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Mechanisms of myeloid leukemogenesis: Current perspectives and therapeutic objectives

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic neoplasm which results in clonal proliferation of abnormally differentiated hematopoietic cells. In this review, mechanisms contributing to myeloid leukemogenesis are summarized, highlighting aberrations of epigenetics, transcription factors, signal transduction, cell cycling, and the bone marrow microenvironment. The mechanisms contributing to AML are detailed to spotlight recent findings that convey clinical impact. The applications of current and prospective therapeutic targets are accentuated in addition to reviews of treatment paradigms stratified for each characteristic molecular lesion – with a focus on exploring novel treatment approaches and combinations to improve outcomes in AML.

Keywords

Acute myeloid leukemia; Leukemogenesis; Epigenetics; Transcription factor; Oncoprotein; Signal transduction; Cell cycle; Novel strategies; Targeted therapy

1. Introduction

Acute myeloid leukemia is a clonal bone marrow disorder reflecting an expansion of a progenitor cell arrested in development, often resulting in hyperproliferation of hematopoietic precursor cells. The SEER-reported age-adjusted incidence of AML has gradually increased over the last few decades, currently at 4 per 100,000 per year [1]. It is primarily a disease of older adults, with the median age at diagnosis of 68 years [2]. Despite recent advances in refinement of predictive and prognostic markers and the assessment of a multitude of novel agents, both FDA-approved and in clinical trials, the mortality rate of AML has remained stable over the past two decades, currently the fifth worst 5-year overall survival when stratified by cancer type at 24.0%, with an approximate median overall survival of 8.5 months [1].

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Complicating the analysis of predictive markers for therapeutic regimens in AML is the existence of clonal hematopoiesis of indeterminate potential (CHIP), in which otherwise healthy patients devoid of overt disease harbor mutations in genes commonly mutated in myeloid neoplasms, such as *DNMT3A, TET2, ASXL1,* and *TP53* [3]. While most patients with CHIP do not exhibit AML or myelodysplastic syndrome (MDS), clonal hematopoiesis was found to be associated with decreased overall survival, with age being the largest contributor to the risk of mutation emergence [3]. Consequently, there is a need to identify the precise mechanisms of mutations implicated in leukemogenesis to tailor rational approaches to therapy and improve outcomes.

Recent years have witnessed dramatic developments in molecular biology, genomics, the understanding of the role of the bone marrow microenvironment, as well as their corresponding effects on leukemogenesis. It is widely recognized that the evolution of AML is unlikely to represent the result of a single biological aberration – but instead the consequence of multiple and synergistic aberrations in epigenetic events, cell cycling, proliferation, signal transduction, and apoptosis. The diverse mechanisms culminating in AML support the notion of a distinctly heterogeneous disease that extends beyond prior and current classifications. In this article, we review and examine the major contributors leading to the development of AML with an emphasis on recent insights and future directions in targeted therapies designed to rationally exploit implicated pathways.

2. Epigenetics

2.1. Overview of epigenetics in AML

Understanding leukemogenesis requires an appreciation of two concepts – the function of proteins of commonly mutated genes and how the relevant genes are expressed. Gene expression is a tightly regulated process with derangements observed frequently in AML. Nucleosomes represent a length of DNA coiled around packing proteins known as histones, which function as transcriptional regulators. Common histone alterations include methylation, acetylation, and ubiquitination – and each histone modification can lead to differential effects on gene transcription or gene repression. In addition to histone alternations, DNA methylation and noncoding RNA species also modify transcriptional activity, and collectively constitute an overarching regulatory process of gene expression known as epigenetic modifications, summarized in Table 1.

DNA methylation is the most well-studied epigenetic modification in AML. Cytosine-guanine dinucleotides, also known as CpGs, control gene expression in clusters that overlap or are adjacent to promoter regions. Methylated CpGs are found in repressed chromatin characterized by transcriptionally silenced activity and are generally associated with tumor suppressor genes. Conversely, unmethylated CpGs promote active transcription and are often found in association with housekeeping genes [4]. While methylated promoters are always repressed, absence of methylation does not always lead to enhanced promoter activity [5]. In neoplastic development, the degree of CpG-island hypermethylation (as observed in leukemia and lymphoma) or global genomic hypomethylation (as observed in colon, lung, or breast cancer) increases with progression from a benign to a malignant state [6].

DNA hypomethylating agents (HMAs) used in treatment of AML, such as decitabine or azacitidine, reduce methylation on a genome-wide scale by forming a covalent bond with DNA methyltransferases (DNMTs) [4], but the effects of these agents are not permanent [7,8]. In the absence of these agents, re-methylation is commonly observed and is thought to culminate in disease relapse. This provides a theoretical basis for the indefinite continuation of hypomethylating agents after an induction period until disease progression. However, the precise mechanisms of azacitidine and decitabine in the treatment of AML have not been definitively resolved. While both agents do result in hypomethylation through inhibition of DNMTs [9], additional potential therapeutic mechanisms include induction of tumor suppressor genes and incorporation into DNA with decitabine or RNA with azacitidine [10,11]. In clinical practice, hypomethylating agents, with or without additional therapies, are used in the treatment of older patients with AML and unfavorable risk cytogenetics, for those who are not candidates for intensive induction as monotherapy, as well as in the post-remission setting.

MicroRNA (miRNA) represent noncoding RNA that targets and down-regulates mRNA [12]. Following nuclear processing, mature miRNA binds to the RNA-induced silencing complex (RISC) and pairs with complementary mRNAs, which results in decreased translation and protein expression. Dysregulated miRNAs can be associated with oncogenesis (oncomiR) or they can down-regulate mRNA encoding for tumor suppressors and influence a diverse range of leukemic processes [13,14]. MiRNAs implicated in leukemia are frequently disrupted by epigenetic silencing via RUNX1-RUNX1T1 while cooperating with the PI3K pathway, resulting in increased signal transduction [15] and dysregulation of transcription factors [16]. Reversing aberrant epigenetic changes may lead to restoration of appropriate miRNA expression, with either hypomethylating agents, or with targeted therapy to disrupt overactive downstream pathways such as PI3K. Despite pharmacokinetic challenges, administration of tumor suppressor miRNA mimics or locked nucleic acid oligonucleotide inhibitors, which feature a fixed conformation of the ribose ring of RNA, provide additional avenues of miRNA modulation [17].

2.2. DNA methyltransferases

The DNMTs regulate epigenetic modifications via methylation of cytosine primarily at CpG dinucleotides to create 5-methylcytosine (Fig. 1A). There are five primary DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L – and of these, DNMT1, 3A, and 3B regulate most of the core processes of DNA methylation in humans. DNMT1 is the primary housekeeper and maintains existing DNA methylation patterns. In contrast, DNMT3A and 3B establish new methylation patterns and are known as *de novo* DNA methyltransferases. Loss of DNMT activity results in altered methylation patterns, culminating in aberrations of critical regulators of HSC differentiation, such as *PU.1, IKAROS,* and *RUNX1* – impairing terminal differentiation and leading to AML [18].

Perturbed transcription of the *DNMT* genes, particularly *DNMT3A/B*, results in a truncated, non-catalytic isoform with a higher frequency of locus-specific irregularities that accelerates tumorigenesis [19–22]. *DNMT* aberrations result in dysregulation of methylation patterns and alteration of gene expression, and a clear link has been demonstrated between

tumorigenesis and methylation of tumor suppressor genes [23]. Mutations in both *DNMT3A* (commonly in codon R882) and *DNMT3B* have been associated with decreased overall survival, increased disease relapse, and a poorer prognosis in patients with AML [24–26]. Smaller studies have suggested that hypomethylating agents may have utility in treating patients with AML and *DNMT3A* mutations, given their superior overall response rate and median overall survival compared to *DNMT3A*-wild type patients, although these results did not reach statistical significance [27].

2.3. TET2 and IDH1/2

Aberrant *TET2* is present in up to 24% of myeloid neoplasms, including primary myelofibrosis, chronic myelomonocytic leukemia, MDS, and AML [28–30]. Similar to the DNMT family, *TET2* and *IDH1/2* mutations also regulate epigenetic expression, although the TET and IDH family proteins are responsible for DNA demethylation. Ten-eleven translocation methylcytosine deoxygenase 2 (TET2) converts 5-methylcytosine to 5-hydroxymethylcytosine (Fig. 1B) [31,32]. Following conversion of 5-methylcytosine by the TET proteins, DNA demethylation subsequently occurs through base excision and opposes the action of DNA methyltransferases [33]. The leukemogenic mechanism appears to be enhancement of HSC self-renewal through aberrant methylation associated with loss of functional TET2 [34]. Consequently, wild-type TET2 acts as a tumor suppressor to maintain hematopoiesis through maintenance of appropriate demethylation, with $TET2^{mut}$ leading to hypermutagenicitiy and a tendency to develop mutations in *FLT3* and *NOTCH1* [35]. Loss-of-function of *TET2* is associated with a poorer prognosis in AML patients with intermediate cytogenetics, particularly when combined with additional negative prognostic markers [36]. In a recent study of cell lines with hypermethylated *TET2*, decitabine reversed *TET2* methylation, resulting in induced expression of *TET2* and decreased chromosomal instability [37]. Consequently, hypomethylating agents appear to hold promise in the treatment for $TET2^{mut}$ or *TET2* silenced AML.

Cytosolic isocitrate dehydrogenase (IDH1) and its mitochondrial homolog (IDH2) are enzymes involved in the citric acid cycle that convert isocitrate to α-ketoglutarate. *TET2* mutations appear to be mutually exclusive with *IDH1/2* mutations and are associated with similar loss-of-function epigenetic defects, as mutations in *IDH1/2* impair *TET2* catalytic function [38]. The leukemogenic mechanism of $IDH1/2^{mut}$ is due to the production of an aberrant metabolite, 2-hydroxyglutarate (2HG), a structural analog of α-ketoglutarate. Thus, 2HG inhibits *TET2* and induces DNA hypermethylation – the dominant feature of $IDH1/2^{mut}$ AML [38–40].

Enasidenib is a small molecule inhibitor of mutated IDH2 and has been evaluated as monotherapy and in combination with hypomethylating agents. Single-agent enasidenib demonstrated an overall response rate (ORR) of 40% in the relapsed or refractory setting and a median overall survival of 8.0–12.4 months [41]. Similarly, ivosidenib is a small molecule inhibitor of mutated IDH1, demonstrating an ORR of 41.6% in the relapsed or refractory setting [42]. Both ivosidenib and enasidenib are FDA-approved for relapsed or refractory AML harboring $IDH1^{mut}$ or $IDH2^{mut}$, respectively. These agents are approved as monotherapy and feature a relatively delayed median time to response compared to more

intensive strategies. The median time to response of enasidenib as salvage therapy was 1.9 months, with nearly half of the cohort achieving maximum response by cycle 4, and 80% achieving maximum response by cycle 6 [41]. Similar findings were observed with ivosidenib, with the median time to response at 1.9 months and the median time to complete remission at 2.8 months [42]. In addition to use in the relapsed or refractory settings, ivosidenib monotherapy is approved in the first-line setting for *IDH1*^{mut} AML ineligible for intensive induction. IDH differentiation syndrome is a significant toxicity associated with IDH inhibitors, with rates of grade 3 or higher toxicity reported at 6.4% [41,42].

Combining hypomethylating agents with IDH inhibitors appears to produce augmented responses, with enasidenib and azacitidine demonstrating a composite response rate (CRR; CR + CRi) of 100% in the front-line setting and the median overall survival was not reached at a median follow-up time of 13.1 months [43]. Similar survival benefits were seen with ivosidenib and azacitidine [44]. On the basis of these findings, ivosidenib has been approved in combination with azacitidine for treatment of *IDH1*^{mut} AML. Multiple clinical trials are underway investigating other combinations of IDH inhibitors with hypomethylating agents [45], the BCL-2 inhibitor venetoclax [46], and intensive induction strategies [47], summarized in Table 2.

2.4. Bromodomain-containing proteins

The bromodomain (BRD)-containing proteins are epigenetic modifiers that bind to acetylated lysine on histones [48]. Because the BRD-containing proteins regulate a multitude of cellular processes, they have been implicated in several stages of leukemogenesis, including potentiation of signal transduction events [49]. This class of proteins represents a diverse family including chromatin remodeling proteins, transcriptional coactivators, and the bromodomain and extraterminal domain-containing (BET) proteins [50]. The BET proteins (BRDT, BRD2, BRD3, and BRD4) are responsible for the regulation of RNA transcription and activation of RNA polymerase II, which modulates cell cycle progression through cyclin T1 and CDK9 [51]. Therefore, up-regulation of the BET proteins, particularly BRD4, results in remodeling of chromatin and activates gene transcription through targeting of aurora B kinase (*AURKB*), *FOS,* and *MYC* [52]. Maintenance of AML was found to operate through BRD4-mediated MYC activation, creating persistent self-renewal, while inhibition of BRD4 resulted in cell cycle arrest and induction of apoptosis [53].

BET inhibitors, such as mivebresib (ABBV-075) and birabresib (MK-8628/OTX015), interfere with the binding of BRD-containing proteins to acetylated histones. In turn, BET inhibitors reverse the effects of BRD up-regulation by promoting cell cycle arrest and MYC suppression [54]. Additionally, BET inhibitors were found to block the transcription of *BCL2* [55] and *MCL1* [56]. As *MCL1* is up-regulated in cells exhibiting resistance to venetoclax, such findings provide a rationale for treatment with a BET inhibitor and a BCL-2 inhibitor – either in combination or in sequence. Furthermore, mutated nucleophosmin impairs transport of BRD4 between the nucleus and the cytoplasm, leading to a loss of repression of BRD4 and an increase in the expression of *MYC* and *BCL2* [57]. This suggests patients with *NPM1* mutations may be sensitive to BET inhibitors, with

or without venetoclax-based combination strategies. Clinical trials are underway evaluating BET inhibitors as monotherapy or in combination approaches, such as with venetoclax [58,59] or azacitidine [60].

2.5. Histone acetyltransferases, deacetylases, and methyltransferases

Unlike the BRD-containing proteins, which function primarily as the “readers” of histone acetylation, histone acetyltransferases are responsible for the addition of acetyl groups to lysine residues. Consequently, histone acetyltransferases can be thought of as the “writers” of histone modifications and histone deacetylases as the “erasers”. Following histone acetylation, recruitment of BET proteins and transcription factors occurs, resulting in assembly of transcription machinery and subsequent gene activation [61]. Malignant cells were associated with relative histone hypoacetylation compared to normal tissues [62], and dysregulated histone deacetylase (HDAC) activity in leukemia has been a subject of intense study. In particular, chimeric proteins frequently seen in AML, such as RUNX1-RUNX1T1 and CBFB-MYH11, recruit HDACs to corepressor complexes and subsequently block transcription of target genes that are ultimately responsible for myeloid differentiation [63].

Inhibitors of histone deacetylation (HDACIs) induce expression of inappropriately silenced genes through induction of a more transcriptionally permissible chromatin structure, resulting in a reversal of a malignant acetylation signature [64]. Following treatment with HDACIs, restoration of differentiation, apoptosis, autophagy, and control of cell-cycle progression occurs [65]. HDACIs as monotherapy have shown limited responses and clinical benefit in AML, with a phase II study of belinostat in relapsed or refractory AML demonstrating no patients achieving a complete or partial response and 25% of patients with stable disease for at least five cycles [64,66]. Combinations of HDACIs with hypomethylating agents, venetoclax, and targeted therapies have been explored to augment clinical response. Despite initial reports of synergism between sequential administration of HDACIs and hypomethylating agents [67,68], this strategy has not resulted in clinical benefit [69]. Phase I trials investigating belinostat, an HDACI, in combination with pevonedistat, a selective NEDD8-activating enzyme inhibitor, are underway following demonstration of reduced tumor burden and improved survival in AML xenograft models [70].

Similarly, methylation of histones plays a critical role in regulation of gene expression responsible for hematopoietic development. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that trimethylates lysine 27 of histone 3 (H3K27me3), creating a repressive transcriptional signature [71]. EZH2 is a member of the polycomb group proteins and composes part of the polycomb repressive complex 2 (PRC2), which functions as a direct inhibitor of gene transcription through chromatin compaction. *EZH2* overexpression in myeloid malignancies has been correlated with increased methylation of the tumor suppressor $p15^{INK4B}$, encoded by *CDKN2B*, and results in poor clinical outcomes [72]. In the context of gain-of-function mutations, EZH2 was shown to function as an oncogene, particularly during disease maintenance [73]. In contrast, in loss-of-function mutations, EZH2 demonstrated tumor suppressor characteristics during AML induction [71,73], and

recent studies have demonstrated that loss of EZH2 correlated with dysregulation of *HOX* gene expression and resistance to chemotherapy in AML [74]. In sum, either gain- or loss-of-function of EZH2 may contribute to leukemic progression.

Inhibitors of EZH2 have been developed, and the most well-studied has been 3-deazaneplanocin (DZNep) [75]. DZNep was shown to induce differentiation and apoptosis in AML and demonstrated synergism with HDACIs, resulting in reduction of *HOXA9* expression [76]. The combination of DZNep and decitabine has also been explored in the preclinical setting, with combination therapy demonstrating reactivation of previously silenced target genes, leading to reduced proliferation in leukemic cells [77]. A dual inhibitor of EZH2 and EZH1, UNC1999, was shown to inhibit growth of *MLL*-rearranged leukemia cells and prolonged survival in murine models [78]. More recently, a second dual inhibitor of EZH1/2, DS-3201 (valemetostat), was discovered to recruit LSCs into the cell cycle, with EZH1/2 inhibition and G-CSF potentiating apoptosis following exposure to venetoclax and a hypomethylating agent [79]. Further utility of EZH2 inhibition in AML remains to be explored in clinical trials.

2.6. Mixed-lineage leukemia

The mixed-lineage leukemia gene (*MLL*, also known as *KMT2A*) encodes for a histone methyltransferase that acts as a master regulator of gene expression during hematopoiesis, and whose protein product controls the *HOX* gene family [80,81]. *MLL* rearrangements are found in approximately 10% of myeloid leukemias [82,83] and are more common in secondary or therapy-related leukemias, particularly after agents that target topoisomerase II. *MLL* rearrangements are also seen in adult and pediatric acute lymphoblastic leukemia, as well as mixed-phenotype acute leukemia, and are associated with poor clinical outcomes [84]. The native function of MLL is to maintain the activity of target genes through what was historically thought to be the histone 3 lysine 4 (H3K4) methyltransferase activity of the MLL protein [85]. However, it has recently been discovered that it is the increase in recruitment and activity of the males-absent on the first (MOF) histone 4 lysine 16 (H4K16) acetyltransferases rather than the MLL-intrinsic histone *methyl*transferase activity that is more directly implicated in maintenance of MLL target genes [86]. Rearrangements of *MLL* are created by the addition of a fusion partner to produce the MLL fusion oncoprotein, similar to the leukemogenesis patterns exhibited by RUNX1-RUNX1T1, although the variety of fusion partners for *MLL*-associated rearrangements is vast with over 70 reported fusion proteins described [87].

Understanding leukemogenesis driven by MLL requires understanding the function of *HOXA9* and *MEIS1. HOXA9* is part of the highly conserved homeobox family responsible for tightly controlling cell differentiation and proliferation, and constitutive expression is critical in maintaining hematopoietic stem cell populations [88]. In leukemias harboring *MLL* rearrangements, *HOXA9* is constitutively expressed during development, leading to persistent replicative immortality of leukemic stem cells [89]. Increased levels of *HOXA9* expression due to the presence of the *MLL* rearrangement are associated with increased histone 3 lysine 79 (H3K79) dimethylation – regulated by the histone methyltransferase disruptor of telomeric silencing 1-like protein, DOT1L [90]. Collectively, these findings

have led to the development of clinical trials investigating small molecule inhibitors that target DOTIL or other modulators of chromatin regulation [91].

MEIS1 is a protein dimerization partner of HOXA9, which enhances and regulates expression of the HOX proteins. Consequently, in the context of MLL-rearranged leukemia, it is partially responsible for the differentiation block, leukemic progression, and self-renewal capacity of the leukemic stem cells [92]. Concomitant expression of *HOXA9* and *MEIS1* or a second cofactor, *PBX3*, promotes AML development [93,94]. Although the interactions between HOXA9, MEIS1, and PBX3 have been the subject of intense investigation over the last three decades, a unifying explanation for the development of leukemogenesis across all possible MLL fusion partners has not yet been fully elucidated.

Additional therapeutic targets in *MLL*-rearranged AML include BRD4 [95], the RAS pathway, and menin, which stabilizes the binding of MLL to chromatin and is critical for MLL fusion-driven gene expression [96,97]. Small molecule inhibitors targeting DOTIL, BRD4, MEK (downstream of RAS), and menin are attractive targets either alone or in combination [98] with additional targeted therapy or cytotoxic chemotherapy to improve outcomes in *MLL*-rearranged leukemia, summarized in Table 3. For example, menin-MLL inhibition has been shown to synergize with FLT3 inhibitors in preclinical models [99] as well as with venetoclax in *NPM1*^{mut} and *MLL*-rearranged AML harboring a *FLT3* mutation [100]. Additionally, as cyclin-dependent kinase 9 (CDK9) comprises a component of the positive transcription elongation factor b (P-TEFb), a target of MLL, combined CDK9 and BET inhibition has shown preclinical activity in *MLL*-rearranged AML [101]. Given the complexify and interactions of the members within the transcription complex and the diverse fusion partners in the oncoprotein, *MLL*-rearranged leukemias represent a highly heterogeneous category, and further studies are needed to facilitate the development of targeted therapies.

2.7. ASXL1

The role of additional sex combs like 1 (*ASXL1*) in hematopoiesis and leukemogenesis has yet to be fully elucidated. Recent work has revealed that ASXL1 regulates histone modifications through the interaction of ASXL1 and a histone deubiquitinase, BRCA1 associated protein 1 (BAP1). BAP1 is an essential component of the polycomb repressive deubiquitinase (PR-DUB) complex, a member of the polycomb group proteins. PR-DUB opposes the ubiquitination of histones catalyzed by the polycomb repressive complex 1 (PRC1) [102], which down-regulates the transcription of genes controlling stem cell pluripotency and regulation of cell death [102]. More specifically, the PR-DUB complex, composed of BAP1 activated by ASXL1 [103], is recruited to DNA and forms a DNA-protein complex which regulates genes responsible for hematopoietic development, including the *HOX* gene family [104] (Fig. 1C). ASXL1 is therefore thought to modulate the balance between polycomb and *HOX* gene expression [105].

ASXL1 mutations promote HSC aberrations while maintaining survival, creating a predisposition to leukemic transformation by cooperating with the acquisition of mutant *RUNX1* [106], *MLL* [106], *NRAS* [107], or loss-of-function of *TET2* [108]. These mutations in *ASXL1* frequently occur as frameshift or nonsense mutations that

characteristically create a C-terminally truncated protein which increases the function of BAP1 in the modification of histones – including enhancement of deubiquitination of histone H2A [102] and down-regulation of methylated histone H3 [108,109]. Ultimately, the complex of mutated ASXL1 and BAP1 leads to gain-of-function synergistic interactions between the two proteins and is thought to confer a myeloid differentiation block by deubiquitinating H2AK119 located at *HOX* gene regions [110].

ASXL1 mutations confer adverse-risk disease with lower complete remission rates and inferior survival [111]. Depletion of BAP1 using CRISPR/Cas9 created a profound reduction in BAP1 – and when $ASXL1^{mut}$ was transduced in BAP1-depleted cells, the differentiation block was reversed [110]. This finding prompted the identification of BAP1 small-molecule inhibitors through biochemical screening that inhibit ASXL1-driven leukemogenesis [112]. These studies set the stage for future pre-clinical work involving small-molecule BAP1 inhibitors for $ASXL1^{mut}$ AML in combination with anthracycline-based or venetoclax-based therapeutic approaches.

3. Transcription factors

3.1. Biallelic CEBPA

The CCAAT-enhancer binding protein alpha (CEBPα) is a transcription factor that regulates differentiation and proliferation in myeloid progenitors. Functional CEBPα directly interacts with coactivators and repressors dependent on cell type and allows progression from the myeloblast to granulocytemacrophage progenitors [113]. *CEBPA* is translated into a 30 kDa isoform and a 42 kDa isoform by initiating translation at different start sites. In mutated *CEBPA*, which occurs in up to 14% of AML, the synthesis of the 42 kDa protein is impaired and the expression of the 30 kDa protein is preserved, conferring a differentiation block during myeloid maturation [114–117].

There are two main types of *CEBPA* mutations: an N-terminal frameshift resulting in production of the 30 kDa protein and a C-terminal mutation in the leucine zipper (bZIP), which affects DNA binding, homodimerization, and heterodimerization [118]. These mutations disrupt the activation of transcription of the granulocyte colony-stimulating factor (G-CSF) receptor and the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, resulting in the differentiation block [119,120]. Additionally, loss of functional CEBPα results in activation of genes responsible for maintaining leukemic cells in their dedifferentiated states [121–123]. Biallelic *CEBPA* mutations produce two mutated 30 kDa mutations (p30/p30 homodimers): one with the N-terminal mutation and the other with the C-terminal mutation, creating a distinct clinical entity with a favorable prognosis when occurring with normal cytogenetics and without additional molecular aberrations [124]. Since biallelic $CEBPA^{mut}$ AML confers a favorable prognosis, current recommendations involve treatment with intensive cytotoxic chemotherapy with cytarabine and daunorubicin, with or without the anti-CD33 antibody-drug conjugate, gemtuzumab ozogamicin. As additional druggable targets are discovered, their implementation into clinical trials will evolve.

3.2. Nucleophosmin

Nucleophosmin (NPM1) is a multifunctional nucleocytoplasmic shuttling phosphoprotein that is found primarily in the nucleolus [125]. NPM1 regulates the assembly of primordial ribosomal constituents and facilitates their transport through the nuclear membrane to the cytoplasm [125,126]. It is also responsible for maintenance of the cell cycle (targeting CDK2-cyclin E during the initiation of centrosome duplication) [127] and promotion of p53-mediated tumor-suppressor activity [128] . *NPM1* is involved in several chromosomal rearrangements that promote leukemogenesis by activating the oncogenic effects of a fused partner, particularly with the retinoic acid receptor α (NPM1-RARα) [129], anaplastic lymphoma kinase (NPM1-ALK) [130], and myeloid leukemia factor 1 (NPM1-MLF1) [131].

In the absence of a partnered gene fusion, it is thought that NPM1^{mut} exerts its leukemogenic effect through aberrant shuttling function, since mutations in *NPM1* appear to increase the concentration of the aberrant protein in the cytoplasm and decrease its concentration in the nucleus. This results in rapid up-regulation of *HOX* gene expression, which is coupled to a gain-of-function interaction between NPM1^{mut} and exportin 1 (XPO1), another protein that facilitates nuclear export [132]. This potentially provides a molecular rationale for induction chemotherapy coupled with an XPO1 inhibitor (such as selinexor) in patients with *NPM1*^{mut} AML. Uniting nuclear transport and transcriptional machinery appears to promote *HOX* expression and resultant AML, partially explaining the contribution of *NPM1*^{mut} to leukemogenesis. New drugs are in development to either restore appropriate nuclear localization of NPM1, suppress *HOX* expression [133], disrupt oligomerization [134], or induce proteasomal degradation to facilitate apoptosis [135].

In patients with AML and normal cytogenetics, *NPM1*^{mut} in isolation is associated with a favorable prognosis and good response to front-line induction chemotherapy [126]. In patients with *NPM1*^{mut} and *FLT3*-ITD mutations with a high allelic ratio (defined as greater than 0.5), the favorable prognosis is abrogated and the risk is reclassified to the intermediate category [111]. *NPM1*^{mut} AML was shown to benefit from high-dose daunorubicin (90 mg/m^2) compared to lower-dose daunorubicin (45 mg/m^2) during induction for patients younger than 60 years, regardless of the presence of a *FLT3*-ITD mutation [136]. However, when stratified by cytogenetic risk, daunorubicin 90 mg/m^2 failed to demonstrate clear benefit in any category in randomized trials compared to daunorubicin 60 mg/m^2, although the study included a large fraction of patients older than 60 years and did not specifically investigate the impact of dose escalation on *NPM1*^{mut} AML [137]. Finally, *NPM1*^{mut} AML strongly expresses CD33 [138], which establishes a molecular rationale for the use of gemtuzumab ozogamicin (GO) in combination with cytarabine and daunorubicin in this subset of favorable risk disease. A recent randomized trial investigating this notion demonstrated significantly fewer relapses in the GO arm, although at the expense of an increased risk of early death [139].

3.3. Core binding factor

Similar to the prognostic significance of *NPM1*^{mut} and biallelic *CEBPA*^{mut}, core-binding factor (CBF) AML is also associated with a favorable prognosis. Runt-related transcription

factor 1 (*RUNX1*, also known as *AML1)* encodes the α subunit of CBF while core-binding factor β (*CBFB*) encodes the β subunit. Both CBFα and CBFβ combine to form the CBF complex that is involved in normal hematopoietic progression [140,141]. A loss of function of the CBF complex results in a differentiation block and culminates in CBF-AML [142]. Specific chromosomal rearrangements are seen in this subset of AML: translocation of chromosomes 8 and 21 or inversion of chromosome 16, resulting in the gene fusion products RUNX1-RUNX1T1 (also known as AML1-ETO) and CBFB-MYH11, respectively. Notably, translocations involving CBFs do not induce AML on their own and are postulated to promote leukemogenesis by partnering with additional mutations – particularly those implicated in cell signal transduction, such as *NBAS, KRAS, FLT3,* and *KIT* [143–146].

RUNX1-RUNX1T1 exerts its effects through recruitment of gene repression complexes, which alter the chromosomal architecture and epigenetic landscape of target genes. In the fusion protein, RUNX1 contains a DNA binding domain that is fused to a transcriptional corepressor, RUNX1T1 [147]. Therefore, RUNX1-RUNX1T1 negatively interacts with transcription factors that regulate hematopoietic progression by binding to DNA with RUNX1 binding sites and repressing genes responsible for hematopoietic differentiation [148–150]. Thus, RUNX1-RUNX1T1 outcompetes RUNX1, resulting in inhibition of RUNX1-mediated gene expression and maintenance of the HSC-like state. Compounds that inhibit altered gene expression due to RUNX1-RUNX1T1 were identified through a bioinformatics search followed by biological activity testing, subsequently revealing that budesonide and dexamethasone demonstrated potent antileukemic activity and synergy with conventional chemotherapy in murine models via inhibition of RUNX1-RUNX1T1 mediated gene expression and restoration of targets of RUNX1 [151]. A phase II clinical trial of dexamethasone in combination with intensive therapy in older adults with AML is ongoing (NCT03609060).

The DNA-binding domain of RUNX1 forms a heterodimer with CBFß, which stabilizes the binding of RUNX1 to DNA [152]. As these proteins unite to form the CBF complex, it stands to reason that disruptions of either *RUNX1* or *CBFB* will have similar effects in patients with AML. Indeed, the fusion between *CBFB-MYH11* represents a disruption of CBFß that stems from the translocation of the smooth muscle myosin heavy chain gene (SMMHC; encoded by *MYH11*) and is associated with a clinical outcome similar to that observed with RUNX1-RUNX1T1 due to the formation of an aberrant core-binding factor complex. The CBFß-SMMHC fusion protein restricts RUNX1 to the cytoplasm, acting as a dominant repressor [153–155], and mediating leukemogenesis through the RUNX1-dependent mechanisms mentioned above. Collectively, CBF-AML is often treated with intensive induction with cytarabine and daunorubicin, with or without gemtuzumab ozogamicin [156].

Direct inhibition of CBFß-SMMHC-RUNX1 has been investigated using a FRET assay to screen for compounds that inhibited oncoprotein interactions with RUNX1. This led to the identification and modification of a series of small-molecule inhibitors against the oncoprotein, one which eventually came to be designated as AI-10-49. When administered to mice transplanted with inv(16) leukemic cells, AI-10-49 significantly improved survival with increased apoptosis of leukemic cells and repression of MYC [157]. Combination

approaches such as synergistic BET inhibition (counteracting BRD4-mediated MYC activation) in conjunction with anti-transcription factor small molecule inhibitors represent a promising area of study for this subset of AML [157].

3.4. Mutated TP53

There are few tumor suppressor genes as well-studied as *TP53*, which encodes for a transcription factor that surveils cellular stress and activates numerous pathways that truncate cell proliferation. The function of intact p53 in hematopoietic precursor cells is to oppose a leukemic phenotype, with either loss- or gain-of-function mutations favoring leukemogenesis and susceptibility to mutational accumulation. Mutations in *TP53* occur in up to 10% of patients with AML [158], with missense mutations among the most common – particularly in R282, which represents a structural mutation. In contrast, truncating or contact mutations, such as R248 or R273, impair p53 binding to DNA [159]. Nevertheless, disruptions of *TP53* encompass an AML subtype with an adverse prognosis due to a higher rate of relapse and poorer survival [160],

Murine models suggest that the loss of a single *TP53* allele may be sufficient for the activation of a multistep leukemogenic pathway [161]. Additionally, down-regulation of DNA repair, apoptosis, and cell cycle regulation due to *TP53*^{mut} may also play a role in the promotion of leukemogenesis. In these circumstances, leukemogenesis is likely not due to a single mutation in *TP53*, but rather the consequence of additional acquired mutations stemming from the dysfunctional p53 protein, resulting in accumulation of cytogenetic abnormalities as well as mutations in epigenetic regulators (*IDH1, IDH2, TET2, DNMT*), transcription factors (*CEBPA, RUNX1, NPM1*), or activating signaling pathways (*FLT3, RAS,* and *KIT*), arising through clonal evolution [162–164].

Patients with *TP53*^{mut} treated with front-line anthracycline-based cytotoxic chemotherapy show poor responses and inferior survival compared to cytogenetically normal, *TP53* wild-type AML [165]. Decitabine has emerged as a preferred therapy backbone in this setting, with studies showing relatively high response rates, but not durable durations of remission [166,167]. Initial findings with decitabine led to alternative strategies to augment responses and improve survival in *TP53*^{mut} AML, culminating in the addition of venetoclax, which appears to exert its effects independently of p53 [168]. Following treatment with azacitidine and venetoclax, suppression of oxidative phosphorylation through disruption of the tricarboxylic acid cycle selectively targeted and eradicated LSCs [169]. The addition of venetoclax demonstrated an overall response rate of 47% in de novo *TP53*^{mut} AML and 24% in relapsed or refractory disease, with the duration of remission still disappointingly short (6.4 months and 3.6 months, respectively) [170]. Further work is needed to delineate prognostic markers for venetoclax-based therapy, particularly in *TP53*^{mut} AML. A recent analysis of venetoclax-based therapy in relapsed or refractory AML demonstrated durable remissions in *NPM1*^{mut} and *IDH2*^{mut}, but resistance to venetoclax was seen in *FLT3*-ITD^{mut} and loss of TP53 [171]. The impact of cooperating mutations in *TP53*^{mut} AML undergoing venetoclax-based treatment is less clear.

Targeted strategies have emerged in the treatment of *TP53*^{mut} AML, with one encouraging innovation being the development of eprenetapopt (APR-246), which binds p53^{mut} and

promotes the regulation of transcription factors involved in apoptosis [172], allowing for reacquisition of cell cycle arrest and apoptosis. Eprenetapopt and azacitidine demonstrated an overall response rate of 64% and a complete response rate of 36% in 11 patients [173]. Resistance to BCL-2 inhibition in AML can be overcome through p53-mediated suppression of the RAS pathway, resulting in MCL-1 phosphorylation and degradation, shown in Fig. 2 [174]. Thus, the addition of venetoclax to eprenetapopt with or without a hypomethylating agent represents a theoretical synergistic regimen, with a phase I trial ongoing (NCT04214860).

MDM2 is a negative regulator of TP53, which inhibits transcription of *TP53* and targets p53 for proteasomal degradation [175,176]. Inhibitors of MDM2, e.g., idasanutlin, result in stabilization of p53, induction of apoptosis, and resultant cell-cycle arrest [177]. The addition of idasanutlin to cytarabine in the relapsed or refractory setting was found to improve the CRR at 38.8% compared to 22.0% with cytarabine alone, although there was no difference in the overall survival [178]. However, idasanutlin was found to overcome acquired resistance to venetoclax, with the combination of venetoclax and idasanutlin demonstrating a CRR of 29% and the median survival was 5.7 months [179]. Attempts to dampen proliferation while promoting apoptosis have led to a trial of MEK inhibition with cobimetinib alongside venetoclax and idasanutlin, which is currently underway (NCT02670044).

While not entirely specific to *TP53* mutants, immunotherapy has shown promise in the treatment of *TP53*^{mut} AML. CD47 is a cell-surface molecule that interacts with SIRPα to inhibit phagocytosis of healthy cells by macrophages, and is constitutively up-regulated in myeloid leukemia cells to evade phagocytosis [180]. Magrolimab, a monoclonal antibody to CD47, results in leukemic cell phagocytosis, and was assessed in combination with azacitidine in treatment-naive AML and MDS, 28% of which had *TP53*^{mut}. In the *TP53*^{mut} cohort, the composite response rate (CR + CRi) was 88%, and the median OS was not reached at a median follow-up time of 4.9 months [181]. Additionally, LSCs were eliminated in 63% of responding patients in the overall cohort. Importantly, as CD47 expression is lost during red blood cell senescence, a notable event is that magrolimab may potentiate clearance of aging erythrocytes and potentially leads to severe or prolonged anemia [182]. Ongoing clinical trials in *TP53*^{mut} AML are summarized in Table 4.

4. Signaling pathways

4.1. Mutated RAS

The rat sarcoma (RAS) proteins are signal transducers that relay to downstream targets to regulate cell proliferation [183]. They are commonly implicated in leukemogenesis as cooperating mutations – partners to additional molecular lesions that accelerate tumor formation and subclonal evolution [184]. Activation of the RAS pathway in this context is generally through one of two mechanisms: autonomous signaling of a protein upstream of RAS (such as FLT3 or KIT) [185] or inactivation of a RAS GTPase-activating protein that serves to hydrolyze GTP, resulting in a persistently active GTP-bound form [186].

The ultimate consequence of persistent RAS signaling is an increased sensitivity to GM-CSF and a skewing of the development of the hematopoietic stem cell toward the common myeloid and granulocyte-macrophage progenitor, producing a strong proliferative advantage [187,188]. However, inhibition of MEK, which targets the pathway downstream of RAS by inactivating MAPK/ERK, has had disappointing results as monotherapy [189]. A potential explanation for these findings is that additional cooperative mutations commonly occur in such patients, leading to expansions of subclonal lines that produce disparate cellular phenotypes. A notable exception is that the addition of a *TET2* disruption to a *RAS* mutation potentiates sensitivity of cells to MEK inhibitors and identifies a subset of AML that may preferentially respond to combined MEK inhibition in combination with other known active agents [190]. The addition of venetoclax to cobimetinib, a MEK inhibitor, modulates sensitivity to apoptosis by increasing the action of the pro-apoptotic pathway through suppression of MCL-1 following inhibition of MEK [191].

Further illustrating the importance of cooperative mutations involving the RAS pathway in myeloid leukemogenesis, it was found that up-regulated MAPK signaling conveyed resistance to enasidenib in *IDH2* mutants [192], providing a molecular rationale for combined use of enasidenib and cobimetinib – with the potential for additional agents (e.g., venetoclax, hypomethylating therapy, or antibody-drug conjugates). Together, such findings raise the possibility that *RAS* mutations, which cooperate with additional drivers of leukemogenesis, may promote resistance to therapy – supporting the notion that combined targeting of appropriate aberrant pathways may lead to more effective therapeutic strategies.

4.2. Mutated FLT3

Among the most common activating signaling mutations in AML are the mutations of *FLT3*. *FLT3* encodes FMS-like tyrosine kinase 3, a transmembrane receptor tyrosine kinase that recognizes an extracellular FLT3 ligand. Following binding of the FLT3 ligand, the FLT3 receptor dimerizes and activates the intrinsic tyrosine kinase through phosphorylation, promoting a cascade of downstream parallel signaling pathways including the RAS and phosphatidylinositol 3-kinase (PI3K) pathways, which lead to cell survival, proliferation, and differentiation [193]. PI3K generates a second messenger, PIP3, which binds and activates the AKT family of serine-threonine kinases, subsequently triggering cell growth through up-regulation of the mammalian target of rapamycin (mTOR) and cell survival through negative regulation of FOXO and other pro-apoptotic proteins [194]. Signaling through the RAS pathway, as discussed above, supports the other arm of leukemogenesis downstream from FLT3 – promotion of cell proliferation (Fig. 3).

In AML, there are two common constitutively activating FLT3 mutations: an internal tandem duplication (ITD) and a tyrosine kinase domain (TKD) mutation. *FLT3*-ITD reflects a duplication of a region in the juxtamembrane domain of *FLT3*, leading to ligand-independent constitutive autophosphorylation, while *FLT3*-TKD mutations reflect aberrations of the inhibitory property of the activation loop, leading to constitutive tyrosine kinase activation [193]. Patients with *FLT3*-ITD^{mut} were shown to have inferior survival and increased risk of relapse when compared to *FLT3* wild-type [195]. In contrast, the prognostic impact of *FLT3*-TKD^{mut} is less well defined [196]. Investigation of FLT3

inhibition with tyrosine kinase inhibitors has led to the FDA approval of midostaurin for newly diagnosed AML in combination with cytotoxic chemotherapy [197] and second-generation gilteritinib in the relapsed or refractory setting [198].

With the advent of more selective TKIs targeting FLT3, the role of FLT3 inhibition during induction has become better defined. However, the use of FLT3 inhibitors during consolidation or maintenance remains to be established and represents an area of active study [199], although the use of FLT3 inhibition following allogeneic stem cell transplant has clearly shown benefit [200]. Nevertheless, resistance to FLT3 inhibition has been problematic. For example, while FLT3 inhibition can be achieved at the level of the tyrosine kinase, constitutive activation of downstream pathways still occurs, such as in PI3K or RAS [201]. This raises the possibility of employing multiple TKIs, either in combination or in sequence, to suppress the constitutive activation of parallel signaling pathways. Indeed, the observation that activated PI3K signaling occurred during sorafenib-mediated FLT3 inhibition in *FLT3*^{mut} AML led to the discovery of FLT3 resistance mediated through the parallel PI3K signaling pathway. Notably, PI3K-mediated resistance was overcome by the addition of the PI3K inhibitor gedatolisib, which induced apoptosis and blocked cell proliferation, demonstrated in Fig. 3 [202]. While monotherapy with PI3K inhibition has not produced clinical benefit [203], combination therapy has not yet been fully explored.

To add to the complexity, recent findings demonstrated that relapsed or refractory AML is associated with a larger *FLT3* allelic burden than newly diagnosed disease [204]. The optimal FLT3 inhibitor and sequence of FLT3 inhibition after disease progression remain unknown in the era of newly discovered targeted agents. For example, BCL-2 inhibition may trigger apoptosis and circumvent resistance to FLT3 inhibitors, with phase I trials demonstrating impressive blast clearance in *FLT3* mutants [205]. Analogous to the downstream action of MEK inhibition with cobimetinib, gilteritinib down-regulates the pro-survival protein MCL-1. This raises the possibility that the combination of gilteritinib and venetoclax may synergistically circumvent MCL-1-mediated venetoclax resistance [206]. These findings provide a rationale for multi-targeted therapy in *FLT3*^{mut} disease. A comprehensive review of FLT3 inhibitors in AML has recently been published which covers these concepts in further detail [207].

With respect to cooperative mutations, a potential therapeutic avenue can be envisioned in *NPM1*^{mut} AML that is concurrently *FLT3* mutated. With inhibition of XPO1, e.g., by nuclear transport inhibitors such as selinexor, NPM1^{mut} remains in the nucleus and promotes nuclear localization of p53 and other tumor suppressor proteins [208]. Therefore, mutations in *NPM1* may confer a vulnerability to selinexor. In support of this concept, simultaneous targeting of XPO1 and FLT3 with selinexor and either gilteritinib or midostaurin demonstrated enhanced antileukemic activity in mouse models [209]. As *NPM1*^{mut} AML has been shown to strongly express CD33, the combination of XPO1 and FLT3 inhibition with gemtuzumab ozogamicin remains to be explored. As noted in studies involving *RAS* mutations, the effect of multiple cooperating mutations requires further investigation to determine the optimal combination of targeted therapies. A summary of ongoing clinical trials in *FLT3*^{mut} AML is provided in Table 5.

4.3. Mutated kit

The KIT (CD117) protein is a receptor tyrosine kinase that is structurally related to FLT3 and binds to stem cell factor (SCF). Following the binding of SCF to KIT at the surface of the cell membrane, autophosphorylation of the intrinsic tyrosine residues ensues and allows for signal transduction through the RAS, PI3K, and JAK/STAT pathways. In *KIT*^{mut} AML, constitutive activation of the tyrosine kinase leads to persistent signal transduction, creating cytokine-independent growth and reduced apoptosis [210].

Similar to *FLT3*^{mut} and *RAS*^{mut}, mutations in *KIT* often cooperate to promote leukemogenesis, although they generally appear later in the process [158]. Constitutively active KIT mutations are commonly found in t(8;21) AML, which features the previously discussed fusion oncoprotein RUNX1-RUNX1T1. Cooperative mutations of *KIT* in t(8;21) AML correspond to a poorer prognosis than t(8;21) alone, resulting in a higher relapse risk and shorter overall survival in adult AML [211], although the same does not appear to be true in pediatric AML [212].

Nevertheless, exposure of *KIT*^{mut} cells to KIT inhibitors such as dasatinib resulted in endocytosis of the KIT receptor tyrosine kinase and activation of the intrinsic apoptotic pathway [213]. These findings suggest that dasatinib added to intensive cytotoxic chemotherapy with or without gemtuzumab ozogamicin may enhance responses in this subset of CBF-AML [214].

4.4. Hedgehog pathway

There are three extracellular proteins that activate the hedgehog pathway in mammals: sonic hedgehog (SHH), desert hedgehog, and Indian hedgehog. These three proteins are ligands for the patched-1 transmembrane receptor (PTCH1), which acts to inhibit a transmembrane protein known as smoothed (SMO) when the hedgehog ligands are unbound to PTCH1 [215]. Therefore, following binding of the hedgehog ligands, PTCH1 will inhibit SMO, whose function is to trigger the activation of the glioma-associated zinc finger (GLI) family of transcription factors [216]. The GLI transcription factors then accumulate inside the nucleus, where they regulate the hedgehog target genes that control cell cycle progression, differentiation, and survival and are crucial in the self-renewal of hematopoietic stem cells [217,218]. In contrast, in the absence of hedgehog ligand binding, the GLI transcription factors remain inactive in the cytoplasm due to a protein complex known as suppressor of the fused (SUFU).

In AML, there exists increased expression of the sonic hedgehog ligand and downstream GLI transcription factors [219]. One of the first advances in modulation of hedgehog signaling showed that treatment of AML cells with decitabine reduced the levels of hedgehog-interacting proteins and partially reduced SMO activity in leukemic cells by reducing cell proliferation [220]. Further studies prompted the development of SMO inhibitors. Treatment with inhibitors of SMO, such as glasdegib, allows leukemic cells to overcome cytarabine resistance [221]. Sensitivity of the hedgehog pathway to azacitidine was identified through an RNA interference assay, which demonstrated azacitidine-sensitizing hits to *SHH*, *SMO*, and *GLI* with the administration of the SMO inhibitor,

erismodegib, in combination with azacitidine. This combination demonstrated inhibition of long-term repopulation capacity in AML and MDS [222]. These findings paved the way for the clinical rationale behind co-administration of glasdegib and low-dose cytarabine, and similarly, with glasdegib and azacitidine [223].

Constitutive activation of the hedgehog pathway may cooperate with persistently aberrant FLT3 signal transduction to promote leukemogenesis [224]. Treatment with the combination of FLT3 and SMO inhibitors resulted in a synergistic reduction of malignant hematopoietic stem cell growth [224]. The importance of these findings is that they support the strategy of targeting parallel pathways in AML. In addition, hyperactivation of the hedgehog pathway maintains chemoresistant cells [225]. While inhibition of the hedgehog pathway represents a prospective therapeutic avenue to overcome drug resistance, a caveat is that eradication of leukemic stem cells is significantly more challenging than eliminating the more differentiated leukemic clones.

5. The bone marrow microenvironment

One of the prominent contributors to AML is the environment in which hematopoietic stem cells (HSCs) reside – the bone marrow. The bone marrow architecture is characterized by specialized stem cell niches at the perivascular and endosteal sites of the intramedullary space, where the bulk of HSCs are found [226]. In addition to *de novo* leukemia, alterations occurring in the bone marrow niche are also thought to contribute to the pathogenesis of MDS and myeloproliferative neoplasms that predispose to leukemia [226,227]. For example, in the perivascular distribution of the bone marrow, granulocytes and megakaryocytes regulate the release of mature progenitors to the peripheral vasculature. However, in primary myelofibrosis, the marrow microenvironment becomes fibrotic and subsequently dysregulated, leading to the release of leukemic blasts [227]. This exemplifies the importance of the bone marrow microenvironment in the pre-leukemic state, potentially culminating in AML.

In contrast to the vascular niche, which regulates the release of matured bone marrow precursors, the endosteal niche represents the site where most HSCs are located and thereby functions as a concentrated stem cell reserve. In MDS, marrow microenvironment alterations are well documented, including intensified vascularization resulting in increased microvessel density often observed in AML [228,229]. However, data are still in conflict regarding whether or not angiogenesis represents a mechanism causally responsible for progression of MDS to AML [230–233]. For example, microvessel density, expression of associated vascular endothelial growth factor receptor 2 (VEGFR2), angio-poietins, and basic fibroblast growth factor (FGF) were lower in AML arising from MDS (secondary AML) than from *de novo* AML [231,232]. This suggests neoplastic independence from angiogenesis, a phenomenon that could have clinical implications in the treatment of this distinct subset of AML arising from multilineage dysplasia.

In a more general sense, it is recognized that residual leukemic stem cells (LSCs) residing in the bone marrow represent a major determinant of treatment failure and lead to early relapse [234]. The $CD34^+$ $CD123^+$ LSCs usually reside in the G_0 phase of the cell

cycle and exhibit multiple mechanisms of drug resistance, including synthesis of proteins such as BCL-2 as well as P-glycoprotein, a broad-spectrum efflux pump responsible for extrusion of xenobiotics and multiple anti-neoplastic agents [235,236]. These residual leukemic cells alter the bone marrow microenvironment, fueling a self-sustaining cycle by generating leukemic clones that have lost the ability to respond appropriately to antiproliferative signals from the surrounding niche [237,238]. The significance of this point can be illustrated through the CXCL12-CXCR4 pathway: CXC motif chemokine ligand 12 (CXCL12) binds to CXC motif chemokine receptor 4 (CXCR4) and serves as a pivotal mediator for stem cell homing, migration, quiescence, and survival within the bone marrow microenvironment [239]. Blocking CXCL12 or its receptor, CXCR4, with either small molecule inhibitors (such as BPRCX807) or monoclonal antibodies has been of recent interest, particularly in combination with targeted therapy, hypomethylating agents, or intensive cytotoxic chemotherapy [238,240]. Similar pharmacological applications can be used for alternative interactions between leukemic and stromal cells, including blockade of VLA-4/VCAM1 [241] and CD44 [242], both of which regulate homing and retention of HSCs in the osteogenic niche. Particularly, CD44, a cell adhesion molecule, appears to promote CXCL12-mediated resistance to venetoclax-mediated apoptosis and preclinical studies have demonstrated that *CD44* knockout sensitized AML cells to venetoclax [243].

Additionally, the up-regulation of cytokines in the leukemic bone marrow niche promotes clonal proliferation and inhibits apoptosis through a paracrine mechanism [244] as well as by modulation of mesenchymal stromal cell transcriptosomes by leukemic blasts to suppress normal cell cycle progression [226]. Another relevant phenomenon is the up-regulation of BCL-2 by stroma-supported leukemic blasts, which antagonizes apoptosis and promotes cell survival. This event has been directly targeted by BH3-mimetics such as venetoclax in the treatment of both the acute and chronic leukemias [245]. In sum, the vascular and endosteal leukemic cell niches, in cooperation with contributions from nervous system signaling, aberrant signal transduction, neoangiogenesis, marrow remodeling [226], and metabolic dysregulation [246] may all play roles in facilitating leukemogenesis in the bone marrow [229,247]. Consequently, each of these processes may provide opportunities for therapeutic interventions [229].

Finally, distinct from the above processes, down-regulation of immune detection in the marrow microenvironment has been hypothesized to contribute to a phenomenon of immune escape, protecting leukemia cells from immune destruction [248]. The interaction between programmed cell death-1 (PD-1) and its ligand (PD-L1) functions as an immune checkpoint to promote self-tolerance through suppression of T-cell inflammatory activity. Lower PD-L1 expression in $CD34^+$ cells was found to be associated with prolonged survival in untreated AML patients [249]. However, blockade of PD-1 and PD-L1 via monoclonal antibodies has not shown impressive results as monotherapy in patients with relapsed AML [250]. Hypomethylating agents were shown to induce PD-L1 expression in AML and up-regulation of PD-L1 is postulated to be a mechanism of resistance to these therapies [249]. Concurrent treatment with anti-PD-L1 nivolumab and azacitidine produced a composite response rate of 22% and a median survival of 6.3 months in relapsed or refractory AML, which compared favorably to a cohort of historical controls that received HMA-based salvage [251].

6. Other novel approaches and future directions

In addition to the previously described mechanisms and corresponding strategies in AML, several new avenues are beginning to be explored. For example, the ubiquitin-proteasome system, which controls timely destruction of intracellular proteins, has emerged as an attractive therapeutic target. The cullin protein family are scaffold proteins that provide support for ubiquitin ligases, which control the ubiquitination and destruction of mediators of cell cycle progression, signal transduction, or DNA damage response [252]. Neddylation regulates the activity of these cullins through the NEDD8 conjugation pathway, controlled by the NEDD8-activating enzyme (NAE) [253]. NAE inhibitors were shown to activate the intrinsic and extrinsic apoptotic pathways [254], and NAE inhibitors such as pevonedistat have been evaluated in combination with venetoclax and azacitidine in clinical trials [255]. A clinical trial of pevonedistat and belinostat is ongoing (NCT03772925).

Additional novel strategies include inhibition of the cyclin-dependent kinases (CDKs) – particularly CDK7 and CDK9. CDK7 functions as a catalytic subunit of the CDK-activating kinase (CAK), which functions to activate multiple other CDKs and also activates RNA polymerase II [256]. A multi-target CDK 7/12/13 inhibitor, THZ1, was evaluated in AML cell lines with RUNX1-RUNX1T1 and demonstrated loss of proximal (5′) pausing of RNA polymerase and concomitant suppression of RNA polymerase at the distal (3′) end of genes [257]. Similarly, CDK9 is a transcriptional regulator and comprises the super elongation complex which controls RNA polymerase II. Fusion partners of MLL also constitute the super elongation complex and therefore inappropriately unite CDK9 to *HOX* promoters, contributing to aberrant CDK9 function. Inhibition of CDK9 with AZD3573 revealed significant MCL-1-mediated apoptotic induction, which was potentiated by venetoclax [258]. Additional preclinical work in this setting demonstrated that concurrent treatment with venetoclax and A-1467729, another small-molecule inhibitor of CDK9, exhibited synergistic cell death [259]. More recently, CDK9 inhibitors were found to result in a reduction of secondary AML burden in xenograft models, with improvement of the overall survival after three weeks of treatment [260]. Additionally, CDK9 inhibitors were found to demonstrate synergistic lethality with anti-BCL-2 strategies in the context of secondary AML [260]. As novel targets are discovered that contribute to or maintain a leukemic state, their application to specifically defined genetic sub-types of AML should present opportunities for newer precision medicine-based treatment strategies. A summary of the mechanisms of leukemogenesis and potential targets is available in Table 6.

7. Conclusions

Future trials investigating novel combinations involving the aforementioned pathways will be necessary to develop innovative treatment regimens and improve survival in AML. Building upon the backbones of either anthracycline- or venetoclax-based regimens (e.g., venetoclax and azacitidine) appear to be the most promising avenue to improve patient outcomes, with careful selection of novel drugs that are either synergistic or act to delay or mitigate the development of drug resistance or disease relapse. In addition, if tolerable and justified by underlying leukemogenic aberrations, one can envision future rational three-agent regimens involving targeted agents – for example, with venetoclax, CDK9 inhibitors,

and FLT3 antagonists for the treatment of *FLT3*^{mut} *MLL*-rearranged AML. Notably, as investigations continue to expand the armamentarium of novel agents to be combined with established therapeutic backbones, care must be taken to assess the risk and benefits of such approaches in the light of potential additional toxicities. Further preclinical work coupled with careful conduct of clinical trials will be necessary to develop such innovative treatment strategies for AML.

The numerous paths that lead to the genesis of leukemia are frequently intertwined, and cooperating mutations involving epigenetic regulators, oncoproteins, transcription factors, tumor suppressors, and activating signaling mutations contribute to the enormous complexity of leukemogenesis. A better understanding of these mechanisms will be essential for the implementation of developing new and more effective treatment strategies for this highly heterogeneous disease. It is likely that the development of rational combinations of such inhibitors offers the best chance for overcoming resistance and improving survival in AML. Consequently, the results of novel combination therapies and the discovery of future targets are eagerly anticipated.

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Practice points

- There are numerous mechanisms which cooperate to promote leukemogenesis. These include aberrations in epigenetics, transcription factors, signal transduction, DNA damage control, and cell cycling.
- As understanding of these mechanisms improves, discovery of potential targets will facilitate the development of novel therapies.
- Given the relatively modest activity of many targeted therapies and the frequency of multiple cytogenetic or molecular lesions in AML, combination strategies involving targeted agents should be vigorously explored.
- In addition to the use of targeted therapies during induction or treatment of relapsed disease, consideration should be given to evaluating novel agents during consolidation or maintenance to delay relapse.
- The use of novel combination regimens in the setting of specific genetically defined AML sub-types is likely necessary for optimal activity.
- Care must be taken to select combinations of novel therapies with established approaches to minimize toxicity and maximize efficacy.

Research agenda

- Improvement in the understanding of the molecular mechanisms of leukemogenesis.
- Identification of new mechanisms or signaling pathways that can be therapeutically targeted.
- Identification and/or development of novel small molecule inhibitors that disrupt or truncate the aforementioned mechanisms.
- Demonstration of preclinical activity of novel agents as monotherapy and in synergy with existing conventional or targeted strategies.
- Identification of subsets of AML that optimally respond to newer agents.
- Demonstration of superiority of novel combination strategies to current standard of care strategies.

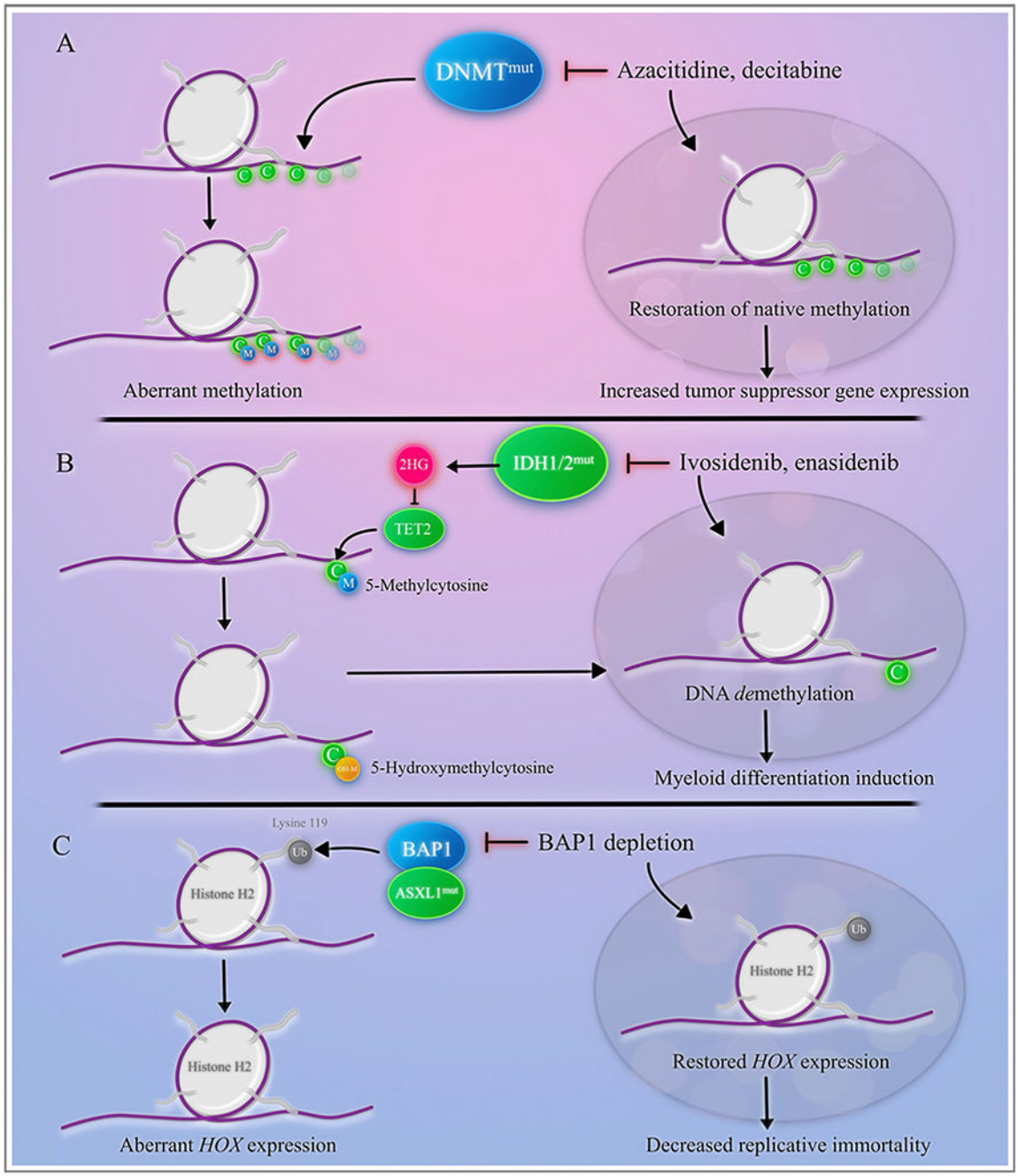

Fig. 1.

Epigenetic regulators in acute myeloid leukemia.

A. The DNMT family converts cytosine residues to 5-methylcytosine and are responsible for somatic methylation patterns. DNMT^{mut} creates aberrant methylation patterns and disruption of gene expression. Hypo-methylating agents, including azacitidine and decitabine, restore methylation through inhibition of DNMT^{mut}, allowing for increased tumor suppressor gene expression.

B. TET2 modifies 5-methylcytosine to 5-hydroxymethylcytosine, resulting in DNA demethylation. TET2 is inhibited by 2-hydroxyglutarate (2HG), produced by mutated IDH1 or IDH2. IDH1 and IDH2 inhibitors indirectly restore methylation patterns and result in myeloid differentiation C. ASXL1 cooperates with BAP1 to deubiquitinate lysine 119 on histone H2, contributing to a gain-of-function effect and resulting in a differentiation block. Depletion of BAP1 restores *HOX* gene expression by decreasing *HOX*-mediated replicative immortality.

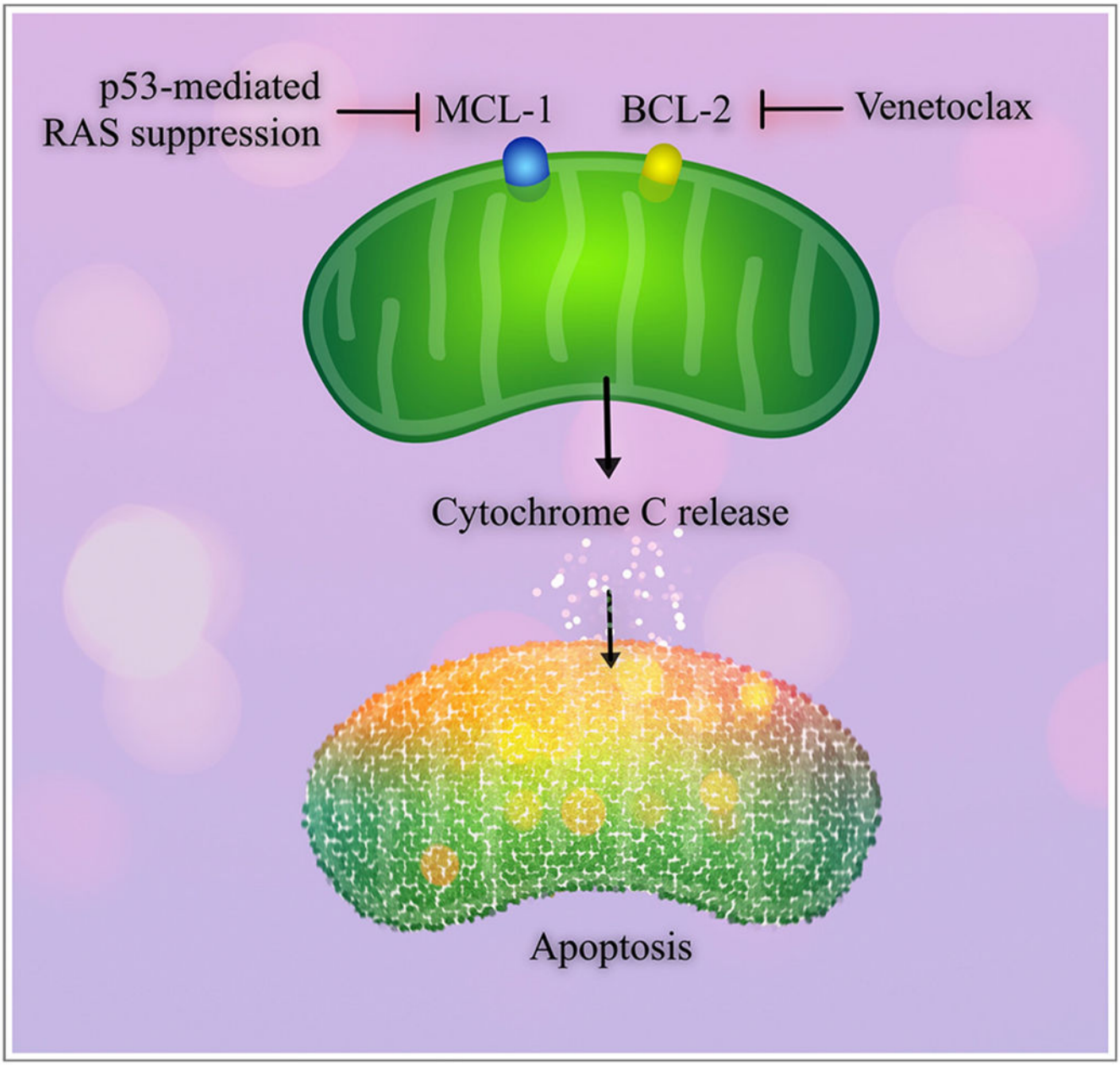

Fig. 2.

Pro-survival proteins and apoptosis in acute myeloid leukemia.

MCL-1 and BCL-2 are pro-survival proteins that are frequently targeted or dysregulated in AML. Venetoclax is a BCL-2 inhibitor that results in apoptosis through mitochondrial cytochrome C release. BCL-2 resistance can occur through up-regulation of MCL-1, which can be overcome through suppression of the RAS pathway by p53.

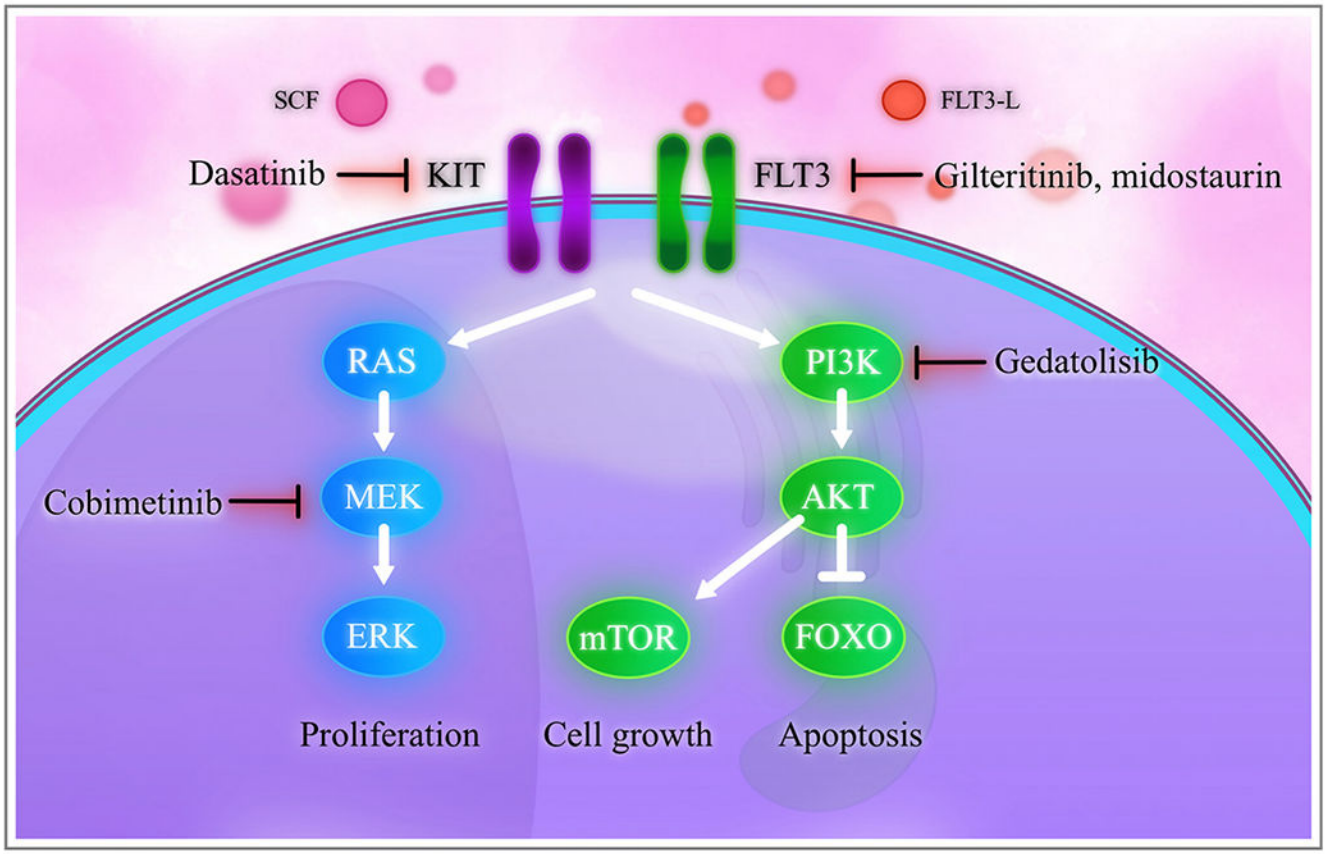

Fig. 3.

Signaling pathways in acute myeloid leukemia.

Stem cell factor (SCF) binds to the KIT tyrosine kinase receptor, triggering activation of downstream RAS and PI3K. Similarly, the FLT3 ligand (FLT3-L) binds to the FLT3 tyrosine kinase receptor and activates RAS and PI3K. RAS cascades into MEK and ERK, resulting in cell proliferation. PI3K activates AKT, which leads to mTOR-driven cell growth. AKT also inhibits FOXO and other pro-apoptotic proteins, leading to cell survival.

Table 1

Major regulators of epigenetics in AML.

Role in epigenetics	Implicated proteins or molecules
DNA methylation	• DNMT family
DNA demethylation	• TET family • IDH1/2 (via supporting catalytic function of TET proteins)
Histone methylation	• EZH2 • KMT2A (MLL) • DOT1L
Histone demethylation	• LSD1 (reviewed elsewhere)
Histone acetylation	• Histone acetyltransferases
Histone deacetylation	• Histone deacetylases (HDACs)
Histone deubiquitiniation	• ASXL1 • BAP1
MicroRNA	• miR-9 (reviewed elsewhere)

Table 2

Clinical trials in *IDH1*^{mat} or *IDH2*^{mat} AML.

Mutation	Study regimen	Trial identifier
IDH1^{mut} or *IDH2*^{mut}	AML	
IDH1^{mut}	Ivosidenib or placebo + azacitidine	NCT03173248
IDH1^{mut}	Ivosidenib and CPX-351	NCT04493164
IDH1^{mut}	Olutasidenib with azacitidine or cytarabine	NCT02719574
IDH2^{mut}	Enasidenib and venetoclax	NCT04092179
IDH1^{mut} or *IDH2*^{mut}	Ivosidenib or enasidenib with 7 + 3 followed by cytarabine or mitoxantrone/etoposide consolidation	NCT02632708

Table 3

Clinical trials in *MLL*-rearranged AML.

Mutation	Study regimen	Trial identifier
MLL-rearranged AML		
MLL-rearranged or *NPM1*^{mut}	DSP-5336 (menin-MLL inhibitor)	NCT04988555
MLL-rearranged or *NPM1*^{mut}	SNDX-5613 (menin-MLL inhibitor)	NCT04065399
None specified; relapsed or refractory AML	KO-539 (menin-MLL inhibitor)	NCT04067336
MLL-rearranged AML	EPZ-5676 (DOT1L inhibitor)	NCT01684150

Table 4

Clinical trials in *TP53*^{mut} AML.

Mutation	Study regimen	Trial identifier
TP53^{mut} AML		
TP53^{mut} AML	Eprenetapopt + azacitidine	NCT03931291
TP53^{mut} AML	Decitabine monotherapy	NCT03063203
TP53^{mut} AML	Magrolimab + azacitidine versus venetoclax + azacitidine or intensive chemotherapy	NCT04778397
None specified; relapsed or refractory AML	Idasanutlin, venetoclax, and cobimetinib	NCT02670044
TP53^{mut}/*TP53*^{wt} and *FLT3*^{mut} AML	Siremadlin (TP53/MDM2 inhibitor) + midostaurin	NCT04496999

Table 5

Clinical trials in *FLT3*^{mut} AML.

Mutation	Study regimen	Trial identifier
FLT3^{mut} AML		
FLT3-ITD^{mut}	Gilteritinib + gemtuzumab ozogamicin	NCT05199051
FLT3-ITD^{mut}	Quizartinib and 7 + 3	NCT04676243
FLT3-ITD^{mut}	Quizartinib + venetoclax	NCT03735875
FLT3^{mut} AML	Gilteritinib + azacitidine	NCT02752035
FLT3^{mut} AML	Gilteritinib + azacitidine and venetoclax	NCT04140487
FLT3^{mut} AML	Gilteritinib + decitabine, cedazuridine, and venetoclax	NCT05010122
FLT3^{mut} AML	Gilteritinib + CPX-351	NCT05024552
FLT3^{mut} AML	Gilteritinib + atezolizumab	NCT03730012
FLT3^{mut} AML	Gilteritinib versus midostaurin	NCT03836209
FLT3^{mut} AML	Crenolanib versus midostaurin	NCT03258931
FLT3^{mut} AML	CLN-049 (FLT3-CD3 bispecific antibody)	NCT05143996

Table 6

Mechanisms of leukemogenesis and potential targets.

Class	Target	Mechanism	Potential drugs or combinations
Transcription	Factors RUNX1-RUNX1T1 or CBFB-MYH11	Alteration of epigenetic landscape, negative regulation of transcription factors that regulate hematopoietic progression, cell cycle arrest, repression of tumor suppressors	• AI-10-49 • Anthracycline-based induction with • Anthracycline-based induction with gemtuzumab ozogamicin if favorable risk
	TP53^{mut} (or *TP53* deleted)	Down-regulation of DNA repair, apoptosis, cell cycle regulation; accrual of sequential mutations	• Eprenetapopt (APR-246) • Magrolimab Idasanutlin or other MDM2 inhibition (siremadlin) Hypomethylating agent + venetoclax
Signal Transduction	*RAS*^{mut}	Increased sensitivity to GM-CSF, skewed development of the hematopoietic stem cell to the myeloid line	• MEK inhibition (cobimetinib) • RAS inhibition • With or without cytotoxic chemotherapy or venetoclax-based therapy
	FLT3^{mut}	Activation of RAS and PI3K pathways	• Midostaurin • Gilteritinib • Quizartinib • Crenolanib • Momelotinib (FLT3/JAK inhibition) • With or without cytotoxic chemotherapy or venetoclax-based therapy
	KIT^{mut}	Activation of RAS, PI3K, and JAK/STAT pathways	Dasatinib with or without cytotoxic chemotherapy or venetoclax-based therapy
	Hedgehog pathway	Increased expression of the sonic hedgehog ligand and GLI transcription factors	Glasdegib with hypomethylating agents or low-dose cytarabine
Other Novel Approaches	NEDD8-activating enyzme	Ubiquitination of proteins controlling cell cycle progression, signal transduction, and DNA damage response	Pevonedistat with or without venetoclax-based strategies
	CDK7/9	Activation of RNA polymerase II, *HOX* dysregulation	• THZ1 for CDK7 • AZD3573 for CDK9 • With or without cytotoxic chemotherapy or venetoclax-based therapy
Bone Marrow Microenvironment	CXCL12 or CXCR4	Up-regulation of cytokines, promotion of clonal proliferation, inhibition of apoptosis, BCL-2 up-regulation	• BPRCX807 • AMD3100 • With or without venetoclax-based therapy
	PD-1 or PD-L1	Immune escape; protecting leukemia cells from immune destruction	Anti-PD-L1/PD-1 with or without HMAs
Epigenetics	CARM1 dysregulated	Histone methylation silencing target genes	• HDAC inhibition (belinostat, givinostat, vorinostat) • With or without cytotoxic chemotherapy or venetoclax-based therapy
	miR-9	*HOXA9*-mediated transformation, hypermethylation, reduction of *FOXO*	• Locked nucleic acid oligonucleotide inhibitors/tumor suppressor miRNA mimetics • Hypomethylating agents • PI3K inhibition
	DNMT^{mut}	Aberrant methylation and alteration of gene expression	Hypomethylating agents with venetoclax
	TET2^{mut} or silenced	Loss of conversion of 5-methylcytosine to 5-hydroxymethylcytosine, resulting in dysregulated methylation	Hypomethylating agents for hypermethylated *TET2* with venetoclax
	IDH1^{mut} and *IDH2*^{mut}	Formation of 2-hydroxyglutarate, inhibition of TET2, DNA hypermethylation	• Ivosidenib or olutasidenib for IDH1 • Enasidenib for IDH2 • With or without hypomethylating agent backbones, venetoclax under investigation
	ASXL1^{mut}	Increases the function of BAP1 in the modification of histones, *HOX* dysregulation	• Small-molecule inhibitors of BAP1 • With or without cytotoxic chemotherapy or venetoclax-based therapy

Class	Target	Mechanism	Potential drugs or combinations
	BET proteins	Dysregulated transcriptional activation through RNA polymerase II, DNA repair, and MYC signal transduction	• Mivebresib • Birabresib • Both with or without venetoclax-based therapies
	Histone deacetylases	Blocking transcription of genes responsible for differentiation due to aberrant histone acetylation	• Belinostat • Vorinostat • Pracinostat • In combination with additional targeted therapy, hypomethylating agents, or venetoclax
	EZH2^{mut}	Methylation of p15^{INK4B}, loss of CEBPα myeloid differentiation, dysregulated *HOX* gene expression	• DZNep with or without HDACI or hypomethylating agents • UNC1999 • GSK126 • DS-3201 with or without venetoclax-based therapies
	KMT2A^{mut} (or *MLL* rearranged)	*HOXA9* constitutive expression due to DOT1L dysregulation, expression of *MEIS1* with *PBX3*	• DOT1L inhibitor • MEK inhibitor • BET inhibition (BRD4) • Menin inhibition • With cytotoxic chemotherapy or venetoclax-based therapy
Transcription Factors	Biallelic *CEBPA*^{mut}	Disruption of G-CSF and GM-CSF receptors, activation of genes responsible for maintaining leukemic cells in dedifferentiated states	Anthracycline-based induction with gemtuzumab ozogamicin if favorable risk
	NPM1^{mut}	Aberrant shuttling between nucleus and cytoplasm, up-regulation of *HOX* expression	• Selinexor • Menin inhibition • Anthracy cline-based induction with gemtuzumab ozogamicin if favorable risk