AML1/ETO and its function as a regulator of gene transcription via epigenetic mechanisms

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INTRODUCTION

Acute myeloid leukemia (AML) has long served as a prime model for our understanding of the initiation and propagation of cancer [1]. This is a reflection of the diagnostic accessibility of leukemic cells, the long tradition of implementing routine cytogenetics into the diagnostic workup, as well as the low number of mutations that drive the disease process compared to other cancer entities [2]. The last decade has yielded astonishing progress on dissecting the genetic landscape that lies at the root of AML [3]. Critical advances in the understanding of the molecular mechanisms driven by specific genetic lesions have resulted in key therapeutic advances, such as the FDA approval of FLT3- and IDH1/2-directed therapies [4, 5]. Among the different genetic and biological subtypes of AML, the disease entity with the translocation (8;21) (q22;q22) maintains a prominent position. The functional role of the resultant oncofusion protein AML1/ETO (also termed RUNX1/RUNX1T1) has been studied since its discovery in the early 1990s, and yet new aspects of its function continue to emerge. This is especially relevant since—in contrast to the PML/RARA oncofusion found in APL—a specific, biologically driven treatment approach resulting in a high rate of cure, remains elusive for this AML subtype.

The translocation was the first balanced chromosomal translocation ever described in leukemia or any other cancer [6], and AML harboring t(8;21) constitutes one of the most frequent recurring genetic subtypes of AML [3], especially in childhood AML [7]. It serves as a unique example of how one cytogenetic abnormality can define a distinct leukemia entity: t(8;21) leukemia is associated with a distinct morphology (i.e., relatively large blasts with a basophilic cytoplasm, azurophilic granules, and perinuclear clearing, presence of Auer rods), immunophenotype (i.e., frequent aberrant expression of CD19, PAX5, and CD56) and recurrent cooperating mutations including KIT, FLT3, NRAS, and both ASXL1 and ASXL2 [8]. The AML1/ETO fusion represents one of the first fusion genes employed for minimal residual disease monitoring [9]. Together with AML with inversion (16) or translocation (16;16), this “Core-Binding Factor” AML displays significantly better outcomes with standard chemotherapy followed by high-dose cytarabine consolidation than most other AML subtypes. Despite a cure rate of 60% or higher (including allografting in patients that relapsed after standard chemotherapy) in patients 60 years and younger, the relapse rate and outcome are still strikingly inferior to APL, particularly in elderly patients who are not candidates for standard chemotherapy due to significant comorbidities. The conundrum of a tantalizingly growing understanding of the functional AML1/ETO and the lack of real improvements in outcome (with the exception of continuously improved outcomes in allogeneic stem cell transplantation) continues to drive basic research of t(8;21) leukemia.

In this review, we will focus on recent results highlighting the role of AML1/ETO as an epigenetic modifier, which provides a strong rationale to treat the disease with so-called epigenetically active agents, and we will emphasize recent findings on cooperating oncogenes that can be targeted by kinase inhibitors.
Outlining the sequelae of AML1/ETO-mediated epigenetic dysregulation becomes all the more difficult to target the function of aberrant transcription factors directly.

AML1 (RUNX1), ETO (RUNX1T1), and AML1/ETO: structure and functions

The transcription factor AML1 (RUNX1) represents a crucial regulator of physiologic hematopoietic differentiation [10] and is recurrently mutated in a wide variety of hematologic malignancies [11]. Together with other lineage-specifying transcription factors including members of the ETS and GATA family, AML1 coordinates definitive hematopoiesis in a spatial and temporal manner [12]. The full-length protein is comprised of a N-terminal Runt-homology domain responsible for DNA-binding to target gene promoters and nuclear interaction with the common heterodimeric partner Core-Binding Factor β (CBFβ), as well as a C-terminal transactivation domain (TAD) that consists of a number of activating and inhibitory domains, such as the Ets1 interacting domain (EID) (Fig. 1A, B). The EID domain facilitates protein–protein interactions, including with the histone acetyltransferases P300 and CREBBP (CBP). An adjacent inhibitory domain of AML1, located towards the C-terminus of the activation domain, acts to limit the interaction between AML1 and transcription factors such as ALY and YAP [13, 14].

Endogenous ETO (MTG8, RUNX1T1) encodes a Zinc-binding protein also named CBFA2T1, a nuclear protein which functions as a transcriptional co-repressor through its close association with transcription factors and by recruiting other corepressors and histone deacetylases. The ETO protein domain structure consists of four highly conserved functional domains termed Nervy Homology domains 1–4 (NHR 1–4): TATA-binding protein-associated factor homology domain (eTAFH = NHR1), the hydrophobic heptad repeat domain (HHR = NHR2), an α-helical domain (Nervy = NHR3), and the myeloid-Nervy-DEAF1 domain (MYND = NHR4). The AML1/ETO 9a variant contains only the NHR1 and NHR2 functional domains, while the AML1(exon 6)/ETO variant contains 64 additional amino acids downstream of the Runt domain with yet unknown functional consequences. mRNA splice variants identified in t(8;21) leukemia include the canonical AML1/ETO, the oncogenic AML1/ETO9a, and other rare variants such as AML1(exon 6)/ETO. C The AML1(exon 6)/ETO variant observes a novel breakpoint between AML1 exon 6 and ETO exon 2. Double-stranded synthetic DNA fragments can be utilized to clone novel AML1/ETO splice variants into the retroviral MSCV-IRES-GFP overexpression construct (ref. [21]) by utilizing intrinsic restriction enzyme sites. D RT-PCR using exon-specific and exon-junction-spanning primers for the AML1 exon 6 splice event following retroviral transfection of 293T cells with the novel AE/AE6 and AE9a/A6 constructs, as well as the previously published AE and AE9a constructs and a no transfection control (Empty). AE = AML1/ETO, AE9a = AML1/ETO9a variant, AE/A6: AML1(exon 6)/ETO variant, AE9a/A6 = AML1(exon 6)/ETO9a variant. This figure includes original work (see “Acknowledgements”).

Fig. 1 Schematic representation of AML1 (RUNX1), ETO (RUNX1T1), and AML1/ETO—structure and function. A The Runt DNA-binding domain of AML1 and almost the whole co-repressor gene ETO are conserved in the fusion gene including its four functional domains termed Nervy Homology domains 1–4 (NHR 1–4): TATA-binding protein-associated factor homology domain (eTAFH = NHR1), the hydrophobic heptad repeat domain (HHR = NHR2), an α-helical domain (Nervy = NHR3), and the myeloid-Nervy-DEAF1 domain (MYND = NHR4). The AML1/ETO 9a variant contains only the NHR1 and NHR2 functional domains, while the AML1(exon 6)/ETO variant contains 64 additional amino acids downstream of the Runt domain with yet unknown functional consequences. B mRNA splice variants identified in t(8;21) leukemia include the canonical AML1/ETO, the oncogenic AML1/ETO9a, and other rare variants such as AML1(exon 6)/ETO. C The AML1(exon 6)/ETO variant observes a novel breakpoint between AML1 exon 6 and ETO exon 2. Double-stranded synthetic DNA fragments can be utilized to clone novel AML1/ETO splice variants into the retroviral MSCV-IRES-GFP overexpression construct (ref. [21]) by utilizing intrinsic restriction enzyme sites. D RT-PCR using exon-specific and exon-junction-spanning primers for the AML1 exon 6 splice event following retroviral transfection of 293T cells with the novel AE/AE6 and AE9a/A6 constructs, as well as the previously published AE and AE9a constructs and a no transfection control (Empty). AE = AML1/ETO, AE9a = AML1/ETO9a variant, AE/A6: AML1(exon 6)/ETO variant, AE9a/A6 = AML1(exon 6)/ETO9a variant. This figure includes original work (see “Acknowledgements”).
translocation represents the most common source of AML1/ETO fusions, rare variants such as inversions (i.e., inv(8)(q22q24)) and insertions (i.e., ins(21q:8) and ins(8;21)) involving the derivative chromosome 8 have been described. Next to t(8;21), more than 50 chromosomal translocations have been attributed to AML1 [22], illustrating its far-reaching role in tumorigenesis. This includes t(12;21), which generates the TEL/AML1 (ETV6/RUNX1) fusion gene product and represents the most common chromosomal translocation in childhood acute lymphoblastic leukemia (ALL). Interestingly, the TEL/AML1 and AML1/ETO fusions can be traced in Guthrie cards in healthy neonates and can be detected prenatally, supporting prenatal initiation and a two-hit model of leukemia inception [23]. Another prominent example is the AML1/MDS/EVI1 (RUNX1/MECOM) fusion mediated by the t(3;21), which is recurrently found in patients with therapy-related MDS and AML.

**AML1/ETO splice variants observe differential leukemic potential**

Importantly, the resultant full-length AML1/ETO fusion protein (752 amino acids = aa) itself is not sufficient to drive leukemogenesis, but rather provides a crucial first hit. Early conditional knock-in mouse models demonstrated that the full-length fusion requires additional mutagenic events to induce leukemia on its own. While transgenic mice expressing only the full-length AML1/ETO fusion did not develop leukemia, exposure to the DNA alkylating agent ENU (also known as N-ethyl-N-nitrosourea) resulted in the rapid development of a malignant state that mimicked the morphologic cues found in t(8;21) leukemia [24]. In a seminal study, Yan et al. found that one mouse, transplanted with AML1/ETO-transduced bone marrow cells, developed leukemia even in the absence of mutagenic stress [25]. Sequencing of leukemic cells in this mouse revealed a 1-bp insertion that leads to a C-terminally truncated form of AML1/ETO lacking 200 amino acids (552 aa) in a domain critical for the NCoR/SMRT and ETO interaction. A transcriptional isoform harboring an additional exon, termed exon 9a, of ETO was discovered to result in a similarly truncated AML1/ETO protein [26]. This alternatively spliced isoform called AML1/ETO9a (572 aa) was recurrently found in a multitude of t(8;21) AML samples [27]. Moreover, co-expression of both the full-length and C-terminally truncated AML1/ETO fusion proteins facilitated a substantially earlier onset of leukemia and blocked myeloid differentiation at an earlier stage [27]. These early studies shed light on how fusion proteins arising from alternatively spliced isoforms secondary to a chromosomal translocation can act in concert to drive the development of cancer.

Sequencing of t(8;21) primary AML samples has revealed a plethora of in-frame and out-of-frame transcript variants arising as a result of alternative splicing. For example, one ETO variant containing an additional exon 11a produces a protein with an additional 27 amino acids in-frame instead of the MYND domain at the C-terminal region of the fusion protein. Identified in primary human t(8;21) AML cells, the MYND-less protein variant was associated with the formation of multimers and reduced transcriptional repressor activity [28]. Mannari et al. describe a transcript harboring an alternative exon 6a leading to a protein that only contains the NHR1 domain [29]. As this fusion protein did not exhibit clonogenic potential compared to the leukemogenic AML1/ETO9a fusion, which includes both the NHR1 and 2 domains, the authors conclude that the homo-oligomerization function conferred by the NHR2 domain likely plays a key role in promoting leukemogenesis. In pediatric t(8;21) AML, transcript variants containing multiple in-frame-deletions involving exons 2–5 of AML1 and exon 2 and 3 of ETO were identified, which displayed both activating and repressive effects on AML1-mediated GM-CSF transactivation [30].

While the natural breakpoint observed in t(8;21) leukemia produces an AML1 exon 5 to ETO exon 2 fusion, Solari et al. described a rare novel AML1/ETO fusion transcript tightly associated with BCR/ABL, wherein the breakpoint lies one intron downstream, resulting in a fusion transcript including AML1 exon 6 [21]. While the functional consequences of this novel fusion transcript remain to be explored, the association of AML1/ETO and BCR/ABL in cases of therapy-refractory CML is especially intriguing, as these genetic aberrations can coexist together in vivo. As whole-genome sequencing approaches become more widespread in diagnosing the genetic landscape of AML [31], more such rare AML1/ETO variants may be identified. Detailed sequencing enables the design of synthetic DNA fragments, which can be utilized to modify established retroviral constructs using AML1/ETO intrinsic restriction enzyme sites. In our laboratory, we have leveraged these new technologies to clone and express the previously described AML1(exon 6)/ETO variant (Fig. 1C, D). These constructs allow variant-specific characterization of AML1/ETO function.

Finally, the presence of spliceosomal mutations in myeloid malignancies has been demonstrated to impact the alternative splicing of the terminal exon of AML1 [32], and splicing changes related to exon 6 of AML1 differentially regulate hematopoiesis in mice [33]. Moreover, recent work points toward AML1/ETO itself being a potential regulator of alternative splicing, adding a novel layer of transcriptome organization in t(8;21) leukemia [34].

**AML1/ETO undergoes posttranslational modifications controlling its function**

Posttranslational modifications regulate protein–protein interaction and the functional activity of transcription and thus play an important role in oncogenesis [35]. In a seminal work, it was reported that the histone acetyltransferase P300 acetylates AML1/ETO at lysine 43 (Lys43), thus enhancing AML1/ETO activating functions and self-renewal activity of hematopoietic progenitor cells. Treatment with P300 inhibitors decreases AML1/ETO acetylation, leading to a blockage of AML progression [36]. Furthermore, a recent study demonstrated that AML1/ETO can increase CD48 expression via AML1-ETO/P300-mediated acetylation. CD48, a member of the SLAM family, plays an important role in regulating natural killer (NK) cell-mediated immunosurveillance. By increasing CD48 expression levels, AML1/ETO can inhibit AML immune escape from NK cell recognition and killing [37].

Recently, the interaction between the histone methyltransferase EZH1 with AML1/ETO was shown. EZH1, which is part of the Polycomb repressive complex 2 (PRC2), methylates Lys43 on the NHR1 domain in AML1/ETO, thus enhancing its repressive function on tumor suppressor genes. Hence, loss of Lys43 methylation by point mutation or domain deletion impairs AML1/ETO-repressive activity [38]. These data suggest that P300 and EZH1 compete for binding and modification of Lys43 (acetylation and methylation), which confer opposite functions in AML1/ETO-mediated transcriptional regulation. The protein arginine methyltransferase 1 (PRMT1) has also been shown to interact directly with AML1/ETO-9a and to methylate the arginine residue at position 142 of the AML1/ETO9a variant. Through this interaction, PRMT1 is recruited to AML1/ETO target promoters and methylates H4R3, which enhanced transcriptional activation [39]. Of note, PRMT1 has been shown to interact with the N-terminus of AML1, thereby enhancing its transcriptional activity by inhibiting the interaction with SIN3a [40], suggesting a key role in physiological gene activation of both AML1 and the concomitant AML1/ETO oncofusion protein.

**AML1/ETO recruits multiple chromatin-modifying enzymes to target genes**

A multi-protein complex is recruited by AML1/ETO to target genes, thus epigenetically modifying chromatin and regulating gene transcription. ETO recruits a nuclear co-repressor complex containing HDACs (histone deacetylases) 1–3 via its interaction with NCoR and SIN3A to the promoters of its target genes, acting as a
Inhibitors reversed not only acetylation of H3, H4, and H3K9 but di- and trimethylation of H3K9 and reversed silencing marks, particularly H3K27me3, only in AML1/ETO-expressing cells. Chromatin modifications were found on the LAT2 promoter and introns, a target gene of AML1-chimeric fusion protein complex creating novel binding sites [36]. AML1/ETO interacts with P300 and CBP, promoting histone acetyltransferase activity to its target genes [38]. These data suggest a functional interaction between AML1/ETO and the PR2C, which is also able to recruit DNMTs [33].

AML1/ETO is not only able to act as a repressor but also as an activator of target genes, through its interaction with transcriptional activators (Table 1). AML1/ETO interacts with P300, recruiting the histone acetyltransferase to its target genes. Of note, an increase of histone acetylation was detected in genes activated by AML1/ETO, but not by repressed genes, suggesting recruitment of AML1/ETO-P300 complex to specific genes [36]. Similarly, PRMT1 is recruited by AML1/ETO to its target genes, thus increasing H4R3 methylation on target promoters and activating gene transcription [39]. Therefore, AML1/ETO might behave as an adaptor protein inducing transcriptional stimulation or repression depending on the activated signaling pathways in leukemia cells.

In summary, accumulating evidence implicates AML1/ETO as a highly important epigenetic modifier similar to PML-RARα [54], activating and repressing gene transcription depending on the context of the interacting chromatin-modifying enzymes. These data support a novel mechanistic rationale encouraging the use of epigenetically active drugs such as HDAC and DNMT inhibitors, which are already in clinical use, to treat patients harboring the t(8;21) fusion. Future investigations could utilize compounds that specifically target transcriptional co-activators recruited by AML1/ETO, such as PRMT1 and P300, as a means of exploiting a vulnerability intrinsic to this leukemia subtype.

**Global changes in chromatin modifications, transcription factor binding, and gene expression mediated by AML1/ETO**

A number of recent studies have performed global analyses on the ability of AML1/ETO to reorganize the chromatin and transcription factor binding landscape of human hematopoietic cells (see Table 1). Both AML1 and AML1/ETO localize in a multi-protein complex interacting with other transcription factors that together regulate differentiation of hematopoietic cells and leukemic blasts. The overexpression and depletion of single transcription factors redistributes the localization of this multi-protein complex creating novel binding sites [35]. AML1/ETO interacts with, and mutually stabilizes, CBFB, E proteins like HEB and E2A, E-box-binding transcription factor LYL1, as well as LMO2 and its interacting partner LDB1 in a so-called AML1/ETO-containing transcription factor complex [55]. AML1/ETO competes for the same binding sites as AML1 and C/EBPα. Importantly, AML1/ETO negatively regulates the expression of C/EBPα by inhibiting positive autoregulation of the C/EBPα promoter [56, 57]. As a result, the selective depletion of AML1/ETO results in upregulation of C/EBPα and together with AML1 restores the differentiation-associated transcriptional program of leukemic cells through regulatory elements previously occupied by AML1/ETO [58]. Classically upregulated genes identified in t(8;21) leukemia include p21/Cdkn1a [59, 60], Sox4, IL-17B, CD200, and γ-catenin [61], and cytokine receptors like CSF3R [62]. Downregulated genes include cytokines such as IL-6 [46] and CSF2 [63], transcription factors such as C/EBPα [57] and proteins involved in cell cycle regulation like CKN2A [64] (Table 2).

The chimeric fusion protein AML1/ETO not only modifies chromatin marks locally, as previously described on its target genes IL-3 and LAT2, but also genome-wide. The effected gene expression and chromatin landscape is distinct from other oncogenic fusions harboring RUNX1 such as RUNX1-EVI1 [65]. Twenty-six (ETS) family transcription factors such as ERG and FLI1 guide and facilitate genome-wide binding of AML1/ETO as demonstrated in human cell lines and primary leukemic blasts. Binding of AML1/ETO to most ERG sites decreases acetylation of also trimethylation of H3K4, suggesting an interplay of inactivating and activating histone-modifying enzymes on target genes by AML1/ETO or by HDAC inhibitors [52]. Moreover, AML1/ETO physically interacts with the PRC2 component EZH1, recruiting histone methyltransferase activity to its target genes [38]. These data suggest a functional interaction between AML1/ETO and the PRC2, which is also able to recruit DNMTs [33].

**Fig. 2 The AML1/ETO oncoprotein fusion but not wild type AML1/RUNX1 recruits a repressor complex.** The hematopoietic transcription factor AML1/RUNX1 binds the consensus sequence TTGTTGT on the promoter of its target genes. DNA binding is stabilized by the interaction with CBFB. AML1 recruits the histone acetyltransferases p300 and CBP. The histone acetyltransferases acetylates lysine residues on the histones of its target genes, which induce an open chromatin and activates gene transcription. However, AML1/ETO interacts with NCOR and mSin3, which recruit class I histone deacetyltransferases (HDACs) 1–3. HDACS1–3 deacetylates the lysine residues of histone tails, which change to a closed chromatin conformation resulting in the repression of transcription of target genes. Some evidence supports that DNA methyltransferases (DNMTs) and the polycomb repressor complex 2 (PRC2) including the H3K27 trimethylase EZH2 are directly or indirectly recruited to AML1/ETO target genes.
| Chromatin marks | Transcription factors | Method | Cell type | Reference |
|-----------------|----------------------|--------|-----------|-----------|
| Acetylation     |                      |        |           |           |
| H3K9ac, H3K14ac, H4panAc | ERG, FLI1, CBFβ, HEB, RUNX1, ETO, AML1-ETO, RNAPII | ChIP-Seq | Cell lines, patient t(8;21) AML blasts and normal CD34+ hematopoietic cells | Martens et al. [66] |
| H3ac | AML1/ETO, AML1, LMO2, Pol II, Pu.I, C/EBPα, HDAC2, P300 | ChIP-Seq DNAseI Footprinting | Cell lines, primary cells, normal. CD34+ | Ptasinska et al. [58] |
| H3K9ac | AML1/ETO, AML1, Pol II | ChIP-Seq DNAse I hypersensitivity | Primary cells and cell lines | Ptasinska et al. [67] |
| H3K27ac | AML1/ETO | ChIP-Seq DNAse I hypersensitivity | Cell lines, primary AML cells, AML1/ETO-transduced iPSCs | Mandoli et al. [70] |
| H2A.Zac | AML1/ETO, P300 | Nuclease accessibility coupled with high-throughput sequencing (NA-seq) and ChIP-seq | Cell lines and primary patient blasts | Saeed et al. [69] |
| K43ac | AML1/ETO, P300 | ChIP-Seq | Cell lines | Wang et al. [36] |
| H3K9/14ac | AML1/ETO9a, PRMT1 | ChIP-qPCR | Cell lines | Shia et al. [39] |
| H4ac loss | AML1/ETO, SP1 | ChIP-chip analysis | Transduced human HSPCs | Maiques-Diaz [118] |
| Methylation |                      |        |           |           |
| H3K4me3, H3K4me1, H3K36me3, H3K27me3, H3K9me3 | AML1/ETO | ChIP-seq, DNA hypersensitivity | Cell lines, primary AML cells, AML1/ETO-transduced iPSCs | Mandoli et al. [70] |
| H3K4me3, H3K27me3 | AML1, NCoR, P300 | ChIP-Seq | Cell lines | Trombly et al. [72] |
| H4R3me2a | AML1/ETO9a, PRMT1 | ChIP-qPCR | Cell lines | Shia et al. [39] |
| H3K4me3 | AML1, AML1/ETO and HEB | ChIP-qPCR | Cell lines | Gardini et al. [73] |
| K43me | AML1/ETO, EZH1 | ChIP-Seq | Cell lines | Dou et al. [38] |

The chromatin marks in Wang and Dou et al. refer to site-specific lysine acetylation/methylation of the respective target protein.
histone H3, H4, and of the specific residues H3K9 and H3K14, correlating with decreased gene expression [66]. AML1/ETO induces profound genome-wide changes and global gene transcriptional reprogramming by decreasing acetylation of H3K9 and RNA polymerase II (RNApol II) promoter occupancy. Interestingly, these epigenetic alterations are reversible at a global scale when AML1/ETO expression is altered, suggesting that targeting either function or expression of the fusion protein may represent a feasible therapeutic approach [58, 67]. Ptasinska et al. recently illustrated the importance of AML1/ETO expression levels, demonstrating that knockdown results in extensive changes in transcription factor binding and gene expression, and specifically to C/EBPα and AP-1 mediated alterations in promoter–enhancer interactions [68]. In sum, a multitude of groups have associated AML1/ETO with the recruitment of transcription factors and chromatin-modifying enzymes and consequently, genome-wide histone modifications, supporting the general role of AML1/ETO as an important epigenetic modifier in leukemia [69–73].

**Table 2.** Selected, clinically validated target genes of AML1/ETO (identified with the use of unbiased screening approaches).

| Target gene | Function | Biological process | Reference |
|-------------|----------|--------------------|-----------|
| **Upregulated** | | | |
| SOX4, IL-17RB, CD200, and JUP (γ-catenin) | Transcription factor, cytokine receptor, anti-inflammatory signal, Adherens junction | Transcriptional regulation, inflammation, cell adhesion | Tonks et al. [61] |
| p21<sup>WAF1</sup> (CDKN1A) | Cyclin-dependent kinase inhibitor | Cell cycle, stem cell maintenance | Berg et al. [59], Peterson et al. [60] |
| BCL2<sup>a</sup> | Anti-apoptotic signal | Apoptosis | Martens et al. [66], Klampfer et al. [119] |
| FOXO1 | Transcription factor | Apoptosis, stem cell maintenance | Lin et al. [120] |
| GFI1 | Transcriptional repressor | Transcriptional regulation, G<sub>i</sub>/S-transition, oncogene | Mameth et al. [121] |
| TRKA (NTRK1) | MAPK pathway activation, protein kinase | Neuronal development, myeloid differentiation | Mulloy et al. [122] |
| ZFP36L1 (ERF-1, TIS11b) | Polypeptide chain release factor | mRNA translation | Shimada et al. [123] |
| ARG2, MT2A | Arginine metabolism, metal homeostasis | Immune response, oxidative Stress | Shia et al. [39] |
| CD48 | NK-cell mediated immunosurveillance | Adaptive immune response, leukocyte migration | Wang et al. [37] |
| CSF3R | Cytokine receptor | Regulation of hematopoiesis | Shimizu et al. [62] |
| PAX5 | Transcription factor | B-cell maturation | Ticiac et al. [124] |
| POU4F1 | Transcription factor | Transcriptional regulation, B-lymphoid expression | Fortier et al. [125], Dunne et al. [126] |
| VLA4 (ITGA4) | Cell adhesion and migration | Leukocyte trafficking, Regulation of hematopoiesis | Ponnusamy et al. [127] |
| **Downregulated** | | | |
| IL-3 | Cytokine | Regulation of hematopoiesis | Buchi et al. [46] |
| CSF2 | Cytokine | Regulation of hematopoiesis | Frank et al. [63] |
| CCL3 | Chemokine ligand | Chemotaxis, immune response | Bristow and Shore [128] |
| CEBPA | Transcription factor | Regulation of hematopoiesis | Koschmieder et al. [56], Pabst et al. [57] |
| LAT2 | Adaptor molecule | Regulation of hematopoiesis | Fleglauf et al. [47], Duque-Afonso et al. [49, 52]; Essig [50] |
| p14<sup>ARF</sup> (CDKN2A)<sup>b</sup> | Cyclin-dependent kinase inhibitor | Cell cycle, G<sub>i</sub>/S-transition, stem cell maintenance | Linggi et al. [64] |
| RASSF2 | K-RAS-specific effector protein | Rac GTPase activation, Rac-mediated signal transduction | Stoner et al. [129] |
| Lysozyme (LYZ) | Bacteriolytic enzyme | Antimicrobial humoral response, myeloid differentiation | Claus et al. [130] |
| OGG1 | DNA repair enzyme | Response to oxidative stress | Liddiard et al. [131] |
| PSGL1 (SELPGL) | Cell adhesion and migration | Leukocyte trafficking, regulation of hematopoiesis | Ponnusamy et al. [132] |
| NF1 | GTPase-activating protein | Ras signal transduction | Yang et al. [133] |
| miR 144/451 | Posttranscriptional regulation | Erythroid differentiation | Kohrs et al. [134] |
| SP1 (PU.1) | Transcription factor | Regulation of hematopoiesis | Vangala et al. [135] |

<sup>a</sup>Individual studies have demonstrated downregulation of BCL2 in leukemic cell lines [136].

<sup>b</sup>Referring to the alternate open reading frame (ARF) which does not function as a CDK4/6 inhibitor.
transcription factors and mediators of cell cycle progression. Dependency of AML1/ETO-driven leukemogenesis on other highlights potential therapeutic vulnerabilities exposed by the overexpression of the apoptosis program in t(8;21) AMLs [70]. Importantly, targeted AML1 knockdown of malignant phenotype [78]. Together these promising findings highlight potential therapeutic vulnerabilities exposed by the dependency of AML1/ETO-driven leukemogenesis on other transcription factors and mediators of cell cycle progression. Novel experimental techniques such as differentiation models utilizing induced pluripotent stem cells, are now being employed to further model AML1/ETO induced oncogenesis [79].

Cooperating oncogenic events in AML1/ETO positive AML
In the last decades, CBF leukemias have served as a crucial model for the two-hit hypothesis of leukemogenesis. Using unbiased approaches based on next-generation sequencing technologies, several mutations have now been identified in CBF leukemias that play a cooperative role in promoting leukaemia (Fig. 3) [8, 80]. Overall, at least one additional mutation was identified in 95% of t(8;21) patients, with a mean of 2.2 driver mutations per patient [81].

Approximately two-thirds of CBF leukemia cases harbor activating mutations in NRAS, KIT, FLT3, KRAS, PTPN11, and/or loss-of-function mutations in NF1 [80]. Importantly, the mutational load at diagnosis is prognostic, as patients with a higher burden of co-mutations have a significantly higher relapse rate with a trend towards inferior survival [82]. While NRAS represents the most frequently mutated gene in CBF leukemias—mutated in one-third of all patients—it is not associated with a worse clinical outcome. A relative preponderance of the NRAS mutation was observed in inv(16) compared to t(8;21) leukemia. Moreover, the spectrum of NRAS mutations differed between both CBF leukemias, and exon 61 mutations were more frequently observed in CBF/MYH11 AMLs (i.e., harboring inv(16) or t(16;16)).

Kit mutations are present in about 25% of CBF leukemias and are associated with inferior outcomes [81, 83]. The Kit exon 17 mutation is particularly enriched in AML1/ETO-positive leukemias. In a large study of CBFB/MYH11 AMLs, both the c-Kit exon 8 and FLT3-TKD mutations represented markers of poor prognosis [84]. Interestingly, AML1/ETO epigenetically trans-activates c-Kit expression by binding and then recruiting the histone acetyltransferase P300 to the c-Kit promoter [85, 86]. Activating c-Kit mutations confer oncogenic cooperativity by augmenting DNA repair and reverting apoptosis, offering a potential mechanistic explanation for the increased chemo-resistance observed in t(8;21) patients with cooperating c-Kit mutations [87]. Using Kit as a therapeutic target, addition of the multi-receptor tyrosine kinase dasatinib to conventional chemotherapy demonstrated a safe profile and promising efficacy in a phase Ib/IIa clinical trial [88].

Cooperating genetic lesions contribute to AML1/ETO-driven leukemogenesis. Gene mutations conferring a positive prognostic impact are highlighted in green, while gene mutations with a poor prognostic are indicated in red. Gene mutations with an equivocal prognostic impact are shown in gray. The relative incidence of the respective mutation is depicted in percent. Mutations occurring more frequently in t(8;21) CBF-AML are portrayed to the left, while mutations with a preponderance in inv(16)/t(16;16) CBF-AML are portrayed to the right.

Fig. 3 Cooperating genetic lesions contribute to AML1/ETO-driven leukemogenesis. Gene mutations conferring a positive prognostic impact are highlighted in green, while gene mutations with a poor prognostic are indicated in red. Gene mutations with an equivocal prognostic impact are shown in gray. The relative incidence of the respective mutation is depicted in percent. Mutations occurring more frequently in t(8;21) CBF-AML are portrayed to the left, while mutations with a preponderance in inv(16)/t(16;16) CBF-AML are portrayed to the right.

The role of AML1/ETO in leukemogenesis
But how do the global changes in the epigenetic network facilitated by the AML1/ETO oncoprotein contribute to leukemogenesis? Recently, Martinez-Soria et al. identified Cyclin D2 (CCND2) as a crucial transmitter of AML1/ETO-driven leukemic propagation, illustrating that AML1/ETO cooperates with AP-1 to drive CCND2 expression, resulting in G1 cell cycle progression and leukemic propagation. The authors demonstrated that pharmacologic inhibition of CCND2 impaired leukemic expansion in a murine model [76]. An unconventional oncogenic partner in the co-expression of MEIS2 with AML1/ETO induced AML in a patient-derived AML model [74]. A further interdependency was identified for TAF1. Not only does knockdown of TAF1 alter the association of AML1/ETO with chromatin, it is indeed required for leukemic cell self-renewal [75]. Moreover, reduction of TAF1 promoted differentiation and apoptosis of AML cells harboring the AML1/ETO fusion, implicating the transcription factor as a potential therapeutic target. Leukemic growth was also demonstrated to be dependent on the DNA-binding protein MEIS2, as the co-expression of MEIS2 with AML1/ETO induced AML in a murine model [76]. An unconventional oncogenic partner in AML1/ETO-driven leukemic growth was identified in Hif1α, a transcription factor critical for the cellular response to oxygen deprivation in malignant cells [77]. High Hif1α levels were correlated with increased AML1/ETO levels, and predicted inferior survival in t(8;21) AML patients.

Perturbations of the transcription factors AML1 and ERG prevent the overexpression of the AML1/ETO oncogene and the onset of the apoptosis program in t(8;21) AMLs [70]. Importantly, targeted knockdown of AML1 in AML1/ETO-positive cells decreases cell-cycling and induces apoptosis, suggesting that a fragile balance between AML1 and AML1/ETO must be maintained to sustain the malignant phenotype [78]. Together these promising findings highlight potential therapeutic vulnerabilities exposed by the dependency of AML1/ETO-driven leukemogenesis on other transcription factors and mediators of cell cycle progression. Novel experimental techniques such as differentiation models
Early in vivo studies suggest a mechanism wherein dasatinib may induce differentiation of t(8;21) AML blasts into neutrophilic granulocytes [89]. A promising recent report revealed that inhibition of mutated c-KIT using avapritinib in AML1/ETO-positive leukemia restored sensitivity to PARP inhibition via downregulation of BRCA1/2 [90].

Among epigenetic regulators, the polycomb-associated protein ASXL2 is mutated in about 20% of AML1/ETO-positive pediatric and adult patients [80, 91]. While recurrent gene mutations have a positive prognostic impact in children [92], the results were equivocal in adults [93]. In a murine model studying ASXL2 loss in the context of the AML1/ETO oncopfusion, ASXL2 functions as a haploinsufficient tumor suppressor when mice are challenged with either the full-length (AE) or short (AE9a) splice isoform of AML1/ETO [94]. Mechanistically, ASXL2, AML1 and AML1/ETO displayed an overlap in target gene expression. While global chromatin accessibility was not altered between conditions, a significant increase in chromatin accessibility at putative enhancers of key leukemogenic loci including HoxA and Meis1 was observed in mice lacking Asxl2 [94].

The transcription factor ZBTB7A is recurrently mutated in 6–23% of t(8;21) AML patients and is rarely found in CBFB/MYH11 AMLs [80, 95, 96]. To date, a prognostic impact of the mutation on clinical outcomes has not been demonstrated. The mutation disrupts the transcriptional repressor potential and anti-proliferative effect of ZBTB7A [96]. A potential mechanism includes the de-repression of glycolytic genes upon ZBTB7A deletion or mutation, which results in increased glycolysis, and thus provides more energy to the tumor cell [97, 98]. However, this addiction to glycolysis may be exploited therapeutically, as a recent study demonstrated that loss of ZBTB7A sensitized leukemic blasts to metabolic inhibition with 2-deoxy-D-glucose [99].

The mutation in the lysine 63-specific deubiquitinating enzyme BRCC3—found selectively in about 5% of t(8;21) AMLs and associated with excellent clinical outcomes—was recently functionally characterized [100]. BRCC3 mutations resulted in an impaired interferon response and diminished inflammasome activity. This may abrogate the strong activation of interferon signaling conferred by AML1/ETO, which has been demonstrated to negatively affect the leukemic potential of the oncopfuson [101]. On a cellular level, the inactivation of BRCC3 led to a higher sensitivity to doxorubicin due to an impaired DNA damage response, offering an explanation for the favorable outcomes of BRCC3 mutated AML patients. Other gene recurrently mutated in about 5% of CBF leukemia include the transcription factors WT1 and MGA, and the epigenetic regulators EZH2 and KDM6A [80]. Further mechanistic studies are required to delineate the functional implications of cooperating and competing mutations in the pathogenesis of AML1/ETO leukemia.

Conclusions and outlook

Despite more than two decades of preclinical and translational research on AML1/ETO, there still remain a number of open questions that need to be addressed in order to introduce novel therapeutic approaches into the treatment of patients with t(8;21) positive AML. We have learned that gene repression by AML1/ETO is mediated via HDAC activity. While early preclinical studies demonstrated antineoplastic activity for HDAC inhibitors [102, 103] and hypomethylating agents [104] in AML1/ETO expressing cells, the clinical use of HDAC inhibitors in AML has been disappointing to date [105–107]. These poor response rates do not support further development of this approach. Possibly, other, as yet unidentified chromatin-modifying enzymes are also involved in the pathogenesis of the disease, and drugs targeting these may yield superior results.

The “high-hanging fruit” remains direct disruption of the AML1/ETO recruited protein complex, though DNA-binding proteins have historically been difficult to target due to a lack of high-throughput screening methods [108]. While challenging, potential therapeutic strategies include exploiting the stability of the mutant oncoprotein either by targeting molecular chaperones (e.g., Calpain B or Hsp90), serine proteases (e.g., Cathepsin G), or via proteasome inhibitors like bortezomib [109–113]. The development of Runt domain inhibitors (RDIs), which disrupt CBF binding and function, represents a further promising approach [114]. Targeting the posttranslational modifications that control the function of the oncopfuson represents an alternative concept. For example, the site-specific acetylation of the NHR1 domain of ETO facilitated by P300 could be inhibited by means of RNA interference or chemical inhibition. Both in vivo and in vitro models demonstrated reduced levels of effector proteins required for cell renewal upon P300 inhibition, pointing towards P300 as an attractive drug target [36]. Recently, Yang et al. report the development of an oral P300/CBP histone acetyltransferase inhibitor using an artificial-intelligence-assisted drug discovery pipeline, which demonstrated efficacy in preclinical studies [115].

However, the broad landscape of cooperating genetic lesions makes it unlikely that directly targeting the AML1/ETO recruited multi-protein complex represents the panacea of CBF leukemia. Novel therapeutic strategies must navigate this individual genetic landscape, taking advantage of the interactive proteins, epigenetic mechanisms, and molecular pathways that jointly drive the neoplastic transformation of hematopoietic cells. The addition of the multikinase inhibitor dasatinib to a conventional chemotherapy backbone represents a prominent example of such an approach—exploiting the frequent occurrence of KIT mutations and higher KIT expression levels in CBF leukemia. Early clinical trial data has been encouraging, demonstrating excellent outcomes for both younger and older patients irrespective of KIT mutational status [88, 116]. These data have prompted a large randomized Phase III trial that is currently ongoing (NCT02013658). Depending on the outcome of c-KIT inhibition, further development of this indirect targeting of hematopoietic function using more specific and potent KIT inhibitors may be warranted. The fact that AML1/ETO-driven disease depends on cyclin D2 may confer a therapeutic avenue for palbociclib, a selective inhibitor of the cyclin-dependent kinases CDK4 and CDK6 [74, 117]. As synergistic effects were observed upon addition of a tyrosine kinase inhibitor, combinatorial strategies may enhance therapeutic efficacy.

The discovery and validation of multiple, biologically highly relevant target genes of AML1/ETO underlines the importance of this leukemia as a very useful model to study the function of a chimeric transcription factor oncogene. Elucidating the mechanism of epigenetic regulation at the heart of this disease entity will be critical to achieve the long-term goal of further improving the prognosis of patients afflicted with this cancer.

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