Review

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Understanding the molecular basis of acute myeloid leukemias: where are we now?

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Practice points

- Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults.
- AML can arise *de novo*, develop from the progression of myelodysplastic or myeloproliferative disorders or result from previous anticancer therapy.
- Leukemogenesis is a multicause, multistep and multipathway process.
- The major progress in the understanding of the complex pathogenesis of AML is being translated into therapeutic approaches.
- In the future tailor-made treatment schemes may become the clinical standard.

Althoughthe treatment modalities for acute myeloid leukemia (AML) have not changed much over the past 40 years, distinct progress has been made in deciphering the basic biology underlying the pathogenesis of this group of hematological disorders. Studies show that AML development is a multicause, multistep and multipathway process. Accordingly, AMLs constitute a heterogeneous group of diseases. The thorough understanding of the molecular basis of AML is paving the way for better therapeutic approaches. Multiple novel drugs are being introduced and new, more efficient and less toxic formulations of conventional therapeutics are becoming available. Here, we review the recent advances in the comprehension of the molecular processes that lead to the onset of AML and its translation into clinical practice.

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Acute myeloid leukemia (AML) develops as the consequence of a series of genetic and epigenetic changes in hemopoietic stem cells (HSCs) or precursors. The lesions alter normal hemopoietic growth and differentiation, resulting in an accumulation of abnormal, immature myeloid cells in the bone marrow and peripheral blood, while normal hemopoiesis is suppressed. AML blasts proliferate, but do not differentiate. Whereas the features of increased proliferation and maturation defects have been thoroughly studied over the years, research into normal hemopoiesis inhibition has lagged behind. In the past, cytopenias concurrent with AML were attributed to the reduced space in the bone marrow. Current research shows that normal hemopoiesis defects are caused by AML blasts. In fact, in AML patients the number of HSCs appears to be normal or even increased [1]. The expression of myeloproliferative leukemia, the thrombopoietin scavenging receptor, on AML blasts predicts peripheral blood neutropenia and thrombocytopenia [2]. The systemic loss of hemopoietic function is also partially the consequence of AML exosome-directed microRNA trafficking to HSCs [3].

AML is a heterogeneous group of diseases; hence, no single prevalent mutation is present in all or even in the majority of patients. The top ten genes mutated at frequencies > 5% are *FLT3*, *NPM1*, *DNMT3A*, *IDH1/2*, *TET2*, *AML1*, *TP53*, *NRAS*, *CEBPA* and *WT1* [4]. The genetic heterogeneity is reflected morphologically: AML blasts exhibit maturation defects corresponding to specific stages of hemopoietic differentiation. The growing knowledge of underlying lesions forms the basis for the current WHO classification of AML [5] that has replaced the morphology-based French–American–British (FAB) classification [6].

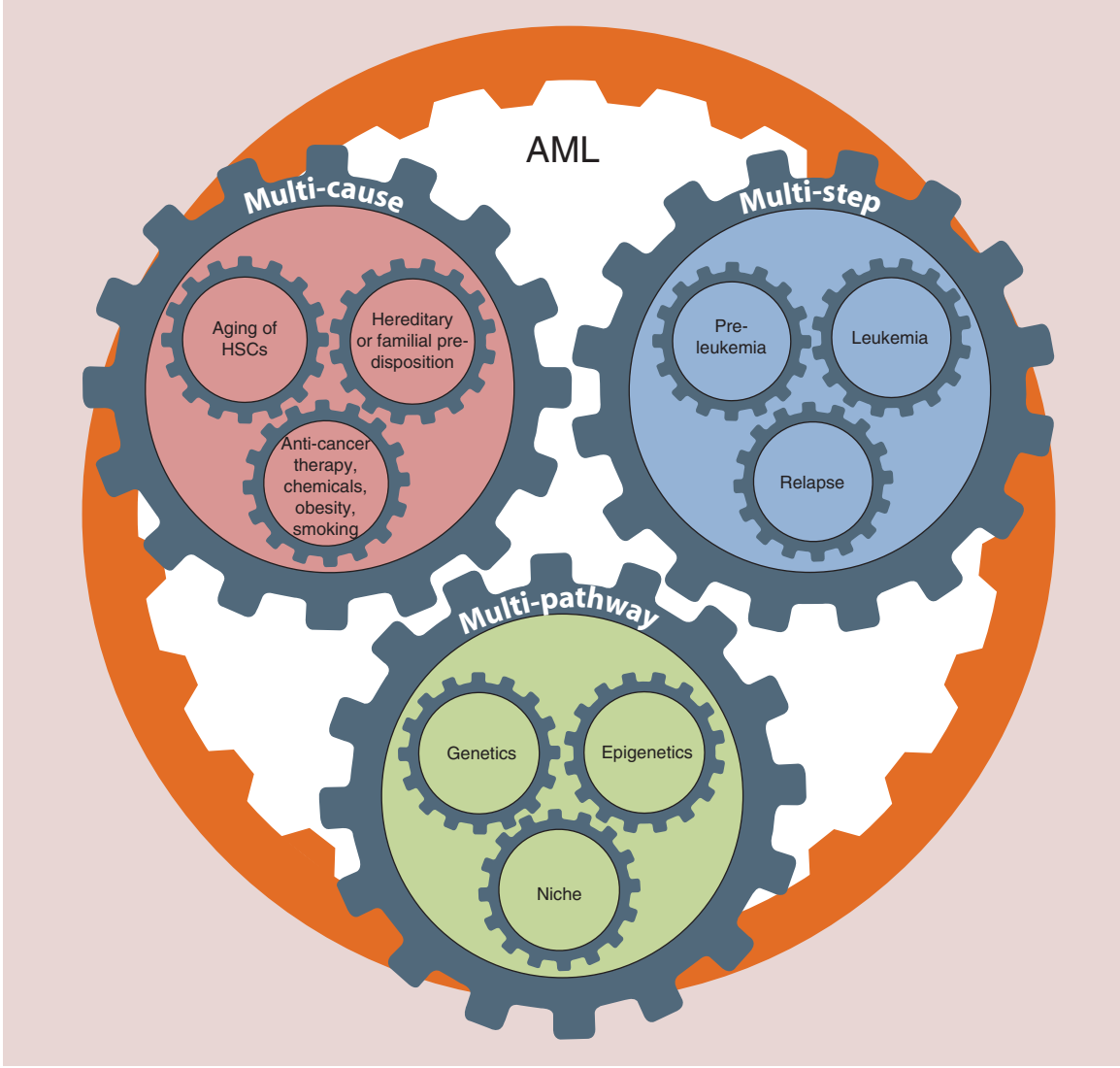

Figure 1. Acute myeloid leukemia pathogenesis: acute myeloid leukemia development is a multicause, multistep and multipathway process.
AML: Acute myeloid leukemia.

AML appears to be maintained by a pool of self-renewing malignant cells denominated leukemic stem cells (LSCs) [7].

In this review, some aspects of the AML pathogenesis are analyzed (Figure 1). AML is a subject of continuous study with over 2000 articles published in 2016 alone containing the MESH term 'AML.' Here we discuss the causes, steps and processes necessary for AML generation and conclude with remarks concerning the translation of basic research into clinical practice.

Multicause process

Even though acquired genetic abnormalities are recurrent in leukemic blasts, the direct and exact causes of AML are unknown in the majority of cases. Risk factors are related to both aging of individuals and to some specific genetic and environmental exposures. Several causes play a role in AML onset.

Aging of stem cells, AML & longevity trade-off

HSCs acquire, throughout their lifespan, DNA mutations and micro-environmental alterations that may be responsible for hematological disease development [8,9]. Cellular quiescence of HSCs constitutes a possible mechanism

Table 1. Therapy-related myelodysplastic syndrome risk factors and their consequences.

Risk factors	Consequence
Alkylating agents/radiation	-5 or 5q-
	-7 or 7q-
Topoisomerase II inhibitors	*MLL*-fusion formation
	AML1 mutations
	PML/RARA
Single nucleotide polymorphisms in *GST, MSH1/2, RAD51, CYP3A4, NQO1, EPHX1*	Alterations in drug metabolizing enzymes, DNA repair or detoxification pathway members
Chemical exposure (vinyl chloride, benzene and formaldehyde)	Hemotoxicity

for avoiding DNA damage, chromosomal aberrations and mutations [10]. The expansion and predominance of mutant clones within the pool is a frequent event during aging [8]. There are two main features of aging HSC. First, the alteration of lineage potential in favor of myelopoiesis causing decreased immune competence and increased incidence of myeloid malignancy is observed [11]. Second, loss of proliferative and self-renewal potential occurs and may be due to DNA damage that leads to the reduction of regenerative ability [8]. Different molecular mechanisms such as telomere shortening [12], replication stress [13], chromatin changes, hypermethylation of CpG islands [14], metabolic alterations and defects in DNA repair and chromosome segregations contribute to DNA damage, mutation accumulation and genomic aberrations during aging [10]. A recent study has also shown an association between mutations in various components of the RNA-splicing machinery and age-related malignancies [15].

Therapy-related AML, chemical/radiation exposure & smoking

Chemotherapy, ionizing radiation and chemical exposure have been linked to genetic changes in HSCs and precursor cells. Primary malignancy therapy-related myelodysplastic syndrome/AML (t-MDS/AML) develops after several months or years [16]. The loss of all or parts of chromosomes 5 and/or 7 are the most frequent cytogenetic abnormalities occurring after the exposure to alkylating agents and/or radiation. Otherwise, balanced chromosomal rearrangements involving *MLL*, *AML1* and *PML/RARA* genes may form after treatment with compounds targeting DNA–topoisomerase II [17]. Individuals carrying a single nucleotide polymorphisms in the *NQO1* detoxifying gene [18], in drug-metabolizing enzymes [19] or genes coding for components of DNA repair pathways such as *MSH1/2* [20,21], *RAD51* [22] or *CYP3A4* [22] are more likely to develop treatment-related (t)-AML.

The exposure to vinyl chloride [23] or benzene [24] plays a role in the pathogenesis of AML as well as mutations in *EPHX1* enzyme involved in benzene metabolism [25]. Recently, Chakraborty *et al.* [26] have demonstrated a possible correlation between the onset of t-MDS/AML and telomere length regulation in autologous hemopoetic cell transplantation setting. Cells from patients who underwent autologous hemopoetic cell transplantation and subsequently developed t-AML/t-MDS showed an initial increase in telomere length followed by accelerated telomere shortening [26]. t-AML risk factors are summarized in Table 1.

In addition, cigarette smoking [27,28] and obesity [29] prove to be significant risk factors for the development of AML in adults.

Familial predisposition & MDS evolution

Familial AMLs are generally rare. Congenital or hereditary disorders such as Down syndrome, ataxia telangiectasia, Bloom syndrome, Fanconi anemia, congenital neutropaenia as well as germline mutations concerning *CEBPA*, *AML1*, *TP53*, *ANKRD26*, *DDX41*, *ETV6*, *GATA2*, *SRP72*, *TERC* and *TERT* genes have been linked to a higher predisposition to AML [30]. Similarly, patients affected by clonal hematological disorders, including MDS and myeloproliferative neoplasms, may develop AML in the course of disease.

Multistep process

The classical two-hit model of leukemogenesis has been postulated by Gilliland and Griffin [31]. In this model, two lesions, each belonging to a different class, cooperate to cause AML and neither is sufficient to do it alone. Class I mutations (e.g., *FLT3-ITD*, *c-KIT* or *NRAS* mutations) bestow a proliferative advantage without blocking differentiation. Class II mutations (i.e., AML-specific fusion genes in the original model) interfere with hematopoietic differentiation and subsequent apoptosis. The initiating lesions are thought to be class II mutations, in other words, fusion genes, whereas class I mutations are typically later events [31]. For many years this model provided

Table 2. Functional categories of genes recurrently mutated in acute myeloid leukemia.

Functional category	Genes
Signal transduction genes	*FLT3, NRAS, KRAS, c-KIT, PTPN11*
DNA modification genes	*DNMT3, IDH1/2, TET2*
Chromatin modifiers	*MLL-fusions, ASXL1, EZH2, MLL-partial tandem duplication*
Multi-function	*NPM1*
Chimeric or mutated transcription factors	*PML/RARA, AML1/ETO, CBFB/ MYH11, CEBPA, AML1*
Tumor-suppressor genes	*TP53, PHF6, WT1*
Spliceosome genes	*SF3B1, SRSF2, U2AF1*
Cohesin complex genes	*SMC1A, SMC3, RAD21, STAG2*

a fitting explanation for the pathogenesis of AML, a disease in which differentiation is blocked and proliferation is increased. However, some of the mutations encountered in AML cannot be classified as type I or type II, thus rendering the two-hit model overly simplistic.

More recently whole-genome and whole-exome sequencing studies [4,32–36] have demonstrated that at least one potential driver mutation exists in nearly all AML patient samples and that a complex interplay of genetic lesions contributes to AML pathogenesis in individual patients [4]. Indeed, it is becoming evident that human AML evolution is a multistep process albeit the timing, intricacy and order of events prior to the diagnosis are much less clear. Clonal evolution within each AML patient appears to be a dynamic process consisting of continuous acquisition and loss of specific mutations often at different times [4,34,37,38]. Such process is subjected to selective pressure exerted by the host microenvironment and, similarly to Darwinian selection, enables the survival of the fittest clones [39]. The end result is simultaneous evolutionary convergence and divergence among clones and subclones during the clinical course of the disease [34,40]. Notably, the genetic composition of subclones has been shown to have a functional significance [41]. Within the same AML patient individual clones may display distinct morphology, surface markers and engraftment potential in immunocompromised mice [41].

Sequencing studies helped to get insight into the clonal evolution of AML from the insurgence of a primary preleukemic clone and its subsequent fate: the onset of overt leukemia and of relapse [34]. In the past, the term 'preleukemia' was used to describe conditions with a propensity for progression to AML [42]. A current understanding of preleukemia is the existence of cells carrying a subset of genetic/epigenetic variants ultimately present in AML blasts without displaying any differentiation defect [43]. Preleukeamic lesions have been reviewed by Shlush and Minden [43] and include mutations in genes such as *DNMT3A*, *TET2*, *IDH1/2*, *ASXL1* and many others. A recent study has suggested that nonleukemic HSCs and progenitor cells may have a competitive fitness advantage after induction chemotherapy, due to the presence of specific aging-acquired mutations, thus allowing them to expand and endure long the completion of treatment. [44]. Indeed, in patients who achieved complete remission, clonal hemopoesis persists as evidenced by a skewed X-chromosome representation [45]. *DNMT3A* mutations existing at presentation are the most common lesions remaining during follow-up after achieving complete remission [45]. Moreover, clonal hemopoiesis harboring AML-associated mutations (mainly in *DMNT3A* and *TET2*) is ubiquitous in healthy adults [46].

In the context of such a background, two scenarios may underlie relapse. It may either constitute the regrowth of the original leukemia, if the treatment were unsuccessful, or its further clonal evolution following the selective pressure of chemotherapy. Clearly, *de novo* generation of AML through accumulation of additional novel mutations in the preceding preleukemic clone is also possible though technically it cannot be described as relapse. Mutational analysis on relapsed disease shows that clonal structure of AMLs changes markedly post therapy, with alterations occurring at both genetic and epigenetic levels [34,47].

Finally, massively parallel sequencing studies enabled the discovery of hitherto unknown mutations in *DNMT3A* and *IDH1* genes as well as in cohesin complex and spliceosome machinery genes [4].

Common AML mutations grouped by functional categories are listed in Table 2.

Multipathway process

AML is not a single disease, but a group of disorders, underscored by alterations in multiple intracellular processes and pathways. AML-associated mutations often involve epigenetic regulators (frequently present in the preleukemic clone) such as *DMNT3A*, *TET2*, *WT1*, *IDH1/2*, while chromosomal abnormalities target numerous transcription

factors (TFs) and transcriptional activators [4]. Mutations in the cohesion complex seem to contribute to leukemogenesis through the modulation of the chromatin accessibility in HSCs and progenitor cells [48]. The deregulation of long noncoding RNAs adds a further level of gene expression deregulation [49]. As a consequence, changes in chromatin status and altered transcription resulting in inhibition of some and activation of other signaling pathways are observed (Figure 2) . Numerous networks that control normal hemopoesis, including RAF/MEK/ERK, JAK/STAT or PI3K/AKT/NFKB, are deregulated [50]. Moreover, LSCs reactivate evolutionary conserved signaling pathways, such as Notch, Wnt or Hedgehog [51]. Last, but not least, LSCs interact with the hemopoietic niche in several ways [52]. On the one hand, LSCs induce microenvironmental reprogramming of the niche that may act as a sanctuary in which LSCs acquire a therapy-resistant phenotype [52]. On the other hand, mutations occurring in stromal cells may by themselves cause AML [53,54].

It is out of the scope and possibility of this review to discuss every facet of AML pathogenesis. Instead, we touch upon the limited and in our arbitrary opinion, most interesting and surprising findings concerning the processes and pathways involved in AML.

Epithelial-to-mesenchymal transition program may play a role in leukemogenesis

Epithelial-to-mesenchymal transition (EMT) is not a single pathway, but a process through which epithelial cells lose cell-to-cell attachment and acquire a motile phenotype that allows them to move to and colonize distant sites. The loss of E-cadherin is considered to be fundamental for EMT. TFs that repress E-cadherin directly or indirectly are considered EMT inducing. SNAI1, SNAI2, ZEB1, ZEB2, TCF3 and KLF8 transcriptionally repress E-cadherin by binding to its promoter, whereas factors such as Twist, GSC, TCF4, SIX1 homeobox protein and FOXC2 TF repress E-cadherin indirectly [55]. EMT is physiologically relevant during organogenesis and to the progression of solid tumors [56]. Until now, it seemed counterintuitive to study EMT in the context of AML cells that are intrinsically endowed with migratory properties. However, induction of EMT also generates cells with stem cell properties [57]. To date, a possible involvement of the EMT program in hematological tumor generation has not been sought directly. Nonetheless, altered expression of some of its modulators has been described [58]. For example, Li *et al.* identified ZEB2 EMT-inducing TF as a novel AML dependency through an RNAi screen [59]. ZEB2 depletion impaired proliferation of human and mouse AML cells, and led to aberrant differentiation of human AML cells through transcriptional repression of myeloid differentiation and cell adhesion and migration genes [59]. Another study showed that knockdown of ZEB1, a further EMT-inducing TF, dramatically reduced leukemic blast invasion in an MLL/AF9-driven leukemia model [60]. The authors identified several EMT-related genes significantly associated with poor overall survival in AML patients [60]. In our hands, the expression of AML1/ETO fusion protein in EML C1 murine hematopoietic stem/progenitor cell line and in primary murine stem/progenitor cells, led to an upregulation of three different EMT signatures. In particular, AML1/ETO downregulated E-cadherin and upregulated Zeb2 causing a phenotype of decreased adhesion and increased motility reminiscent of EMT [61].

Taken together, the findings may suggest that the machinery involved in EMT is deployed to achieve a more mobile and less adherent phenotype of leukemic cells. Data obtained by Stavropoulou *et al.* suggest an instinctive connection between EMT induction and aggressive forms of AML with extensive liver and lung infiltration [60,62].

Wnt activation in AML

Wnt pathway is an evolutionary conserved signaling pathway. It plays a pivotal role in HSC maintenance [63], in differentiation of blood cells and its constitutive activation is a common feature of AML [64–66]. In particular, canonical Wnt signaling is known to be active in AML expressing specific oncogenes such as MLL/AF9, AML1/ETO and PML/RARA [66–68]. In mouse models of AML induced either by co-expression of the Hoxa9 and Meis1a oncogenes or by the MLL/AF9 fusion oncoprotein it has been shown that Wnt signaling is specifically required for the self-renewal of LSCs [68]. Curiously, AML can be induced by an activating mutation in the β-catenin gene, the major Wnt signal transducer, in osteoblasts [53].

Two recent studies extend the findings regarding Wnt signaling activation in AML. We have recently reported that canonical Wnt signaling is active in the patient-derived OCI-AML3 cell line expressing a leukemogenic cytoplasmic mutant of the NPM1 (NPMc+) protein and in primary AML-NPMc+ patients' blasts. Furthermore, ectopic expression of NPMc+ activates canonical Wnt signaling during zebrafish development [69].

Lazzaroni *et al.* [70] reported a novel mechanism for Wnt activation in AML via a genetic rearrangement in blast cells involving intron 1 (IVS1) of the WNT10B locus flanked at the 5′ end by nonhuman DNA. The resulting WNT10B^{IVS1} transcript is expressed mainly in intermediate/unfavorable risk AML patients. Blasts from

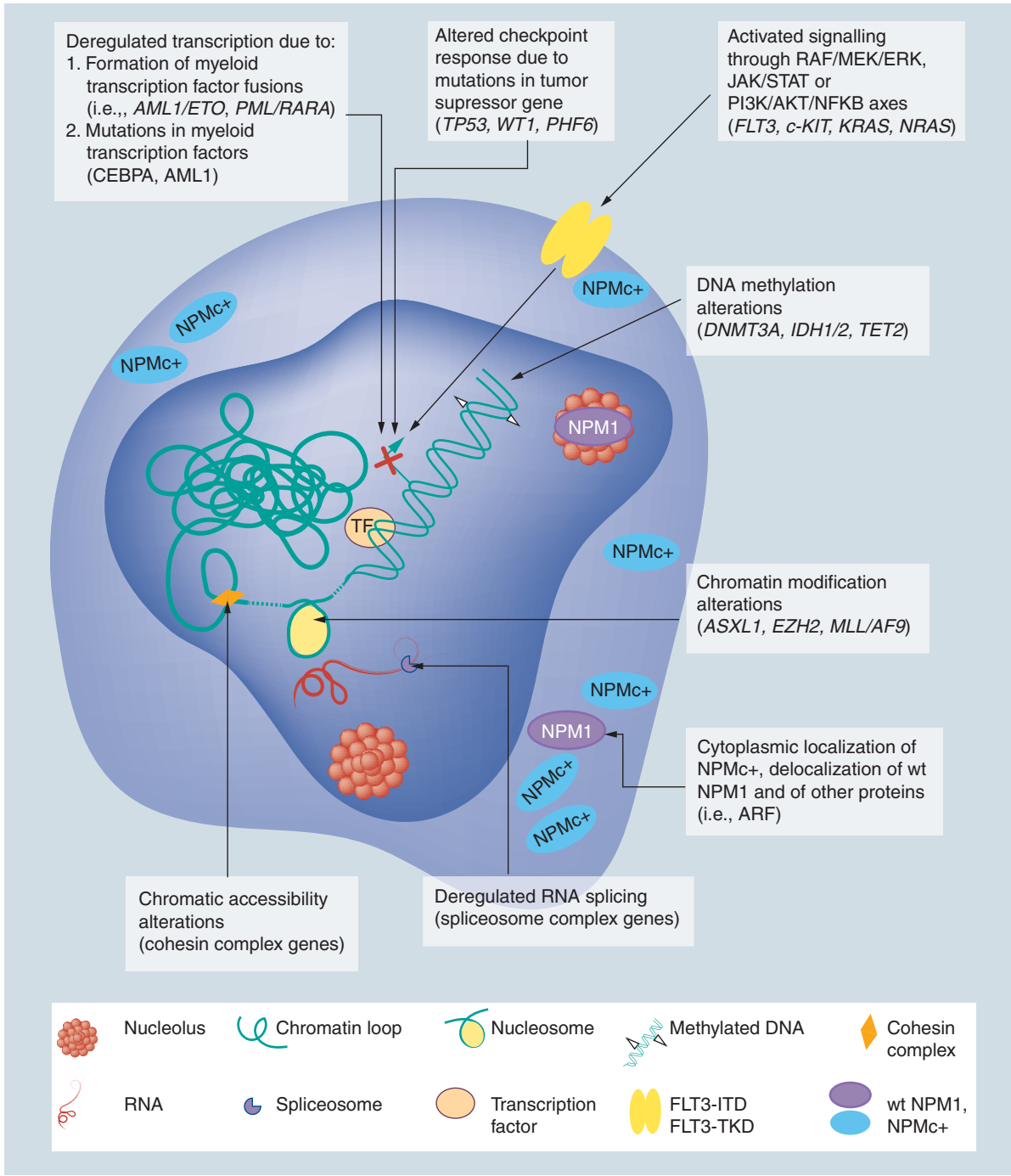

Figure 2. The molecular consequences of common acute myeloid leukemia mutations.
Mutations in myeloid transcription factor (TF) and TF fusions caused by chromosomal rearrangements such as translocations lead to transcriptional deregulation and impaired hematopoietic differentiation (top left). Mutations in tumor suppressor genes influence the transcription and checkpoint responses of the cell (top center). Mutations in signaling genes (e.g., *FLT3* receptor) confer a proliferative advantage through the RAF/MEK/ERK, JAK/STAT and PI3K/AKT/NFKB signaling axes (top right). *DNMT3A*, *IDH1/2* and *TET2* mutations acting through the 2-hydroxyglutarate oncometabolite production deregulate DNA methylation (top middle). Mutations in genes responsible for the cellular epigenetic regulation (e.g., *ASXL1* and *EZH2*) lead to alterations in chromatin modification (H3 and H2A histone methylation on K79, K27 and K119 lysine residues, respectively), while *MLL/AF9* fusion gene through aberrant methylation upregulate HOX gene expression and thus expands stem cells and blocks differentiation (bottom middle). Mutations of *NPM1* gene, encoding a multifunctional shuttling protein, result in the formation of cytoplasmic mutant of NPM1 known as NPMc+ and cause delocalization of NPM1-interacting proteins, influence ribosome biogenesis and TP53 stability (bottom right). Mutations in spliceosome complex genes (*SRSF2*, *SF3B1* and *U2AF1*) are involved in deregulated RNA processing including intron retention (bottom center). Cohesin complex gene mutations, in other words, *SMC1A*, *SMC3*, *STAG2* and *RAD21*, trigger increased chromatin accessibility and enhanced binding of AML1 and GATA2 TFs enforcing stem cell programs (bottom left).

another patient carried a genomic transposable short form of human WNT10B (ht-WNT10B), possibly involved in a nonrandom microhomology-mediated recombination generating $WNT10B^{IVS1}$. Interestingly, the expression of WNT10B in zebrafish embryos promotes the accumulation of hemapoietic precursors [70]. Incidentally, Wnt signaling is one of the multiple pathways involved in the execution of the EMT program [71].

The issue of differentiation block

Lack of appropriate myeloid maturation is a key feature of AML. A recent study proposes that myeloid differentiation constitutes a requirement for AML initiation. LSCs often share features of lineage-restricted progenitors and the relative contribution of differentiation status to LSC transformation is unclear. Using MLL/AF9 and MOZ/TIF2 murine AML models, Ye *et al.* [72] showed that myeloid differentiation to granulocyte macrophage progenitors is crucial for LSC generation. Disrupting granulocyte macrophage progenitor formation by deleting the CEBPA lineage-restricted TF blocked normal granulocyte production and prevented AML formation. However, restoring myeloid differentiation in CEBPA mutants using inflammatory cytokines re-established AML transformation capacity [72].

Using Hox9a AML model system, Sykes *et al.* [73] performed high-throughput phenotypic screen and identified dihydroorotate dehydrogenase enzyme as a target in AML. The inhibition of this metabolic enzyme involved in pyrimidine synthesis caused differentiation of leukemic cells. Importantly, inhibitors of dihydroorotate dehydrogenase showed therapeutic potential in a range of murine and xenotransplant AML models, independently of oncogenic driver [73]. Clearly, both findings have a potential of being exploited in new therapeutic approaches.

Where are we now: major lines of research

Despite major progress in understanding the biology of AML, a high relapse rate is still a foremost problem in clinical hematology. The progress has been made both in the fields of the basic biology and the translation of molecular findings into better patient care [74,75]. For example, the discovery of mutations in genes such as *IDH* and *FLT3* gave rise to the development and implementation of specific inhibitors [76,77]. Indeed, CALGB 10603/RATIFY study provided the first evidence for a survival advantage attributable to therapy with the midostaurin FLT3-inhibitor [78]. Still, more work has to be done to improve the survival of AML patients.

High-throughput identification of novel so-called 'druggable' targets in AML represents a possible way of approaching the quest for innovative treatment modalities. Until recently, RNA interference loss-of-function screens in combination or not with chemotherapeutic agents were commonly used to study tumor vulnerabilities [79–82]. These studies identified Brd4 [79], WEE1 [80], GSK-3a [81] and ROCK1 [82] as potential targets. More recently, Tzelepis *et al.* [83] optimized a genome-wide clustered regularly interspaced short palindromic repeats screening platform. A total of 492 AML-specific cell-essential genes were identified including several established therapeutic targets such as DOT1L, BCL2 and MEN1 [83]. Accordingly, early development of inhibitors targeting DOT1L, BET, WEE1, CDK and others has been undertaken [84].

Evidently, the success of targeted treatments depends on the presence of the very target in any individual AML. Mutational profiling has to be implemented on a large scale into clinical practice allowing for assigning tailored therapeutic schemes for any given patient. Integrated genomic analysis is also needed for more precise evaluation of prognosis.

Current AML research focuses on elderly patients, since AML incidence peaks at around 65 years of age. There are fewer therapeutic options for this group of frequently frail patients with comorbidities. At the same time, age should not be regarded as an obstacle for treating fit elderly patients with the modalities reserved for the group of young patients. Novel agents alone or in combination with low-dose standard cytotoxic drugs are being tested [85].

Conclusion

Although objectively, the overall outcome of AML treatment is still poor, identification/design of new, smart agents and clinical trials of the ones already identified are under way and should drastically change the prognosis of the disease in the near future. Continued research into the biology and therapy of AML is essential.

Future perspective

In the years to come the diagnosis of AML will have to be comprehensive of detailed molecular analysis. Any patient will be assigned to a genetic subgroup and consequently to an appropriate risk group and will receive personalized treatment to better match the mutations present in the AML blasts.

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