications are being developed for clinical use. Histone acetylation is traditionally considered to be a hallmark of actively transcribed genes, and is dynamically regulated by histone acetyltransferases and histone deacetyltransferases (HDACs) (118). Four classes of HDACs mediate deacetylation of histones and even other proteins. Increased levels of or abnormal recruitment of HDACs by transcription factor complexes contribute to tumorigenesis, and leukemogenesis, by reducing lysine acetylation, and gene expression levels (119).

While MDS and AML patients do respond to HDACi treatment, the response is relatively low compared to the 20–30% response rate reported in DNMTi trials (120–127). But, as with DNMTi therapy, the mechanisms of action for HDAC inhibitors are not well-delineated, which makes understanding and interpreting patient responses very difficult. HDAC inhibition does increase histone acetylation levels, but this effect is not predictive of clinical response. These compounds might affect multiple targets in the same cell, since transcription factor activity is also regulated, in part, by acetylation and deacetylation (126). The different HDACs under investigation could thus have widely differential effects on gene expression patterns. For instance, the HDAC vorinostat increases reactive oxygen species levels independent of its effects on histone acetylation (121), while panobinostat can reduce EZH2 expression levels and selectively kill AML cells (126).

Aberrant epigenetic signatures in myeloid diseases show both altered DNA methylation patterns and histone modifications. It is possible that effective therapy and epigenetic repatterning might require agents affecting both aspects of the epigenome. This has been well demonstrated in vitro (128, 129), and has contributed to the design of several recent clinical trials combining DNMTi and HDACi to treat AML and MDS (114, 129–132).

**DOT1L inhibition in MLL-rearranged leukemia**

DOT1L is a histone H3K79 methyltransferase that mediates oncogenesis in MLL-rearranged AML (133). Small molecule inhibitors of DOT1L have been recently developed, and
these compounds reduce H3K79 methylation levels and effectively kill MLL-transformed cells (134–136). Importantly, they also reverse MLL-mediated leukemogenic gene expression. Based on these successful preclinical efforts, a phase I clinical trial of EPZ-5657, a DOT1L inhibitor, is currently underway in leukemia patients (136, 137).

### IDH inhibitors

Due to the clear evidence supporting IDH1/2 mutations playing a critical role in oncogenesis, small molecule inhibitors of mutant IDH activity and 2-HG oncometabolite production have been developed. AGI-6780, an inhibitor of IDH2-R140Q, allosterically binds to the IDH R140Q dimer interface. This activity reduces 2-HG levels, due to blocked mutant IDH2 activity. This inhibition is sufficient to induce differentiation in primary AML cells (138). Another IDH inhibitor developed by Agios, AGI-5198, is efficacious against IDH1-R132H activity. Treating glioma cells with AGI-5198 reduced H3K9 methylation levels and induced consequent gene expression changes which promoted differentiation and decreased cell growth (139). Additional compounds have been developed which differentially inhibit IDH activity. Treating primary AML cell lines with one of these compounds, HMS-101, which blocks the isocitrate binding pocket of IDH, reduces colony formation and increases apoptosis. This effect was not seen in normal human CD34+ stem/progenitor cells treated in parallel (140). Thus, IDH1/2 inhibition in myeloid diseases can reverse the epigenetic derangement resulting from neomorphic mutant IDH1/2, and potentially have tremendous clinical benefit for patients.

### Conclusions, unanswered questions, and future challenges

Many myeloid disease alleles have well-delineated roles in normal hematopoiesis. However, we still do not fully understand the molecular role of many of these recently described myeloid disease alleles in the pathophysiology of myeloid malignancy. We also do not know why these different disease alleles are observed in different clinical phenotypes, and if they contribute to a wide spectrum of disease phenotypes via similar or divergent processes. In vivo modeling of these mutations, and in-depth profiling of patient samples drawn from all the disease phenotypes will provide deeper insight into these questions, particularly as models are developed in which hematopoietic cells are engineered to have combinations of disease alleles observed in MPN, MDS, and AML patients.

The molecular basis for transformation by many of these epigenetic regulators is still largely unknown. Specifically, many of the key target genes whose expression is disrupted by a given mutant epigenetic regulator are not known. Greater insight is needed in delineating the key deregulated loci and what relevance and role they have in leukemogenesis and myeloid transformation. However, such endeavors will not be without their challenges. Gene expression at a particular locus is affected by a number of spatial and temporal complexes, and is highly influenced by the three-dimensional nature of chromatin (141). Conventional RNA-seq and ChIP-seq methods cannot capture this, and it is very difficult to interpret the effects of a given epigenetic mark on gene expression. The application of Hi-C and other chromatin capture technologies in the not-too-distant future should greatly improve our understanding of how specific marks modify expression of target genes (142–143). Taken together, these data suggest that despite the importance of these genomic discoveries, there remains much to elucidate regarding how these alleles contribute to disease initiation and maintenance and how this knowledge can be used to develop novel, mechanism based therapies.

### References

84. Schmietter S, et al. ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. Leukemia 2013;27:82–91.
90. Abdel-Wahab O, Kilipovaar O, Patel J, Busque L, Levine RL. The most commonly reported variant in ASXL1 (c.1934dupG;p.Gly646TrpfsX12) is not a somatic alteration. Leukemia 2010;24:1656–1657.


