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The Role of Somatic Mutations in Acute Myeloid Leukemia Pathogenesis

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Acute myeloid leukemia (AML) is characterized by attenuation of lineage differentiation trajectories that results in impaired hematopoiesis and enhanced self-renewal. To date, sequencing studies have provided a rich landscape of information on the somatic mutations that contribute to AML pathogenesis. These studies show that most AML genomes harbor relatively fewer mutations, which are acquired in a stepwise manner. Our understanding of the genetic basis of leukemogenesis informs a broader understanding of what initiates and maintains the AML clone and informs the development of prognostic models and mechanism-based therapeutic strategies. Here, we explore the current knowledge of genetic and epigenetic aberrations in AML pathogenesis and how recent studies are expanding our knowledge of leukemogenesis and using this to accelerate therapeutic development for AML patients.

Acute myeloid leukemia (AML) is a clonal disorder initiated in hematopoietic stem and progenitor cells (HSPCs) that is characterized by impaired myeloid differentiation and abnormal proliferation at the expense of the normal hematopoietic system. AML is the most common form of acute leukemia in adults; an estimated 19,940 people will be diagnosed in 2020, and 11,180 patients will die of the disease (Siegel et al. 2020). AML is the 10th leading cause of cancer deaths in the United States. AML is most common in older adults, with a median age at diagnosis of 68 yr. Treatment of AML remains a significant challenge owing to the diverse molecular mechanisms contributing to different AML subtypes, and limited therapeutic options. Even though two-thirds of fit patients with newly diagnosed AML achieve morphological remission with intensive chemotherapy consisting of cytarabine and an anthra-

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cycline, the majority of these patients eventually relapse (Döhner et al. 2017; Kishtagari et al. 2020).

The first whole-genome sequencing of a cancer patient was of a patient with cytogenetically normal AML, reported more than a decade ago (Ley et al. 2008). Since then, gene discovery studies have identified an extensive catalog of recurrent somatic mutations in different AML subtypes (Mardis et al. 2009; Patel et al. 2012; Cancer Genome Atlas Research et al. 2013; Papaemmanuil et al. 2016; Tyner et al. 2018). These discovery studies have led to a greater understanding of AML biology, including stepwise accumulation of genetic alterations and recurrent mutations in epigenetic modifiers in AML. The types of mutations found in AML include chromosomal rearrangements (such as translocations or inversion), gain or loss of chromosomes (aneuploidy), total or partial gene deletion, point mutation, insertion, or gene duplication/amplification. AML can develop after an antecedent myeloid malignancy (secondary AML [s-AML]), after leukemogenic therapy (therapy-related AML [t-AML]), or without an identifiable prodrome or known exposure (de novo AML). The therapeutic strategy in "fit" patients with AML (primarily influenced by patient-related factors such as advanced age, performance status, and pretreatment comorbidities) is divided into induction and postremission therapy. Induction refers to the use of cytotoxic therapy (combination of cytarabine and an anthracycline) to induce remission, preferably without measurable residual disease (MRD). Standard postremission strategies include lower-intensity cytotoxic therapy and hematopoietic stem cell transplantation (HSCT). The use of different therapies in the postremission treatment is largely determined by prognostic risk stratification (Table 1). The treatment of AML patients who are "unfit" for intensive therapy include low-dose cytarabine, hypomethylating agents, and combination treatment strategies with novel agents (e.g., venetoclax, glasdegib). Recent data suggests remarkable efficacy for venetoclax/azacytidine in older adults with AML who are not candidates for intensive therapy, such that this regimen is quickly emerging as a new alternative for first-line therapy for older adults with AML (Pollyea et al. 2018).

In this review, we provide an overview of AML pathophysiology through the prism of genomic and epigenomic alterations and discuss how these mechanisms are perturbed in leukemogenesis. Acute promyelocytic leukemia (APL), a distinct subtype of AML, will not be addressed in this review, as we would posit it is a unique malignancy with distinct mechanisms of transformation and therapeutic options. In essence, we will discuss the pathogenesis of AML by addressing the role of recurrent cytogenetic abnormalities in AML pathogenesis, the underlying molecular events that contribute to AML, how our evolving understanding of the molecular basis of AML is refining prognostic schema, and how these insights are leading to new therapeutic approaches.

CHROMSOMAL ABNORMALITIES

In AML, detection of chromosomal abnormalities by cytogenetic analysis is critically important for diagnosis, risk stratification, and therapeutic decision-making (Fig. 1). Chromosomal abnormalities are detected in ~50% of patients with AML at the time of diagnosis. Chromosomal abnormalities most commonly contribute to leukemogenesis through the production of a fusion oncoprotein and/or by altering *cis*-regulatory elements. These large-scale copy-number alterations can serve as founding events and drive disease evolution in AML. Detection of t(8:21)(q22;q22.1), inv(16)(p13.1q22), t(16;16)(p13.1;q22), or translocations generating *PML-RARA* fusion transcripts (APL) allow the diagnosis of AML, regardless of the percentage of myeloblasts in the bone marrow or peripheral blood.

Core Binding Factor Rearrangements

The inv(16)(p13;q22) or t(16;16)(p13.1;q22) and t(8;21)(q22;q22.1) translocations lead to the expression of the *CBFB-MYH11* and *RUNX1-RUNX1T1* fusion oncogenes, respectively. In steady state hematopoiesis, core bind-

Table 1. The European LeukemiaNET (ELN) 2017 risk stratification of acute myeloid leukemia (AML)

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3-ITD* or with *FLT3-ITD*$^{\text{low = allelic ratio < 0.5}}$
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3-ITD*$^{\text{high = allelic ratio > 0.5}}$
	Wild-type *NPM1* without *FLT3-ITD* or with *FLT3-ITD*$^{\text{low}}$ (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3-ITD*$^{\text{high}}$
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

Data reprinted from Döhner et al. (2017), with permission from The American Society of Hematology, © 2017.

ing factor beta (CBFβ) and RUNX1 heterodimerize to bind DNA and recruit lineage-defining transcription factors to regulate hematopoietic differentiation (Tahirov et al. 2001). In CBF-AML, chromosomal rearrangements alter this transcriptional complex, thus dysregulating normal hematopoiesis. These rearrangements are present in ∼15% of AML cases. They are the most responsive AML subtypes to intensive chemotherapy, including postremission therapy with high-dose cytarabine (HiDAC) such that patients with these fusion oncoproteins are treated with induction chemotherapy followed by HiDAC consolidation as standard of care (Bloomfield et al. 1998; Byrd et al. 2004). Patients with CBF-AML usually have a relatively good outcome with overall survival rates of 60%–70% in adults. However, the expression of CD56 and the activating mutations in *KIT* have been correlated to greater risk of recurrence and shorter survival. The *KIT* mutations are found in 20%–25% of t(8;21)(q22;q22) and in ∼30% of inv(16) (p13;q22) patients (Paschka et al. 2006). However, the role of *KIT* mutations in prognostication and in molecularly targeted therapies for AML requires further prognostic and therapeutic studies. Both *CBFB-MYH11* and *RUNX1-RUNX1T1* fusion transcripts are well-established markers for MRD monitoring by real-time quantitative polymerase chain reaction (qRT-PCR) to guide postremission therapy (Schuurhuis et al. 2018).

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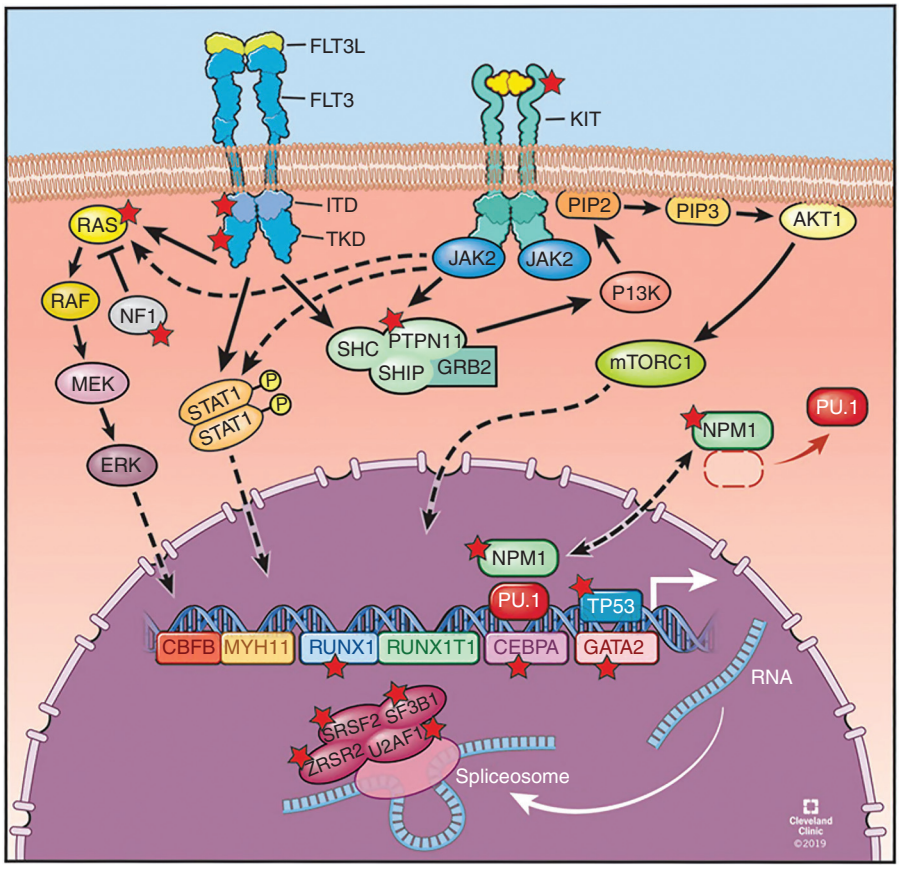

Figure 1. Overview of frequently mutated cellular pathways in acute myeloid leukemia (AML). These include (1) *CBFB-MYH11* and *RUNX1-RUNX1T1* fusion oncogenes; (2) nucleophosmin 1 (*NPM1*); (3) transcription factors *RUNX1, CEBPA,* and *GATA2*; (4) tumor suppressors *TP53*; (5) signal transduction, including *FLT3-ITD, FLT3-TKD, KIT, CBL, NRAS, KRAS, PTPN11,* and *NF1*; and (6) RNA splicing: *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*. Mutated genes are highlighted with a red star. (Refer to the main text for detailed explanations of the depicted processes.) (Figure reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2019–2020. All rights reserved.)

KMT2A (*MLL1*) Rearrangements

The mixed-lineage leukemia gene (*KMT2A*, also known as *MLL1*) encodes a histone H3 lysine 4 (H3K4) methyltransferase that regulates the expression of target genes, including homoeobox (*Hox*) genes. In *MLL1* fusions, *MLL1* retains the DNA-binding amino-terminal domain, and the catalytic methyltransferase activity carboxy-terminal SET domain is replaced by a fusion partner of one of more than 70 partner genes, most commonly *AF4* t(4;11), *AF9* t(9;11), *ENL* t(11;19), *AF10* t(10;11), and *ELL* t(11;19) (Krivtsov and Armstrong 2007). The most common fusion partners of *MLL1* are members of the DOT1L complex and/or the super elongation complex (SEC), which are both involved in transcriptional control. *MLL1*-fusion leukemias are characterized by increased expression of *HOXA9*, *MEIS1*, and *MEF2C*, which encode for leukemogenic transcription factors (Armstrong et al. 2002). *MLL1* fusions occur in as many as 10% of patients with AML and are common in t-AML following exposure to topoisomerase II inhibitors (Strissel et al. 1998). *MLL1*-fusion AML carries a poor prognosis be-

cause of refractoriness to chemotherapy and shorter period to relapse (Meyer et al. 2018). Aberrant recruitment of the DOT1L by *MLL1*-fusion leukemias provided a rationale for development of small-molecule inhibitors (e.g., pinometostat) targeting DOT1L with modest results (Stein et al. 2018). Moreover, recent data showing that *MLL1* fusions, but not wild-type *MLL1*, requiring menin as a co-factor to activate downstream target gene expression has led to the development of small-molecule inhibitors of the MLL1/menin interaction, which are now entering clinical trials (Yokoyama et al. 2005; Grembecka et al. 2012; Borkin et al. 2015; Krivtsov et al. 2019; Klossowski et al. 2020). Another mechanism by which *MLL1* plays a role in leukemogenesis is through an in-frame partial tandem duplication (*MLL1-PTD*) spanning exons 3 to 9, exons 3 to 10, or exons 3 to 11 (Steudel et al. 2003). *MLL1-PTD*s occur in 3.2%–11% of adult AML and more frequently present in AML with normal karyotype and trisomy 11 (Basecke et al. 2006). They confer a worse prognosis with shortened overall survival. *MLL1-PTD* AML has a distinct gene expression signature, and concomitant *DNMT3A* and *NRAS* mutations were associated with adverse clinical outcomes (Hinai et al. 2019).

Rare Translocations and Chromosomal Alterations

inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM(EVI1)

These rearrangements result in overexpression of the proto-oncogene *MECOM (EVI1)* at 3q26.2, through juxtaposition of the *EVI1* gene with a distal *GATA2* enhancer, and simultaneously confers *GATA2* haploinsufficiency (Gröschel et al. 2014). It is a distinct entity, which is present in de novo AML or AML arising from prior myelodysplastic syndromes (MDS), and is associated with aggressive disease with minimal response to standard therapies and short survival (Lugthart et al. 2010; Rogers et al. 2014). These rearrangements occur in 1%–2% of all AML cases. Mutations in genes activating RAS/receptor tyrosine kinase signaling pathways are reported in a significant proportion of the cases with these rearrangements; this may provide a target for a rational treatment strategy in this adverse-risk patient group (Gröschel et al. 2015).

t(6:9)(p23;q34.1); DEK-NUP214

This rearrangement results in a fusion of oncogene *DEK* on chromosome 6 with the nucleoporin gene *NUP214* on chromosome 9. The resulting nucleoporin chimeric fusion protein interacts with exportin-1 (XPO1)/CRM1 and may affect the export of nuclear proteins (Saito et al. 2016). It is a distinct entity reported in ∼2% of AML cases and is associated with a very poor prognosis. The majority of the patients with these rearrangements have a concomitant mutation in *FLT3-ITD*. Outcomes are dismal with standard chemotherapy with the only chance of long-term remission achieved for patients who can undergo allogeneic hematopoietic stem cell transplantation in first complete remission (Díaz-Beyá et al. 2020; Kayser et al. 2020). Given the fusion chimeric protein interaction with XPO1/CRM1, it will be interesting to see whether this fusion imparts sensitivity to XPO1 inhibitors (e.g., selinexor).

Complex Karyotype, -5/5q, -7/7q, -17/17p, +8/8q

Somatically acquired chromosome copy or segment gains, chromosomal monosomies, as well as the accumulation of karyotypic abnormalities, classified as complex karyotype, defined as three or more chromosomal abnormalities, are reported in ∼5%–10% of patients with AML. These alterations carry an adverse prognosis and are associated with a poor response to cytotoxic chemotherapy. Common abnormalities include those involving chromosomes 5 and 7, particularly in AML arising out of the background of MDS, and in t-AML arising in patients treated with alkylating agents and/or radiation therapy for a previous cancer. About 50% of patients with complex karyotype abnormalities have mutations in *TP53,* and it is hypothesized that *TP53* inactivation allows cells to

tolerate and persist in a setting of ongoing chromosomal instability. Moreover, it remains unclear whether the poor prognosis of complex karyotype AML is due to chromosomal alterations, inactivation of the p53 pathway, or both molecular events acting in concert. Haploinsufficiency of the following genes have been implicated in myeloid malignancies with monosomy 5 or large deletions of 5q [-5/del(5q)], including *RPS14*, *APC*, *CTNNA1*, *HSPA9*, *EGR1*, and *CSNK1A1* (Joslin et al. 2007; Liu et al. 2007; Ebert et al. 2008; Wang et al. 2010; Chen et al. 2011). Haploinsufficiency or loss of heterozygosity of candidate genes involved in -7/del(7q) include *SAMD9/9L*, *DOCK4*, *EZH2*, *CUX1*, *MLL3*, and *LUC7L2*, each of which has been suggested to contribute to myeloid transformation (Zhou et al. 2011; McNerney et al. 2013; Nagamachi et al. 2013; Chen et al. 2014; Hosono et al. 2014). Loss of chromosome 17p, including the *TP53* locus at 17p13.1, is associated with complex karyotype as well as abnormalities in chromosomes 5 and 7. In contrast to AML with balanced translocations, leukemias that develop in the context of 17p alterations are characterized by greater genomic instability (Liu et al. 2016). It is important to note that *NF1* is also on chromosome 17 and that some of the events involving 17q result in haploinsufficiency/loss of *NF1* and augmented MAPK signaling (Parkin et al. 2010). Other rare rearrangements, such as t(3;17)(q26;q22), involving fusion of the *EVI1* gene located on chromosome 3q26.2 and *MSI2* (MUSASHI [MSI] family of RNA binding protein) located on 17q are found in AML (De Weer et al. 2008). Somatic acquisition of trisomy 8 is seen in ~10% of patients with AML. They contribute to leukemogenesis, at least in part, via amplification of *MYC*, which is located at chromosome 8p24 and is implicated in a spectrum of malignancies including AML (Sloand et al. 2007).

RECURRENT MUTATIONS IN AML

NPM1

More than 50% of patients with normal karyotype AML harbor mutations in nucleophosmin 1 (*NPM1*) (Fig. 1). NPM1 is a multifunctional nucleus and nucleolar phosphoprotein involved in pleiotropic cellular functions (Falini et al. 2005). *NPM1* gene mutations typically occur as insertions in exon 12, causing a frameshift mutation with an added nuclear export signal (NES) motif at the carboxy-terminus; NPM1c protein product thus accumulates aberrantly in the cytoplasm (Falini et al. 2005; Sportoletti et al. 2015). Multiple recent publications have demonstrated that mutant NPM1 is aberrantly localized in the cytoplasm (NPM1c) and this mislocalization is essential for maintaining active chromatin marks that may help maintain expression of key target genes including *HOXA* and *HOXB* genes and *MEIS1*, thereby inhibiting differentiation of leukemic cells (Brunetti et al. 2018; Gu et al. 2018). It has been suggested that NPM1c shuttles PU.1 (SPI1) into the cytoplasm, which abrogates PU.1 mediated repression of *HOX/MEIS1* transcription (Gu et al. 2018). The World Health Organization (WHO) classification of hematopoietic tumors recognizes *NPM1*-mutant AML as a distinct entity (Table 1) (Arber et al. 2016; Döhner et al. 2017). Strikingly, the majority of *NPM1*-mutated AML have mutations in *DNMT3A* and *FLT3*. AML patients with *NPM1* mutations without mutations in *DNMT3A* and *FLT3* carry a favorable prognosis, but the co-mutations *FLT3-ITD* and *DNMT3A* confer an adverse prognosis (Döhner et al. 2017). In *NPM1*-mutant AML, prediction of relapse risk by MRD assessment by qRT-PCR demonstrated that persistent *NPM1* positivity in blood was the sole predictor of relapse in multivariate analysis irrespective of the co-mutational status (Ivey et al. 2016; Dillon et al. 2020). This further supports its inclusion into daily clinical practice to better guide postremission therapy (Schuurhuis et al. 2018). *NPM1* mutation is considered to be a leukemia-initiating event and thus represents an ideal therapeutic target. In a recent study, using *Npm1c/Dnmt3a* mutant knock-in mice and cell lines, a model of AML development, showed *NPM1*-mutant induced self-renewal properties in myeloid progenitor cells, which act as leukemia-initiating cells. In mice, oral administration of the small molecule inhibitor,

which targets the interaction between the histone methyltransferase MLL1 and adaptor protein menin, eradicated preleukemic *Npm1c*-mutant myeloid progenitor cells, prevented AML development, and extended survival. This study demonstrates that the elimination of at-risk preleukemic cells using targeted inhibitors may be a promising strategy (Uckelmann et al. 2020).

Mutations Disrupting Transcription Factor Function in AML (Fig. 1)

Transcription factors tightly regulate normal hematopoiesis. Abrogation of specific transcription factor activity results in block in differentiation of hematopoietic progenitor cells and the development of hematologic malignancies, including AML.

RUNX1

RUNX1 encodes the sequence-specific master hematopoietic transcription factor Runt-related transcription factor 1 (RUNX1), which is an essential regulator of hematopoiesis by maintaining the balance between cell lineage specification, self-renewal, and proliferation (Ito et al. 2015). RUNX1 has a highly conserved "Runt" homology domain (RHD), the motif responsible for heterodimerization with CBFβ, which facilitates the specificity and affinity of RUNX1 binding to target genes (Crute et al. 1996). More than 50 mutations in *RUNX1*, both point mutations and chromosomal rearrangements, have been reported in various hematologic malignancies, including AML. Loss-of-function somatic mutations in *RUNX1*, which are mechanistically distinct from rearrangements, occur in ∼5%–15% of all patients with AML and confer a poor prognosis (Gaidzik et al. 2016). Mutations in *RUNX1* are enriched in patients with intermediate-risk (including normal karyotype AML) disease (Patel et al. 2012; Papaemmanuil et al. 2016). *RUNX1* mutations are usually secondary events that drive disease progression, and they are mutually exclusive with *NPM1* and *CEBPA* mutations (Hirsch et al. 2016; Papaemmanuil et al. 2016). They are associated with older age and lower complete response (CR) rates and inferior overall survival (OS) (Mendler et al. 2012). Germline mutations in *RUNX1* are associated with familial platelet disorder with propensity to myeloid malignancy (FPDMM), characterized by thrombocytopenia, platelet functional/ultrastructural defects, and a 20%–60% rate of transformation to overt myeloid malignancies (Song et al. 1999).

CEBPA

The transcription factor CCAAT/enhancer-binding protein α (CEBPA) is a master regulator involved in cell fate decisions, including myeloid-lineage commitment (Avellino and Delwel 2017). CEBPA is expressed as two isoforms: the full-length (p42) and the amino-terminally truncated isoform (p30) that lacks the full *trans*-activation potential of p42. *CEBPA* mutations in AML harbor *CEBPA* amino-terminal nonsense or frameshift mutations, resulting in the increased expression of the dominant-negative p30 isoform, diminishing p42 availability to promote myeloid differentiation. Carboxy-terminal in-frame mutations interrupt DNA binding and homodimerization properties of the proteins and often co-occur on a separate allele from amino-terminal mutations. This pattern of mutations produces p30/p30 homodimers as the functional CEBPA transcription factor entity in *CEBPA*-mutant AML (Pabst et al. 2001; Kirstetter et al. 2008). These loss-of-function *CEBPA* mutations are reported in 5%–15% of AML patients and occur mostly in younger patients with normal karyotype AML (Fröhling et al. 2004; Cancer Genome Atlas Research et al. 2013). AML patients with biallelic *CEBPA* mutations, with one allele harboring an amino-terminus mutation and the other allele having a mutation in the carboxyl terminus, exhibit a distinct gene expression signature and a favorable prognosis with the majority of younger patients achieving a cure with standard chemotherapy alone (Table 1) (Wouters et al. 2009; Fasan et al. 2014). Biallelic *CEBPA* mutations have a JAK-STAT pathway activation signature, and a high percentage of these patients also harbor gain-of-function mutations in *CSF3R* and increased sensitivity

to JAK kinase inhibitors (such as ruxolitinib) ex vivo (Lavallée et al. 2016; Maxson et al. 2016). Germline mutations in *CEBPA* are associated with autosomal dominant familial AML with 100% penetrance (Smith et al. 2004).

GATA2

GATA2, a member of the zinc finger transcription factor family, is a critical transcriptional regulator of hematopoietic stem and progenitor cell differentiation and self-renewal (Crispino and Horwitz 2017). Homozygous *Gata2* knockout in mice are embryonically lethal because of the failure of definitive hematopoiesis (Tsai et al. 1994). Somatic mutations in *GATA2* are infrequent in AML, occurring in <5% of AML overall, and are clustered in normal karyotype AML with concurrent biallelic *CEBPA* mutations (Greif et al. 2012). These mutations include frameshifts, amino acid substitutions, insertions, and deletions scattered throughout the gene but most often clustered in the region encoding the zinc finger domains, leading to impaired DNA binding and affecting transcriptional activity. In its capacity as a tumor suppressor, heterozygous germline mutations in *GATA2* in coding or regulatory element regions cause a spectrum of hematopoietic disorders and a predisposition to MDS/AML, often with subsequent acquisition of somatic *ASXL1* mutations (Hahn et al. 2011; Ostergaard et al. 2011; West et al. 2014).

Mutations Altering Signal Transduction (Fig. 1)

Deregulation of signaling pathway components is frequently reported in AML. This is associated with pro-proliferative states by constitutive activation.

FLT3-ITD and FLT3-TKD

Mutations in FMS-like tyrosine kinase 3 (FLT3) occur in approximately one-third of all patients with newly diagnosed AML. These mutations can occur either as an in-frame internal tandem duplication within the juxtamembrane domain of the receptor (*FLT3-ITD*), seen in ~20%–25% of AML, or as point mutations most commonly in the activation loop of the catalytic domain (*FLT3-TKD*). Both mutations lead to autoactivation of FLT3-kinase activity and activation of downstream signaling pathways, which lead to proliferation and block in differentiation (Meshinchi and Appelbaum 2009). AML patients with mutations in *FLT3-ITD* have an adverse prognosis compared to patients with *FLT3-TKD* or wild-type *FLT3*, and the majority of these patients relapse after chemotherapy and allogeneic HSCT (Thiede et al. 2002; Sengsayadeth et al. 2012). The poor prognosis is particularly influenced by co-mutation status (*DNMT3A* mutations, absence of *NPM1* mutations) and the allelic ratio of mutant *FLT3-ITD* to wild-type (Table 1) (Patel et al. 2012; Papaemmanuil et al. 2016). The advent of *FLT3* inhibitors (midostaurin, gilteritinib, quizartinib, and crenolinib) in various stages of clinical development represents a paradigm of targeted therapy in AML and has changed practice. These agents act through the competitive inhibition of the ATP-binding site in the FLT3 receptor; however, they vary substantially in their inhibitory properties, pharmacokinetics, and toxicity profiles. There are currently two FDA-approved small-molecule inhibitors (midostaurin and gilteritinib) of FLT3-kinase activity. In randomized clinical trial, comparing midostaurin in combination with chemotherapy or placebo with chemotherapy has been shown to significantly improve survival of untreated *FLT3*-mutant AML patients (age 18–60 yr; 74.7 vs. 25.6 mo) (Stone et al. 2017). Gilteritinib, a next-generation tyrosine kinase inhibitor, is approved for the treatment of adults who have relapsed and/or have refractory AML with *FLT3-ITD* and *FLT3-TKD* mutations (Perl et al. 2019). Of note, therapy has substantive-agent activity, whereas midostaurin has not shown significant activity when given as monotherapy.

KIT

The *KIT* gene encodes a 145-kDa transmembrane glycoprotein that is a member of the type III receptor tyrosine kinase (RTK) family (Yarden et al. 1987). Upon binding of stem cell

factor (KIT ligand), the monomeric KIT receptor dimerizes and becomes autophosphorylated at key tyrosine sites and activates diverse signal transduction cascades essential for cell proliferation/survival, self-renewal, and differentiation (Malaise et al. 2009). Somatic gain-of-function *KIT* mutations occur in 10% of AML patients, but they are clustered in patients with CBF-AML rearrangements (t(8;21)/*RUNX1-RUNX1T1*, inv(16)/*CBFB-MYH11*) and may confer a more adverse prognosis in this otherwise favorable AML subtype (Paschka et al. 2006).

CBL

Casitas B cell lymphoma (CBL) protein functions as E3 ligases that ubiquitinate and negatively regulate receptor tyrosine kinases, including FLT3 and KIT (Mohapatra et al. 2013). Mutations in the E3 ubiquitin ligase *CBL* have been reported in ~1%–3% of AML patients. Mutations in *CBL* result in the loss of ubiquitin E3 ligase activity and resultant increased tyrosine kinase signaling by decreased proteasome-mediated degradation. In preclinical models, there is evidence that *FLT3-ITD* mutations cooperate with *CBL* to promote the rapid induction of AML (Taylor et al. 2015). Although, mutations in *CBL* cause biochemical loss of function, their functional consequences resemble canonical gain-of-function mutations (Sanada et al. 2009). The prognostic significance of these mutations in AML is unclear given the lack of robust data.

NRAS and KRAS

The Ras family of small GTPases, NRAS and KRAS, function as binary molecular switches by cycling between an active GTP-bound (RAS-GTP) and an inactive GDP-bound (RAS-GDP) confirmation that regulate downstream signaling effectors, such as Raf and PI3K (Schubbert et al. 2007). Somatic gain-of-function mutations in *RAS* are among the most common oncogenic drivers in cancers leading to constitutive activation of receptor tyrosine kinases (Li et al. 2018). *NRAS* (~12%) and *KRAS* (~5%) mutations are seen in a subset of AML patients at the time of diagnosis (Bacher et al. 2006). They are frequently acquired at the time of progression from MDS to AML and are associated with poor survival. In a recent study, the presence of *NRAS* and *KRAS* mutations in patients with AML receiving standard cytotoxic therapy was associated with decreased OS and event-free survival (EFS) (Ball et al. 2019). Mutations in epigenetic modifiers (*TET2/IDH/WT1*) often co-occur and cooperate with *NRAS* mutations to promote leukemogenesis (Papaemmanuil et al. 2016). Preclinical studies suggest this cooperativity and dependence on activated Ras signaling may be therapeutically exploited as they are preferentially sensitive to MAPK kinase (MEK) inhibition in mouse models and patient samples (Kunimoto et al. 2018). The emergence or co-occurrence of *NRAS* and *KRAS* mutant clones is a common and clinically relevant mechanism of resistance to *FLT3* inhibitors, *IDH* inhibitors, and BCL-2 inhibitors (Amatangelo et al. 2017; McMahon et al. 2019; DiNardo et al. 2020).

PTPN11

The non–receptor protein tyrosine phosphatase PTPN11 (also known as SRC homology 2 domain–containing phosphatase 2 or SHP2) is implicated in several signaling pathways including RAS, JAK-STAT, PI3K, and others (Tartaglia et al. 2003). Mutations in *PTPN11* are present in ~5%–10% of AML cases and enriched in patients older than 60 yr with secondary AML (Makishima et al. 2017). These mutations co-occur and cooperate with *AML1-ETO* (Hatlen et al. 2016) and *MLL-AF9* (Chen et al. 2015) rearrangements.

NF1

The neurofibromin 1 gene (*NF1*), encodes neurofibromin, a GTPase-activating protein and negative regulator of Ras. Inactivating mutations in *NF1* leading to loss of neurofibromin and resulting in prolonged activation of the RAS/RAF/MAPK signaling pathway and ultimately an increased cellular proliferation (Cichowski and Jacks 2001). Recurrent somatic inactivating mutations in *NF1* have been recently reported in ~5% patients with AML (Eisfeld et al. 2018). In

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the same study, *NF1*-mutant AML patients had poor outcomes with lower complete remission rates and shorter overall survival when treated with standard chemotherapy.

Mutations in Epigenetic Modifiers: *DNMT3A, TET2, IDH1/2,* and Polycomb-Group Genes (Fig. 2)

One of the hallmarks of AML pathogenesis is disruption of epigenetic regulation. The mutations in genes encoding epigenetic modifiers are commonly acquired early and are present in the initiating clone. This has led to an increasing interest in the development of epigenetic therapies for AML.

DNMT3A

DNA methyltransferase 3A (DNMT3A) is a member of the DNA methyltransferase family, which includes DNMT1, DNMT3A, DNMT3B, and DNMT3L (Okano et al. 1998). It catalyzes de novo DNA methylation at the C-5 position of cytosine bases of unmethylated CpG dinucleotides. DNA methylation is a critical epigenetic modification and plays an essential role in maintaining hematopoietic homeostasis. Consistent with its function, *Dnmt3a*-null HSCs up-regulate genes implicated in HSC self-renewal and multipotency, while down-regulating expression of genes associated with HSC differentiation (Challen et al. 2011). Mutations in *DNMT3A* are observed in ~30% of AML cases.

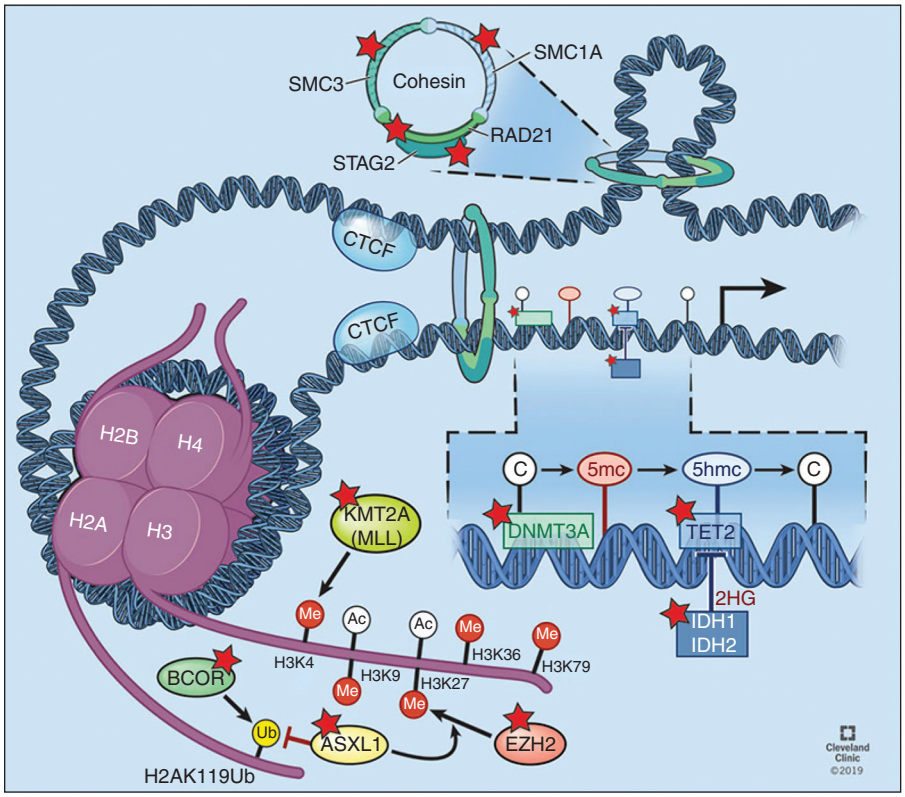

Figure 2. Overview of frequently mutated epigenetic modifiers. This includes (1) DNA cytosine modifications *DNMT3A*, *TET2,* and *IDH1/2*; (2) histone post-translational modifications *ASXL1, BCOR/L1,* and *MLL1 (KMT2A)*; and (3) Cohesin complex *SMC1A, SMC3,* and *RAD21*, bound to *STAG1/2.* Mutated genes are highlighted with a red star. (Refer to the main text for detailed explanations of the depicted processes.) (Figure reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2019–2020. All rights reserved.)

DNMT3A-mutant AML patients are older and most commonly present with a normal karyotype (Ley et al. 2010; Cancer Genome Atlas Research et al. 2013). The mutations in *DNMT3A* include nonsense, frameshift, and missense alterations that are enriched within the methyltransferase domain. The recurrent mutation at codon R882 is the most frequent *DNMT3A* mutation in AML patients, the resultant protein exerts a dominant-negative effect on wild-type *DNMT3A* methyltransferase activity (Holz-Schietinger et al. 2012; Cancer Genome Atlas Research et al. 2013; Russler-Germain et al. 2014). Mutations in *DNMT3A* frequently co-occur with mutations in *NPM1* and *FLT3-ITD*, which collectively confer adverse risk (Marcucci et al. 2012; Bezerra et al. 2020). *DNMT3A* mutations at codon R882 have been shown to promote anthracycline resistance through a proximal defect in nucleosome remodeling and resultant impaired DNA damaging sensing (Guryanova et al. 2016). Clonal hierarchy studies show that mutations in *DNMT3A* are one of the founder mutations shaping the course of leukemia evolution and progression (Welch et al. 2012), making this an attractive target for the development of novel therapeutic approaches. This is further underscored by studies showing that the *DNMT3A* is the most frequently mutated gene in clonal hematopoiesis (CH) (Genovese et al. 2014; Jaiswal et al. 2014; Shlush et al. 2014; Xie et al. 2014). Although essential insights into the role of *DNMT3A* mutations in leukemogenesis have emerged from human and preclinical studies, the fundamental mechanism(s) by which mutant *DNMT3A* leads to increased self-renewal in HSCs has not been delineated.

TET2

The mammalian ten-eleven-translocation (TET) family proteins TET1, TET2, and TET3, are α-ketoglutarate-dependent dioxygenases. The TET enzymes, in the presence of oxygen, reduced Fe^{2+}, and ascorbic acid, catalyze the stepwise oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), with resultant loss of DNA methylation (Tahiliani et al. 2009; Ko et al. 2010; He et al. 2011; Ito et al. 2011). *TET2*, but not *TET1* or *TET3*, loss-of-function mutations are common across a spectrum of hematologic malignancies (Delhommeau et al. 2009; Jankowska et al. 2009). Somatic mutations in *TET2* are seen in ∼20% of patients with AML, especially in patients with a prior history of MDS or myeloproliferative neoplasm (MPN) (Abdel-Wahab et al. 2009). In AML, *TET2* mutations co-occur with *NPM1*, *FLT3-ITD*, *NRAS,* and *DNMT3A* and are largely mutually exclusive with *IDH1/2* neomorphic mutations (Papaemmanuil et al. 2016; Desai et al. 2018). *TET2* mutations are among the most common mutations seen in clonal hematopoiesis (CH) (Busque et al. 2012).

Tet2$^{-/-}$ mice develop myeloid and lymphoid neoplasms, after long latencies, through the accumulation of numerous mutations, including *Apc*, *Nf1*, *Flt3*, *Cbl*, *Notch1*, and *Mll2* (Pan et al. 2017). Increased production of inflammatory mediators (IL-6) and bacterial translocation across gut mucosa may enhance leukemogenesis in *Tet2*$^{-/-}$ mice (Zhang et al. 2015; Meisel et al. 2018). Recent studies also indicate aberrant expression of diverse metabolic pathways, such as BCAT1, can decrease TET function by inhibiting α-ketoglutarate (Raffel et al. 2017). Supplementation of vitamin C (ascorbate), a co-factor of α-ketoglutarate-dependent dioxygenases like TET enzymes, has recently been shown to enhance residual *TET2* function in the heterozygous mutant state (Agathocleous et al. 2017; Cimmino et al. 2017). A clinical trial evaluating the safety and efficacy of high-dose vitamin C for *TET2*-mutated myeloid neoplasms is ongoing. Despite our mechanistic understanding of the function of TET proteins, how loss-of-function mutations in *TET2* initiates and maintains leukemogenesis is largely unknown.

IDH1 and IDH2 Mutations

High-throughput sequencing of AML patient samples identified neomorphic mutations in cytosolic (*IDH1* at R132) or mitochondrial (*IDH2* at R140Q or R172) isoforms of isocitrate dehydrogenase, which are critical for the oxidative

carboxylation of isocitrate to α-ketoglutarate. These mutations contribute to leukemogenesis through the production of the oncometabolite R enantiomer of 2-hydroxyglutratrate (R-2-HG), which, in turn, impedes epigenetic regulation via competitive inhibition of dioxygenases, including the TET family of enzymes and histone lysine demethylases (KDM), by competing with α-ketoglutarate (Mardis et al. 2009; Figueroa et al. 2010; Xu et al. 2011). This leads to a repressive chromatin landscape via DNA and histone hypermethylation, and consequent block in cellular differentiation (Lu et al. 2012; Losman et al. 2013). Mutations in *IDH1* and *IDH2* are present in ∼5%–10% and ∼15%–20% of patients with newly diagnosed AML, respectively. Mutations in *IDH1* and *IDH2* mutually exclusive and occur as early clonal events (Papaemmanuil et al. 2016). *IDH1* and *IDH2* mutations are more frequently observed in patients with trisomy 8 and normal karyotype AML (Medeiros et al. 2017). First-in-class, oral, selective inhibitors of mutant *IDH1* (ivosidenib) and *IDH2* (enasidenib) are U.S. Federal Drug Administration (FDA)-approved for the management of *IDH1/2*-mutant adults with refractory or relapsed AML (Stein et al. 2017; DiNardo et al. 2018). Several mechanisms of resistance to enasidenib (*IDH2* inhibitor) leading to late relapse have already been described, including acquisition of *IDH1*-mutated subclones (Quek et al. 2018) or development of additional noncatalytic second-site mutations of *IDH2* (Intlekofer et al. 2018).

ASXL1

Additional sex combs-like 1 (*ASXL1*), located at 20q11.21, is one of the three mammalian homologs of the *Drosophila* additional sex combs (*Asx*) gene. *Asx* functions as an epigenetic regulator that was originally identified as an enhancer of Trithorax (TrxG) and Polycomb group (PcG) genes (Micol and Abdel-Wahab 2016). ASXL1 interacts with BRCA1-associated protein 1 (BAP1) to form the Polycomb repressive deubiquitinase (PR-DUB) complex, which removes monoubiquitin from histone H2A at lysine 119 (H2AK119Ub) (a repressive mark) to activate genes targeted by PRC1 (Scheuermann et al. 2010). ASXL1 also interacts with the PRC2 complex to mediate histone H3 lysine 27 (H3K27) trimethylation (Abdel-Wahab et al. 2012; Inoue et al. 2013). It is yet to be determined if the observed changes in H3K27 methylation with ASXL1 loss are caused by direct interaction between ASXL1 and PRC2 or are secondary to depletion of H2AK119Ub and subsequent failure of PRC2 recruitment (Balasubramani et al. 2015). Somatic mutations in *ASXL1* are frequently detected in clonal hematopoiesis and various myeloid malignancies, suggesting they are the initial events in the leukemic transformation. *ASXL1* mutations occur in ∼10%–20% of patients with AML and are enriched in those with underlying myelodysplasia (Metzeler et al. 2011). *ASXL1* mutations coexist with those mutations in the splicing factor (*SRSF2*, *U2AF1*), signal transduction (*NRAS*, *JAK2*, *NF1*), and transcription factor (*RUNX1*) (Papaemmanuil et al. 2016). *ASXL1* mutations are more common in older patients with AML and confer a poor prognosis (Schnittger et al. 2013). A mechanistic understanding of how *ASXL1* mutations in myeloid malignancies drive leukemogenesis remains to be fully elucidated

BCOR and BCORL1

BCL6 corepressor (*BCOR*) and its closely related homolog, BCL6 corepressor like 1 (*BCORL1*), are located on the X-chromosome and are targeted by somatic mutations in AML, MDS, chronic myelomonocytic leukemia (CMML), and aplastic anemia (Damm et al. 2013; Yoshizato et al. 2015; Papaemmanuil et al. 2016). BCOR/BCORL1 function as components of PRC1.1, a noncanonical PRC1 complex. PRC1.1 is responsible for the deposition of monoubiquitination on lysine 119 of histone 2A (H2AK119ub1) and mediates transcriptional repression by recruitment of PRC2 and subsequent deposition of H3K27me3 mark (Blackledge et al. 2014; Di Carlo et al. 2019). In mice, loss of *Bcor* function results in enhanced self-renewal of myeloid progenitor cells and cooperates with $Kras^{G12D}$ to initiate leukemia in vivo (Kelly et al. 2019).

Loss-of-function somatic mutations in *BCOR* and *BCORL1* have been reported in ~1%–4% of AML, and carry an unfavorable prognosis (Grossmann et al. 2011; Damm et al. 2013).

Splicing Factor Mutations (*SF3B1, SRSF2, U2AF1, ZRSR2, U2AF2, PRPF8,* and *LUC7L2*)

Almost all human genes are subject to alternative pre-mRNA splicing, a primary source of diversity in messenger RNA species and protein isoforms, which is orchestrated by the macromolecular spliceosome complex (Fig. 1) (Lee and Rio 2015). Recent advances in high-throughput sequencing technologies have unexpectedly identified somatic mutations in the core spliceosomal proteins and associated RNA splicing factors in MDS, CMML, and AML (Anczuków and Krainer 2016). This includes mutations in *SF3B1* (splicing factor 3b subunit 1), *SRSF2* (serine/arginine rich splicing factor 2), *U2AF1* (U2 small nuclear RNA auxiliary factor 1), and *ZRSR2* (zinc finger RNA binding motif and serine/arginine rich 2). These mutations are mutually exclusive, heterozygous point mutations, suggesting synthetic lethal interactions if they were to co-occur (Yoshida et al. 2011; Lee et al. 2018). Splicing machinery mutations are most common in AML arising out of antecedent myeloid malignancy (secondary AML) (Lindsley et al. 2015). It has been shown that mutations in *SRSF2* cooperate with *IDH2*, with resultant synergy between RNA splicing and epigenetic regulation in promoting leukemogenesis (Yoshimi et al. 2019). Several groups have developed therapeutic strategies targeting the core spliceosome (SF3B1 inhibitors), splicing regulatory factors (PRMT5 inhibitor, RBM39 degraders, and SRPKs/CLKs inhibitors), and modulating pathologic splicing events (oligonucleotide-based approaches) (Lee et al. 2016; Obeng et al. 2016; Seiler et al. 2018; Fong et al. 2019; Wang et al. 2019).

Cohesin Complex

The cohesin complex is a tripartite ring including three structural proteins, SMC1A, SMC3, and RAD21, bound to either STAG1 or STAG2 (Fig. 2). This complex has many functions, most notably an essential role in aligning and stabilizing sister chromatids during metaphase (Nasmyth and Haering 2009). Cohesin complex also plays a critical role in the regulation of gene expression by cooperating with the DNA-binding protein CTCF in maintaining topologically associated domains (TADs) and regulating three-dimensional (3D) genome organization (Wendt et al. 2008). Heterozygous loss-of-function mutations in the cohesin complex occur in 12%–20% of AML and MDS patients and are more prevalent in high-risk MDS and secondary AML (Kon et al. 2013; Thota et al. 2014). These mutations are not associated with aneuploidy. Several studies have reported that the haploinsufficiency induced by the cohesin mutations enhances HSPC self-renewal and block hematopoietic differentiation (Mazumdar et al. 2015; Mullenders et al. 2015; Viny et al. 2015). *STAG2*, which is X-linked, is the most commonly mutated cohesin gene across many hematopoietic and solid tumors. A recent study demonstrated a specific role for *STAG2* (not by *STAG1*) in balancing self-renewal and differentiation in HSPCs (Viny et al. 2019).

Mutations in Tumor-Suppressor Genes: *TP53, WT1, PHF6*

TP53

The tumor-suppressor gene *TP53* is the most frequently mutated gene in human cancer and has many cellular functions, which are discussed in detail elsewhere (Fig. 1) (Kastenhuber and Lowe 2017). Mutations in *TP53* occur in 5%–20% of patients with newly diagnosed AML. There is an increased frequency of *TP53* mutations in AML patients with therapy-related myeloid neoplasms, complex karyotype, monosomal karyotype, and relapsed disease. One possible postulation for *TP53* pathogenesis in therapy-related myeloid neoplasms is that *TP53* mutations are acquired in some HSPCs during normal aging (clonal hematopoiesis) and are then selected for upon exposure to cytotoxic chemotherapy, ultimately resulting in

the expansion of HSPCs with *TP53* mutations (Wong et al. 2015). The majority of *TP53* mutations are missense mutations within the central DNA-binding domain, leading to loss of function. The resulting loss of function of p53 favors genomic instability and impaired apoptosis, which can contribute to resistance to chemotherapy. AML patients with *TP53* mutations have been associated with an inadequate response to conventional cytotoxic therapies and inferior overall survival rates (median of 5–9 mo) (Stengel et al. 2017). Historically, the efforts to target p53 therapeutically have been challenging. The landscape has been changing with the development of novel therapeutic strategies targeting the *TP53* pathway. One such promising approach is APR-246, a methylated derivative of PRIMA-1 that is converted to Michael acceptor methylene quinuclidinone (MQ), which can bind covalently to mutant p53 and restore wild-type transcriptional properties to the protein (Lambert et al. 2009). There is an ongoing multicenter phase III randomized study comparing the combination of APR-246 with azacytidine compared with azacytidine alone in *TP53*-mutant MDS and AML (NCT03745716).

WT1

Wilm's tumor 1 (*WT1*) encodes for a transcription factor that regulates cell proliferation, differentiation, apoptosis, and organ development (Call et al. 1990). Somatic loss-of-function mutations target *WT1* in 6%–15% of AML patients. Also, overexpression of wild-type WT1 protein has been observed in AML, further challenging the mechanistic understanding of how *WT1* contributes to leukemogenesis (Rampal and Figueroa 2016). Heterozygous loss of *Wt1* enhances HSCs self-renewal and cooperates with *Flt3-ITD* to induce leukemic transformation (Pronier et al. 2018). Loss-of-function mutations in *WT1* led to marked promoter hypermethylation pattern and reduction in 5hmC levels similar to AML patients with mutations in *TET2* and *IDH1/2* (Rampal et al. 2014). WT1 protein has also been shown to bind directly to TET2 and recruit TET2 to specific genomic sites to activate the gene expression of WT1-target genes (Wang et al. 2015). Mutations in *WT1* are associated with younger age, often co-occur with *FLT3-ITD*, and confer adverse prognostic significance in AML with increased risk of primary refractory disease (Hou et al. 2010).

PHF6

Somatic loss-of-function mutations in the X-chromosome-linked tumor suppressor plant homeodomain factor 6 (*PHF6*) are present in ~3% of AML patients (Van Vlierberghe et al. 2011). PHF6 is involved in chromatin-mediated transcriptional regulation by binding to the nucleosome remodeling and deacetylase (NuRD) chromatin remodeling complex (Todd and Picketts 2012; Liu et al. 2015). The presence of *PHF6* mutations in preleukemic clonal hematopoiesis supports its role in leukemia initiation and HSC self-renewal (Yoshizato et al. 2015; Abelson et al. 2018). In line with this notion, in a mouse model of T-cell acute lymphoblastic anemia (T-ALL), loss of *Phf6* enhances tumor initiation and transformation and leads to increased self-renewal in mouse HSCs (Wendorff et al. 2019). Mutations in *PHF6* are associated with *RUNX1* mutations and with reduced overall survival in AML patients (Patel et al. 2012).

CONCLUDING REMARKS

Mutational studies involving large cohorts of patients with AML have identified a complex mutational landscape, including patterns of significant co-occurrence and mutual exclusivity of mutations. The mechanistic interactions between different mutations have only begun to be elucidated, and we expect these insights can be used to better inform our understanding of molecular pathogenesis of the disease, improve risk stratification, and identify novel therapeutic vulnerabilities. In specific cases, such as co-occurring *FLT3*/*TET2* (Shih et al. 2015) and *SRSF2*/*IDH2* (Yoshimi et al. 2019) mutations, mechanisms of oncogenic synergy have been elucidated; however, the basis for cooperativity between AML disease alleles requires greater investigation. Moreover, bulk sequencing studies

have given us a glimpse into the temporal and spatial sequence of somatic mutations that drive leukemogenesis, from early mutations, which induce clonal hematopoiesis (*DNMT3A*, *TET2, ASXL1*), to disease-defining mutations present in all cells at transformation (*NPM1*), to mutations that can occur in specific subclones and further enhance the fitness of AML cells (*FLT3*, *RAS*). However, the roles of all of the different mutations in this complex sequence of genetic events that contribute to AML are not known, and many AML patients are a complex amalgam of multiple branching clones such that the AML "ecosystem" has not been fully characterized. Moreover, the observations that relapse can come from rarer AML subclones (Ding et al. 2012; Shlush et al. 2017) suggest that a better understanding of the role of specific mutations in AML heterogeneity and drug response is urgently needed.

In addition to novel mechanistic insights, we need to ensure that this knowledge is translated into improved outcomes for our patients. Most AML patients do not present with mutations for which we have a molecularly targeted therapy, and targeted therapy has limited curative potential when given as monotherapy in AML. We need to identify additional mechanisms of leukemic transformation induced by genetic/epigenetic events in AML, such that we can uncover novel therapeutic targets that lead to genotype-based combination therapies for AML patients. It is our hope that the ever-evolving understanding of AML pathogenesis will continue to lead to novel therapies for our patients.

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