Molecular Landscapes and Models of Acute Erythroleukemia

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Abstract
Malignancies of the erythroid lineage are rare but aggressive diseases. Notably, the first insights into their biology emerged over half a century ago from avian and murine tumor viruses-induced erythroleukemia models providing the rationale for several transgenic mouse models that unraveled the transforming potential of signaling effectors and transcription factors in the erythroid lineage. More recently, genetic roadmaps have fueled efforts to establish models that are based on the epigenomic lesions observed in patients with erythroid malignancies. These models, together with often unexpected erythroid phenotypes in genetically modified mice, provided further insights into the molecular mechanisms of disease initiation and maintenance. Here, we review how the increasing knowledge of human erythroleukemia genetics combined with those from various mouse models indicate that the pathogenesis of the disease is based on the interplay between signaling mutations, impaired TP53 function, and altered chromatin organization. These alterations lead to aberrant activity of erythroid transcriptional master regulators like GATA1, indicating that erythroleukemia will most likely require combinatorial targeting for efficient therapeutic interventions.

Introduction
First described in 1917 by Giovanni Di Guglielmo, acute erythroleukemia (AEL) accounts for 1%–5% of cases with acute myeloid leukemia (AML) and is generally associated with a poor prognosis.1 While most cases are identified in aged patients, AEL is occasionally also diagnosed in very young children. The cellular hallmark of AEL is impaired erythroid terminal differentiation and uncontrolled expansion of erythroid progenitor cells. AEL often presents with variable features that complicate the diagnosis which resulted in several changes in its classification over the years. The first French-American-British (FAB) system classified myeloid neoplasms with >30% leukemic blasts and ≥50% erythroid progenitor cells as AML-M6.2 Some AEL patients present with a heterogeneous mixture of myeloid and erythroid features, while some less frequent cases present with >80% of erythroid progenitor cells considered as purely erythroid leukemia (PEL), respectively, called AML-M6a and AML-M6b in the WHO classification of 2008.3 Importantly, AEL patients may develop their disease de novo, but it frequently follows an antecedent myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), or therapeutic exposure to genotoxic agents. This suggests that the disease reflects a continuum of MDS and AML with erythroid hyperplasia. The clinicopathological overlap and the diagnostic difficulties to distinguish MDS from AEL led the WHO in 2016 to reclassify cases previously diagnosed as AEL into either MDS or PEL.4 Multiple studies have shown that leukemic blasts from AEL patients often carry complex karyotypes with frequent loss of chromosomes 5 and 7.5 Only recently, deep sequencing studies have revealed a more extensive genetic landscape of AEL beyond the most prevalent mutations. However, as outlined in the following, it is important to note that the first insights into the biology of AEL emerged already over half a century ago, when researchers (accidentally) developed erythroleukemia models while studying the in vivo activities of avian and murine tumor viruses. Mechanistic studies aiming to understand these unusual phenotypes initiated several more rationale erythroleukemia models. More recently, the established epigenomic AEL roadmap allowed to establish models that are based on the distinct AEL-associated lesions. These models together with completely unexpected erythroleukemia-like phenotypes in genetically modified mice provide a wide experimental platform to elucidate the molecular mechanisms of AEL initiation and maintenance. Here, we discuss how the increasing knowledge of AEL genetics combined with often unexpected findings from various erythroleukemia models allowed to generate new hypotheses on the molecular mechanisms driving AEL that may provide keys for future targeted therapeutic interventions.

From complex karyotypes to epigenomic erythroleukemia landscapes
It has been recognized over 50 years ago that AEL leukemic blasts carry variable chromosomal abnormalities.6,7 Indeed, clonal chromosomal alterations are found in at least 75% of AEL patients and complex karyotypes were detected in at least 50% of patients.8,9 Complex or hypodiploid karyotypes were seen in at least 50% of cases with entire or partial monosomies of chromosome 5 and 7 being the most frequent.10

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Improved technologies have allowed the first targeted and then genome-wide sequencing of AEL patients samples, revealing recurrently mutated genes. Considering all published genetic studies, TP53 is the most frequently mutated gene, identified in about 30% of the patients, associated with complex karyotype and poor outcome. Notably, TP53 mutations have been identified in almost all patients with pure erythroid leukemia (PEL), thus representing a molecular hallmark of human erythroleukemia.13–15 The other recurrent mutations target NPM1, epigenetic regulators (including TET2 and ASXL1 loss of function mutations, DNMT3A or IDH1/2), intermediates of signaling pathways as well as key hematopoietic transcription factors, which were also frequently identified in other AML subtypes.16–23 Signaling mutations have been reported in 25%–50% of AEL patients, including recurrent activating mutations of the JAK-STAT signaling (JAK2V617F, FLT3ITD, or EPOR) or the RAS (NRAS, KRAS, PTPN11, or NF1) pathways and are mostly associated with additional mutations such as TP53 or NPM1. However, no single highly recurrent gene mutation has been reported to date supporting a higher heterogeneity in the type of signaling mutation associated with AEL (Figure 1; Supplemental Digital Table 1, http://links.lww.com/HS/A149).

As the disease frequently develops secondary to MPN or MDS, known driver mutations of these disorders like the BCR-ABL fusion gene and activating mutations of JAK2 are often associated with AEL.10,21,22 Notably, cytogenetic analysis of 75 AEL patients, 4 of 7 cases of pure erythroid leukemia were associated with a BCR-ABL fusion.21 Single BCR-ABL-positive AML-M6 cases were reported to enter into long-term remission upon treatment with the imatinib tyrosine kinase inhibitor.24 However, some cases of secondary AEL following a JAK2V617F mutation in >60-year-old patients and higher representation of NPM1, TET2, or DNMT3A in 20- to 59-year-old patients.

The concept of clonal hematopoiesis of indeterminate potential (CHIP) is now well established with the observation that several mutations are particularly associated with CHIP and can predispose to the development of hematopoietic malignancies, as well as other human diseases.27,28 Increasing evidence suggests that AEL represents the evolution of a continuum between normal hematopoiesis, CHIP, myeloid neoplasm, and acute leukemia. Indeed, recurrent mutations identified in CHIP including TP53, DNMT3A, and TET2 are also frequently found in human erythroleukemia. Genetic analyses of AEL shows that several mutations often present with low-allelic frequency (eg, ASXL1, PTPN11, and WT1) evoking their post-MPN, -MDS, -CHIP origin. In contrast, TP53 mutations in AEL are characterized by a high-allelic frequency in leukemic cells (often with evidence of bi-allelic inactivation) and are also often identified in other cell populations than the leukemic cells (ie, T-cell compartment), supporting the idea that they arise at an early time point during disease development in an immature multipotent hematopoietic progenitor. Together, these results support an early acquisition of genetic lesions (including those associated with CHIP) and acquisition of additional sometimes subclonal alterations compatible with the emergence of AEL directly or indirectly from a CHIP situation. However, this hypothesis will need to be formally proven.

Although rare, AEL can affect pediatric patients. Interestingly, some recurrent translocations have been described in pediatric AEL, which so far have never been associated with other AML forms including t(1;16)(p31;q24) and t(11;20)(p11;q11) leading to expression of NFIA-E TO2 and ZMYND8-REL A fusions, respectively.28–31 Very recently, a novel t(1;8)(p31;q21) translocation leading to the expression of an NFIA-ETO fusion was described in an infant with PEL presenting as erythroleukemia.32 Fusions targeting the nucleopore components like NUP98 or NUP214, like DEK-NUP214 described in other AML types have also been found in pediatric AEL.33 In addition, several less frequent fusions were identified, involving erythroid-associated factors (MYB-GATA1 and APLP2-EPOR), epigenetic factors (ZEB1-KDM4C and SMARCA4-CBS), or signaling pathways like ASNS-PTPN11, SRC-VWC2, RUNX2-STAT3 and PRKAR2B-PIK3C, or PCMI-JAK2.19,19 Of note, most of these fusions are individually very rare, suggesting that many different genetic changes are required for disease development.

Figure 1. Genetic landscape of human AEL. Schematic representation of the most prevalent genetic lesions reported in human AEL patients.14,15,17,18,21 Alteredtranslocations of the TP53 tumor suppressor were found in ≥30% of AEL and in the majority PEL patients. Note: only the studies by Iacobucci et al and Montalban-Bravo et al included patients with leukemia diagnosed as PEL (according to the WHO 2016 classification). AEL = acute erythroleukemia; PEL = purely erythroid leukemia.
alterations can lead to childhood AEL. Taken together, these studies proposed a molecular classification of AEL patients according to genetic and transcriptomic landscapes that could be associated with age at diagnosis and distinct clinical outcomes.

From tumor viruses to rationale erythroleukemia models

The first erythroleukemia models emerged over half a century ago mostly from phenotypes induced by tumor viruses. These models set the stage for more rational models exploring the transforming potential of signaling mediators and transcription factors in cells of the erythroid lineage. In addition to more recent models that explored the transforming activity of genetic alterations found in AEL patients, unexpected erythroleukemia phenotypes in genetically modified mice provided insight into epigenetic regulation of erythroid differentiation (Figure 2).

Tumor virus-induced erythroleukemia models

Avian leukemia viruses

The first hematopoietic malignancies-inducing oncogenes have been discovered by studying the in vivo activity of avian viruses. The avian erythroblastosis retrovirus (AEV), encoding for viral oncogenic variants v-ErbA and v-ErbB of cellular genes (respectively, the thyroid hormone receptor alpha [TRα] and a mutated epithelial growth receptor) was found to induce fatal erythroleukemia in young chicken. AEV blocks terminal differentiation of committed erythroid progenitor cells. Further functional studies led to the hypothesis that v-ErbA cooperates with activated cellular stem cell factor receptor Kit and v-ErbB to efficiently arrest terminal erythroid maturation. Molecular studies delineated a more general mechanism of oncogenesis based on the inability of altered nuclear receptors to efficiently respond to physiological concentrations of ligands, which was also shown to be the driving force of other AML forms such as acute promyelocytic leukemia mediated by retinoid acid receptor alpha (RARA) fusion proteins.

The E26 avian retrovirus, which induces massive erythroblastosis in newborn chicken, encodes for a fusion between a portion of the viral gag sequences to truncated mutated forms of the transcription factors MYB and ETS1. Functional studies revealed that v-ets is required for the E26-mediated block erythroid differentiation. Interestingly, the Myb-Ets fusion protein seems to inhibit v-ErbA and RARA, indicating overlapping pathways of malignant transformation by E26 and AEV.

In 1957, Friend reported that intraperitoneal injection of cell-free extracts prepared from ascites of mice inoculated with Ehrlich’s carcinoma cells induced a leukemia-like disease. Electron microscopic analysis indicated that Ehrlich’s cells, derived from a spontaneous mouse mammary adenocarcinoma, contained particles similar to what has been seen in virus-infected cells. Intraperitoneal injections of spleen cell suspensions or filtrates into Swiss albino mice resulted in signs of disease in >80% of the recipients. Affected mice had significant infiltrations in hematopoietic organs by cells that looked like erythroid progenitor cells. Subsequent studies suggested that erythropoietin (EPO)-sensitive erythroid progenitors and in particular late burst forming unit or colony forming unit-erythroid (CFU-E) are the targets of the Friend leukemia virus. Permanent cell lines could be established called Friend tumor cells or murine erythroleukemia cells (MELs). Notably, the observation that particular chemicals (eg, dimethyl sulfoxide [DMSO]) are able to induce partial terminal erythroid differentiation made these cells one of the most widely used in vitro platform to study erythroid maturation. Similar to Friend’s, other viruses like the Rauscher or Graffi MuLV were shown to induce an erythroblastosis that phenocopied human erythroleukemia in mice.

Friend virus contains 2 components, the replication-competent Friend murine leukemia virus acting as a helper for the replication-defective spleen focus forming virus (SFFV), which is the erythroblastosis-inducing component. The pathogenic activity is mediated by an env-derived viral 55kDa glycoprotein (gp55) that directly interacts with and activates the EPO

Figure 2. Chronology of AEL mouse models. Schematic timeline of tumor virus-induced, unexpected, and rational erythroleukemia mouse models. r = viral overexpression; tg = transgenic; ge = genome editing; kd = knock-down; MuLV = murine leukemia virus; AEL = acute erythroleukemia.
receptor (EPOR) promoting EPO-independent proliferation and differentiation. Recruitment of a cellular receptor tyrosine kinase receptor stk/RON by gp55 results in activation of downstream signaling effectors including signal activators of transcription (STATs), PI3K/AKT, or MAP kinases.\textsuperscript{46} Provirral integration cloning revealed that Friend virus integrated almost exclusively in a site called SFFV-proviral integration site-1 (Spi-1), which resulted in transcriptional activation of the Spi-1 gene locus by the viral LTR enhancers and in overexpression of the Spi-1 mRNA.\textsuperscript{47}

**Rationale erythroleukemia mouse models**

**Erythroleukemia by overexpression of a Spi-1 transgene**

To model the biological activity of aberrant Spi-1 expression, Françoise Moreau-Gachelin and co-workers established a transgenic mouse model in which a Spi-1 mini-gene was expressed under the control of the SFFV-LTR (Table 1). During an observation period of 12 months, 50% of Spi-1 transgenic mice developed hepatosplenomegaly with extensive erythroid-blast infiltration and occasional tumor cells on the peripheral blood smears. Cells from diseased mice could be grown ex vivo as EPO-dependent cell lines but did not induce the disease upon transplantation, indicating that ectopic Spi-1 expression blocks erythroid differentiation but does not overcome growth factor requirement for survival.\textsuperscript{50} Under hypertransfusion stress, tumor cells (referred as “HS-2 cells”) emerged that were able to proliferate independent of EPO and induce the disease in immunodeficient mice. HS-2 cells carried mutations of the Kit receptor tyrosine kinase leading to constitutive activation of PI3K/AKT and MAPK signaling,\textsuperscript{58} representing a good example of acquired mutation in signaling molecules as a cooperative mechanism contributing to differentiation blockade in cancer. Notably, erythroblasts from the late stage FLV-induced disease harbored allelic losses or missense Trp53 mutations.\textsuperscript{59} Loss of Trp53 alleles also increased penetrance and reduced the latency of erythroleukemia in Spi-1 transgenic mice.\textsuperscript{60}

**Erythroleukemia by constitutive EPOR activation**

SFFV-encoded gp55 glycoprotein binds and activates the EPOR bypassing the cellular requirement for EPO and supporting proliferation and survival of infected erythroid cells.\textsuperscript{61} To address the leukemogenic potential of a constitutively active EPOR, Longmore and Lodish\textsuperscript{48} generated an SFFV in which they replaced gp55 with a mutated EPOR. Injection of this modified SFV-induced polycythemia and splenomegaly in mice.\textsuperscript{62} Transfer of growth-factor-independent erythroblasts isolated from the spleens of these mice rapidly induced an

Table 1. Rationale Genetic AEL Mouse Models

| Year | Gene | Model | Phenotype | Major Findings | Surface Markers on Leukemic Cells | References |
|------|------|-------|-----------|----------------|---------------------------------|------------|
| 1990 | EPOR | SFFV/EPOR transduction | Erythroleukemia | Polycythemia, splenomegaly | n.d. | 46 |
| 1995 | c-MYC | Transgene under control of Gata1 enhancer/promoter | Erythroleukemia | Splenomegaly, erythroid progenitors in peripheral blood, tumor cell infiltration, severe anemia, and moderate thrombocytopenia | n.d. | 49 |
| 1996 | Spi-1 | Classical transgene ("mini gene") | 50% homozygous mice developed a multi-step erythroleukemia within 1.5 to 6 mo of birth. Transplantable into nude recipients | Hepatosplenomegaly with erythroblast infiltration | Increase Ter119<sup>+</sup>, reduced B220<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, Mac1<sup>+</sup>, Gr1<sup>+</sup>, Sca1<sup>+</sup> | 50 |
| 1998 | H-Ras | Transgene under control of Zeta-globin enhancer/promoter mouse | Mesenchymal and epithelial neoplasms, <5% erythroleukemia | Mesenchymal and epithelial neoplasms, <5% showed hepatosplenomegaly with erythroblast infiltration | | |
| 2004 | GATA1<sup>+</sup>, OSE | Transgenic mouse, insertion of Neo cassette before Gata1-GSE region | 50% of GATA1<sup>+</sup>, OSE mice developed disease: 2 phenotypes: myeloid disease after 143 d and lymphoid disease after median latency of 387 d. Transplantable into nude mice | Anemia, thrombocytopenia, erythroblasts, and megakaryocytes in spleens of mice with myeloid disease | Myeloid disease: Kit<sup>+</sup>, CD31<sup>+</sup>, Ter119<sup>+</sup>, CD19<sup>+</sup> | 52 |
| 2007 | EWS-FLI | Inducible transgene in Rosa26, activated by Mx1-IRE (pIpC) | Rapid, highly penetrant (90%–100%) erythroleukemia (+pIpC: 19 d, −pIpC: 95 d). Transplantable in 19/26 sublethally irradiated wild-type and 7/7 NOD/SCID recipients | Anemia, peripheral blood blasts, no thrombocytopenia, hepatosplenomegaly with infiltration of tumor cells | Lympohid disease: Kit<sup>+</sup>, Sca1<sup>+</sup>/CD34<sup>+</sup>/CD19<sup>+</sup>, Kit<sup>+</sup>, CD43<sup>+</sup>, CD71<sup>+</sup>. Many cells Gata1<sup>+</sup> with increased c-Myc expression | 53 |
| 2012 | ERS | MSCI viral overexpression and BM reconstitution | Erythro-megakaryoblastic leukemia, T-cell acute lymphoblastic leukemia. | Hematopoietic malignancies affecting erythroid, megakaryocytic, and T-cell lineage | CD31<sup>+</sup>/Ter119<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup> | 54 |
| 2016 | VENTX | MSCI viral overexpression and BM reconstitution | Lymphoid leukemia, erythroid-megakaryocytic leukemia. Transplantable (30/32) | Accumulation of immature erythroblasts in vivo, cell clones exhibited both erythroid and megakaryocytic differentiation in vitro | CD4<sup>+</sup>/CD8<sup>+</sup>, CD71<sup>+</sup>/Ter119<sup>+</sup>, CD71<sup>+</sup>/CD41<sup>+</sup> | 55 |
| 2019 | CDX4 | MSCI viral overexpression and BM reconstitution | Erythroleukemia-like diseases after a long latency. Transplantable | Anemia, splenomegaly, infiltration of erythroid progenitors. Tumor cells expressed low levels of Gata1 | CD31<sup>+</sup>/Ter119<sup>+</sup>, CD41<sup>+</sup>/Gr-1<sup>+</sup> | 57 |
| 2020 | SKI | MSCI viral overexpression and BM reconstitution | Leukemia of erythroid and myeloid phenotype | Pan-cytopenia associated with accumulation of erythroid and myeloid progenitors in BM, spleen and liver | CD71<sup>+</sup>/Ter119<sup>+</sup>, CD11b<sup>+</sup>/Gr-1<sup>+</sup> | 21 |

AEL = acute erythroleukemia; BM = bone marrow; MSCV = murine stem cell virus.
erythroleukemia-like disease in the recipients. Of note, tumor
cells did neither secrete a pathogenic virus nor did they inte-
grate into Spi-1, but they carried inactivating Trp53 rearrange-
ments. Ablent EPO expression was also found in serially
propagated FLV erythroleukemia cell lines, due to genomic rear-
rangements independent of retroviral integration.62

**Erythroleukemia by targeted expression of master oncocenes**

In vitro studies showed that DMSO-induced MEL cell eryth-
roid differentiation is associated with reduced expression of the
MYC proto-oncogene and that its overexpression inhibited dif-
ferentiation.33,46 To study the in vivo transforming potential of
MYC in the erythroid lineage, Phil Leder and co-workers used
regulatory sequences potentially controlling the expression of
the erythroid master regulator GATA1 in transgenic mice.49

Disease mice presented with splenomegaly with significant
tumor cell infiltration and erythroid progenitors in the periph-
eral blood. Tumor cells showed clonogenic activity in methyl-
celllose (MC) without growth factors and induced the same
disease phenotype when transplanted. Furthermore, tumor cells
expressed erythroid genes (EPOR, globin), but, unlike MEL
cells, exposure to DMSO did not induce terminal differentia-
tion. These observations suggest that aberrant MYC activation
at a particular vulnerable phase of erythroid differentiation is
most likely sufficient to induce erythroleukemia.

In addition to MYC, increased levels of the H-Ras and
K-Ras oncogenes were found in Friend’s murine erythroleu-
kenia.63 Leder and co-workers established another series of
transgenic mice in which the embryonic alpha-like zeta-globin
gene was driving expression of an activated H-Ras oncogene.
Unexpectedly, these transgenic mice (“Tg.AC”) developed multi-
ple mesenchymal and epithelial neoplasms and only few mice
(≤5%) showed hepatosplenomegaly with erythroid blast infiltra-
tion.51 Impaired EPO-induced differentiation of an FLV-induced
erthroleukemia cell line (SK6) by a constitutively active
H-RasG12V mutant also suggested that aberrant RAS activation
can enhance erythroid transformation.66

**Erythroleukemia by aberrant activity of erythroid transcrip-
tional regulators**

**GATA binding protein 1**

The GATA1 gene on chromosome X encodes for a zinc-finger
transcription factor expressed in erythroid, megakaryocytic,
erythroblast, and mast hematopoietic cells as well as in Sertoli
cells of the testes.67 As the generation of germline Gata1-null
alleles resulted in embryonic lethality, Yamamoto and colleagues
aimed to alter Gata1 expression by inserting a neomycin selec-
tion cassette in the promoter region between the so-called double
GATA sequence and the erythroid-specific exon 1 (IE). Whereas
hemizygous mutant male embryos died in utero due to impaired
primitive erythropoiesis, heterozygous female mice survived due
to random inactivation of the X-chromosome. As these mice
expressed about 5% of Gata1 transcripts, this targeted muta-
tion was referred as the Gata1.05 allele. While the decreased
number of erythroid cells and GFU-E formation was observed in
fetal livers, accumulation of primitive erythroid progenitors
was observed as early as E9.5 in mutant mice.68 Heterozygous
Gata1.05/52 female mice developed signs of distress at the age
of 5 months presenting with anemia, thrombocytopenia with
massive accumulation of erythroblasts and megakaryocytes in
their spleens.69 Detailed analysis of a larger cohort of heterozy-
gous female Gata1.05/5 mice revealed a high incidence (~50% pen-
etrance) of leukemia composed of either Kit+ erythroid blasts
(starting at 143 d) or CD19+ lymphoid blasts (starting at 387
d). Tumor cells were Kit+/CD71+/Ter119+CD41−/−CD19+ proery-
throblasts. Tracking of the cells with a GATA1-controlled fluo-
rescent reporter suggested that immature erythroid cells were
already expanding in the hematopoietic organs of GATA-1.05/5
mice at the late embryonic stages.52

Several models indicate that GATA1 activity tightly con-
rols the balance between proliferative erythroid progenitors
and maturing cells. First, in the Gata1.05/5 model, leukemia
development is completely abolished by transgenic expression
of wild-type Gata1. A cell line (“GAK-14”) was established from
Gata1.05/5 diseased mice that maintained an immature
erythroblastic phenotype (CD71+Kitt/Ter119+) when grown on
OP9 stroma cells in the presence of EPO and stem cell factor.
Retroviral overexpression of Gata1 resulted in GAK-14 differ-
entiation into mature erythroid cells when cultured on fetal liv-
er-derived stroma cells.70 Similarly, through expression of the
apoptosis inhibitor BCL2 into Gata1-deficient embryonic stem
cells followed by in vitro erythroid differentiation, Weiss et al.71
generated a stable erythroblastic cell line (“G1E”). Exogenous
Gata1 expression in G1E cells restored erythroid maturation72
and allowed to functionally dissect Gata1 critical domains, post-
translation modifications and target genes.73-74 Collectively,
the observations suggested that impaired GATA1 activity is an
important feature for induction and most likely also mainte-
nance of transformed murine erythroid progenitor cells.75

**ETS transcription factor ERG**

ERG is a member of the E26 transformation-specific family of
transcription factors that contain a highly conserved ETS DNA
binding domain that interacts together with other transcrip-
tion factors to enhancer elements.75 Functional studies in mice
revealed that ERG expression promotes HSC maintenance but
also controls erythromegakaryocytic differentiation.76-79 ERG
was found to bind together with GATA1 to regulatory elements
of key hematopoietic transcription factors like SCL/TAL1.80
The ERG gene is targeted by several chromosomal transloca-
tions associated with AML but also solid tumors.81 High ERG
expression levels have been associated with poor prognosis in
cytogenetically normal AML.82 In addition, increased ERG
gene dosage seems to cooperate with the N-terminal GATA1
mutation (GATA1s) in the transient myeloproliferative disorder
associated with Down’s syndrome.83 ERG is not only highly
expressed in trisomy 21-related but also in sporadic cases of
acute megakaryoblastic leukemia (AMKL). Increased ERG
expression was shown to promote in vitro megakaryopoiesis
and synergize with GATA1 to immortalize hematopoietic pro-
genitor cells.84

Several groups explored the oncogenic potential of increased
ERG expression levels in the hematopoietic system of the mouse.
Brady and colleagues reported that transplantation of fetal liver-derived murine hematopoietic stem and progeni-
tor cells (HSPC) retrovirally overexpressing a human ERG
ORF into sublethally irradiated mice resulted in fully pene-
trant megakaryoblastic leukemia.84 Suzuki and Seto85 found that
transplantation of adult bone marrow (BM) cells from 5-FU-stimulated donor mice retrovirally expressing a human
ERG ORF into lethally irradiated mice induced a leukemia-like
disease characterized by accumulation of CD71+/Ter119+ ery-
throblasts and expansion of CD4+/CD8+ double positive T cells.
Kile and colleagues reported that transplantation of fetal liv-
er-derived or adult mouse bone marrow (BM) from 5-FU
treated donors) retrovirally expressing a murine ERG ORF into
irradiated mice induced a leukemia-like disease. Similar to the
observation by Seto, some mice developed CD4+/CD8+ T-cell
leukemia, others developed nonlymphoid disease composed of
CD71+/Ter119+/− cells in some, but also CD71+/CD41− cells in
other mice.81 Collectively, these studies suggest that abnormal
high ERG expression contributes to hematopoietic malignan-
cies affecting the erythromegakaryoblastic and T-cell lineage.

The Sleeping Beauty (SB) transposon-based mutagenesis sys-
tem allows to identify potentially cooperating genetic lesions
for cancer development. Targeting a conditional SB allele to
the hematopoietic system in mice expressing the constitutively active JAK2V617F resulted in a strong phenotypic selection for an erythroleukemia-like disease.86 The vast majority of SB/JAK2V617F mice developed an aggressive erythroleukemia occasionally coincident with CD4+/CD8+ T-cell ALL. Interestingly, the most prevalent common transposon insertion sites were the genes encoding for the transcription factors ERG and ETS1. Notably, transplantation of fetal liver-derived HSPCs retrovirally expressing an AML-associated TLS-ERG fusion also induced a very similar erythroleukemia as observed in the SB/JAK2V617F mice. Expression of TLS-ERG in SB mice resulted in acceleration of the disease. Interestingly, the Jak2 gene locus was among the most common CIs in this model further underlying cooperation of ERG and constitutively active JAK2 in murine erythroleukemia.86

**Caudal-type homeobox 4**

The caudal-type homeobox family comprises CDX1, CDX2, and CDX4 known as developmental regulators of the clustered HOX homeobox genes.87 In normal hematopoiesis, CDX4 mirrors HOX gene expression with a peak in hematopoietic stem cell and decreasing upon differentiation.88 Ectopic Cdx4 expression in mouse embryonic stem (ES) increased the hematopoietic colony output associated with upregulation of a Hox gene expression.89 However, Cdx4 gene inactivation only minimally affected adult hematopoiesis in mice.67 Retroviral Cdx4 overexpression induced aberrant self-renewal potential in mouse hematopoietic cells in vitro and transplantation induced an AML-like disease in about 50% of mice.82 Feuring-Buske and colleagues recently reported Cdx4 mRNA expression in a small cohort of AEL patients and in 3 established AML cell lines with an erythroid phenotype. Similar to previous studies, retroviral Cdx4 overexpression provided aberrant serial replating potential to BM-derived hematopoietic cells.57,91 Notably, mice transplanted with Cdx4 virally transduced BM-derived HSPCs developed a transplantable erythroleukemia-like disease after a long latency, characterized by anemia, splenomegaly with infiltration of CD71+Ter119+/− erythroid progenitors, occasionally erythroid progenitors in the periphery, and multiorgan infiltrations upon propagation into secondary recipients. Tumor cells were characterized by low expression levels of genes associated with erythroid specification or differentiation including Gata1. Interestingly, leukemic blasts from diseased mice carried some additional mutations in erythroid transcription factors like GATA1 or GATA2. These observations indicate that aberrant Cdx4 expression levels in a permissive progenitor may induce a transcriptional program that interferes with normal erythroid development. However, the direct relevance for the human disease remains unclear, as the transcriptome analysis of large AEL patient cohorts did not highlight Cdx4 alterations.90,91

**EWS-FLI fusion**

The Fli-1 gene encoding for an ETS-transcription factor was identified as an additional common integration site in FLV-induced erythroleukemia.92,93 In addition to FLV-induced mouse erythroleukemia, Fli-1 mRNA expression was found in some human AML cells lines with erythroid phenotypes.74 Experimental Fli-1 overexpression was shown to reduce the expression of GATA1 and to impair induced erythroid differentiation in human and mouse cell lines.82 Apart from FLV-driven mouse erythroleukemia, FLI-1 is better known as fusion partner to EWSR1 (EWS) as consequence of a t(11;22)(q24;q12) chromosomal translocation found in Ewing’s sarcoma and other neuroectodermal tumors.65 Interestingly, Mxi1-iCre-controlled hematopoietic expression of a transgenic EWS-FLI-1 ORF in the Rosa26 murine gene locus rapidly resulted in a highly penetrant aggressive transplantable erythroleukemia with tumor cells expressing Kit, CD71, CD43, and Gata1, but no Ter-119 or other lineage markers. Leukemic cells expressed high levels of Myc but did not harbor any gross chromosomal or Trp53 alterations.31 Although the erythroleukemia phenotype does not match the human disease associated with this fusion, these transgenic mice provided an in vivo platform to study strategies for therapeutic targeting of EWS-FLI1-driven tumors.77 Notably, several compounds were found that inhibit Fli-1 transcriptional activities and impaired EWS-FLI1-driven erythroleukemia in mice. Their detailed mode of action and clinical value for human erythroleukemia remains to be elucidated.77,98

**Erythroleukemia by cooperating genetic lesions**

**BCR/ABL and loss of C/EBPα**

Earlier work suggested that AML is the product of signaling mutations (eg, in tyrosine kinases) supporting proliferation and survival that functionally cooperate with mutations in hematopoietic transcription factor mutations blocking differentiation (Table 2).103 BCR-ABL is a constitutively active tyrosine kinase fusion associated with chronic myeloid leukemia (CML) and is also recurrently found in AEL.10 Tenen and colleagues developed a model of progression from chronic to acute disease by retrovirally expressing BCR-ABL in fetal liver-derived HSPCs lacking the myeloid transcription factor CCAAT-enhancer binding protein (C/EBPα).99 Mice transplanted with BCR-ABL-expressing Cebpα−/− cells developed acute erythroleukemia with infiltration of BM and spleens and erythroblasts on peripheral blood smears. Furthermore, tumor cells expressed erythroid regulators such as SCL/TAL1 and GATA1. Notably, similar to tumor cells from diseased BCR-ABL transduced Cebpα−/− mice, the human erythroleukemia cell line K562 (established from a patient with CML in blast crisis) also expresses BCR-ABL and GATA1 and lacks C/EBPα. Functional studies with Cebpα−/− fetal liver progenitors revealed that C/EBPα functions in hematopoietic cell fate decisions by the dual actions of inhibiting erythroid and inducing myeloid gene expression.105

**GATA2 and C/EBPα mutations**

Genetic alterations of the C/EBPα and GATA2 transcription factors regulating myeloid differentiation and HSC self-renewal have been reported in some AEL patients.17,19 A recent mouse model demonstrated that bi-allelic Cebpα mutations led to myeloid leukemia development and that an additional Gata2 mutation enhanced leukemogenesis with a subset of triple transgenic mice (40%) developing leukemia with erythroid and myeloid features.101 Interestingly, the identified leukemia-initiating cells in both models were neutrophil-monoocyte progenitors and molecular characterization of this population revealed distinct function of each cooperating mutations. While bi-allelic Cebpα mutations increased expression of erythroid genes, the Gata2 mutation increased chromatin accessibility at erythroid TF motifs (eg, GATA1, ZFP1, and KLF1) and decreased it at myeloid TF motifs. These findings suggested that the erythroid phenotype of this leukemia model is driven by the aberrant chromatin accessibility at key erythroid TF-regulated loci, controlled by aberrant GATA2 activity.

**JAK2V617F and loss of Trp53**

The majority of BCR-ABL-negative MPNs are driven by somatic activating mutations of the JAK2 tyrosine kinase of which V617F is the most prevalent. A subset of MPN patients progress to AML that is associated with recurrent somatic alterations affecting epigenetic regulators, splicing-related factors and/or the TP53 tumor suppressor.106 To demonstrate potential cooperation, researchers retrovirally overexpressed JAK2V617F in either wildtype or Trp53−/− BM-derived HSPCs and transplanted them into irradiated wildtype recipients.73,100 Mice developed a serially transplantable leukemic phenotype with hepatosplenomegaly with infiltration of CD71+/Ter119+ erythroid progenitor cells. Kurokawa and colleagues
found abnormal karyotypes, such as hyperdiploidy, suggesting increased genomic instability upon Trp53 loss.100 Viral expression of wildtype Trp53 significantly reduced clonogenic activity in vitro and in vivo leukemia induction by Jak2V617F;Trp53−/− erythroblasts suggesting an active role in aberrant self-renewal. Both groups explored therapeutic approaches in their models. Kurokawa and colleagues reported that a potent JAK1/2 inhibitor (INCB18424) reduced the spleen weight but did not affect tumor cells in the BM.100 Levine and colleagues found that treatment with the ruxolitinib JAK2 inhibitor somehow prolonged the survival of secondary recipients of jak2V617F;Trp53−/− erythroblasts; however, treatment with an HSP90 inhibitor appeared more efficient in reducing tumor cell load and restoring normal myelopoiesis.29 Although these studies demonstrated cooperation of jak2V617F and loss of Trp53 in mice, post-MPN erythroleukemia patients mostly develop TP53 DNA-binding domain point mutations rather than loss of both alleles that seemed to be essential for the observed mouse phenotype in these experiments.

**NTRK1 and mutated TP53**

NTRK1 is a member of the neurotrophic receptor tyrosine kinase gene family including the TRK-A, TRK-B, and TRK-C receptors, respectively. NTRKs are target of gene fusions, deletions/ truncations and point mutations not only in hematological malignancies but also in various solid cancers that mostly lead to constitutive activity.107,108 Iacobucci et al found NTRK1 tyrosine kinase domain (H498R, G6117D, and H766R) mutations in tumor cells from 3 AEL patients carrying TP53 mutations. While activation of downstream signaling pathways is likely dependent on both the cell context and the type of NTRK mutation, NTRK fusion expression in hematopoietic progenitors led to increased phosphorylation of AKT and PLCγ1.109 Together with the observation that PI3K-AKT mutations have been found in about 7% of AEL (primarily older adults), aberrant activation of this pathway may be a driver of some human AEL.9 NTRK1 mutations in AEL gained particular attraction as selective small molecule TRK inhibitors (TRKi) such as larotrectinib, which has been shown to have clinical activity in cancer patients.109 To model functional cooperation, Iacobucci and colleagues transplanted wildtype and Trp53 R172H irradiated syngeneic recipient mice. 

**Table 2. Rational AEL Mouse Models Based on Genetic Cooperation**

| Year | Gene | Model | Phenotype | Major Findings | Surface Markers on Leukemic Cells | Reference |
|------|------|-------|-----------|---------------|----------------------------------|-----------|
| 2010 | BCR-ABL—Cegpa+| MSCV-viral overexpression and BM reconstitution | Erythroleukemia after 26–157 d, 100% penetrance. Transplantable (5/6 mice) | Splenomegaly, erythroblasts on PB smears. Tumor cells expressed SCL/TAL1 | CD71+, Ter119- | 96 |
| 2013 | Sleeping beauty— JAK2V617F | SB insertion tagging, MSCV viral overexpression and BM reconstitution | Erythroleukemia (75%), after a median latency of 50 d. Transplantable | Erythroblast infiltration in BM and spleen. ERG and ETS1 were the most frequent integration site | CD71+, Ter119-, some CD41+ cells | 86 |
| 2014 | JAK2V617F—Trp53 | MSCV-viral overexpression and BM reconstitution | Erythroleukemia-like, after 14–100 d. Transplantable | Hematopoiesemegaly, normal platelet counts | Kt+, CD71+, Ter119- | 23 |
| 2017 | JAK2V617F—Trp53 | MSCV-viral overexpression and BM reconstitution | Erythroleukemia, 100% penetrance, median latency 46.5 d. Transplantable | Anemia, hematopoiesemegaly, pulmonary hemorrhage, and expansion of dysplastic erythroid progenitors | CD71+, Ter119- | 100 |
| 2019 | NTRK1H498R— Trp53+/−/+ | MSCV-viral overexpression and BM reconstitution | Erythroleukemia, 100% penetrance, median latency 25 d. Transplantable | Hematopoiesemegaly, infiltration of GATA1+, RUNX1+, Ter119− tumor cells. Tumor cells were sensitive to Larotrectinib | CD71+, Ter119- | 19 |
| 2019 | Gata2—Cegpa+| Cegpa+; Gata225225−/−, knock-in alleles, fetal liver hematopoietic cell transplants | Bi-lineage acute erythroleukemia almost 100% penetrance, after 8–10 mo | Anemia, thrombocytopenia, and splenomegaly | Kit-, CD71+/−, Ter119−, but also some Mac1+ cells | 101 |
| 2020 | Gata1s—Tet2+ | Erythroblasts from Tet2+/− + Gata1s transgenic mice grown in vitro and transplanted | Erythroleukemia, 100% penetrance | Anemia, Hepato-splenomegaly, Infiltration of erythroblasts GATA1+ in BM, spleen, and liver | CD71+/−, Ter119− | 21 |
| 2020 | ERG—TP53R248Q | MSCV-viral overexpression and BM reconstitution (HUPKI), EB sorting from the first degree recipients and injected into the second degree recipients | Erythroleukemia, 100% penetrance with median latency 60 d (24+ recipients) | Anemia, Hepatopoiesemegaly, infiltration of erythroblasts GATA1+ in BM, spleen, and liver | CD71+/−, Ter119− | 21 |
| 2020 | NFI-AETO2— TP53R248Q | MSCV-viral overexpression and BM reconstitution (HUPKI), EB sorting | Pure erythroid leukemia 80%–100% penetrance. Fully transplantable | Anemia, hepatopoiesemegaly, organ infiltration, erythroblasts on blood smears | Kit+, CD71+, Ter119− | 102 |
| 2021 | Trp53Cre/Bmmt3a and Trp53Bcmt1/Rb1−/− Ntix | Lentiviral vectors with different of sgRNA for multiplex genome editing in Cas9-eGFP lineage-negative HSCs and BM reconstitution | Erythroleukemia | AEL cells sensitive to CDK9 inhibitor (LY2857785) | Not yet access | 103 |

AEL = acute erythroleukemia; BM = bone marrow; MSCV = murine stem cell virus.
like other cancers carrying NTRK alterations, treatment with selective TRK inhibitors could be of therapeutic benefit for AEL patients carrying these alterations.

**NFIA-ETO2 and mutated TP53**

The t(1;16)(p31;q24) chromosomal translocation found in PEL from very young children leads to fusion of the nuclear factor 1A (NFIA) to ETO2 (also known as CBFA2/RUNX1 Partner Transcriptional Co-Repressor, 3, CBF2AT3). The transcription factor NFIA has previously been shown to control erythroid fate of hematopoietic progenitors, while ETO2 is as transcriptional cofactor controlling HSC and differentiation of erythroid progenitor cells. We observed that retroviral overexpression of NFIA-ETO2 fusion blocked in vitro erythroid differentiation of MEL cells and primary murine erythroblasts. However, NFIA-ETO2-expressing cells could not be serially propagated in growth-factor containing methylcellulose and transplantation of NFIA-ETO2-expressing BM or fetal liver erythroid progenitor cells into irradiated mice did not result in any disease. In contrast, NFIA-ETO2-expressing erythroblasts harboring one of the most frequent cancer and AEL-associated TP53 mutation, TP53R248Q, could be serially plated in MC and when transplanted into irradiated recipients, induced a fully penetrant transplantable lethal erythroleukemia-like disease characterized by hepatosplenomegaly, anemia, thrombocytopenia, and the presence of erythroid progenitor cells on peripheral blood smear. Molecular studies suggested that NFIA-ETO2 primarily blocks erythroid differentiation by repressing NFIA as well as GATA1 target genes, and that the TP53R248Q mutation endowed cells with aberrant stemness and aberrant activity of the polycomb complex 2 (PRC2). In addition, similar to other ETO-protein containing fusions, NFIA-ETO2 immortalized cells may also be sensitive to small peptides that disrupt ETO-NHR-domain-mediated protein/protein interactions, suggesting a potential therapeutic vulnerability.

**Alterations of BCOR collaborating with TP53 and DNMT3A mutations**

To functionally demonstrate oncogenic cooperation, Iacobucci and colleagues used multiplexed CRISPR/Cas9-mediated genome editing of HSPCs followed by BM reconstitution in irradiated mice. They established 14 genetically different leukemia mouse models in which induction or an AEL associated TP53 mutation, TP53R248Q, could be serially plated in MC and when transplanted into irradiated recipients, induced a fully penetrant transplantable lethal erythroleukemia-like disease characterized by hepatosplenomegaly, anemia, thrombocytopenia, and the presence of erythroid progenitor cells on peripheral blood smear. Molecular studies suggested that NFIA-ETO2 primarily blocks erythroid differentiation by repressing NFIA as well as GATA1 target genes, and that the TP53R248Q mutation endowed cells with aberrant stemness and aberrant activity of the polycomb complex 2 (PRC2). In addition, similar to other ETO-protein containing fusions, NFIA-ETO2 immortalized cells may also be sensitive to small peptides that disrupt ETO-NHR-domain-mediated protein/protein interactions, suggesting a potential therapeutic vulnerability.

**Inactivation of the nuclear receptor interacting SET domain 1 methyltransferase**

Posttranslational modification of the histone tails is one of the key events of epigenetic gene regulation. Hereby, trimethylation of lysine 4 and 36 of histone 3 are generally associated with active transcription, whereas trimethylation of lysine 9 or 27 correlates with repression of a given gene locus. The so-called epigenetic code is based on the interplay of histone lysine methyltransferases (HMT) that set these marks (referred as “writers”) and demethylases (HDM) that remove them (referred as “erasers”). Multiple genes encoding for these epigenetic regulators are targets of recurrent somatic gene alterations that have been shown to contribute to AML initiation and maintenance. H3K36me3 is the product of mono- and dimethylation by the nuclear receptor interacting SET domain (NSD1)–3 family, ASH1L, or SETMAR followed by SETD2-mediated trimethylation. NSD1 is the target of recurrent genomic alterations in human cancers. Highly prevalent putative loss of function NSD1 mutations have been found in head and neck and other solid cancers, and NSD1 expression was reported epigenetically silenced in renal carcinomas. In contrast to solid cancers, NSD1 mutations are rare in hematological malignancies; however, NSD1 was found to be target of a recurrent t(5;11) chromosomal translocation found in childhood AML that results in a fusion with the NUP98 gene. To better understand its function in hematopoiesis, we inactivated NSD1 in human and mouse hematopoietic cells.
Hematopoietic (Varl-iCre-mediated) inactivation of Nsd1 induced a fully penetrant erythroleukemia-like disease characterized by anemia, thrombocytopenia, splenomegaly, and multiorgan infiltrations with occasional erythroblasts on peripheral blood smears. Nsd1−/− erythroblasts formed abnormal serially replating burst forming unit-erythroid in EPO-containing MC. Transplantation of BM cells from diseased mice propagated the disease in wild-type recipients, alone or in competition with normal cells. Despite constitutive expression of the erythroid master regulator GATA1, in vitro erythroid terminal maturation of Nsd1−/− erythroblasts was significantly impaired. Expression of known positively regulated GATA1 targets was decreased, while the regulation of GATA1-repressed target genes was less affected. Retroviral overexpression of GATA1 was able to overcome the terminal differentiation block in vitro. Similarly, retroviral expression of wildtype, but not a catalytically inactive Nsd1H3K36M mutant, was also able to rescue the terminal maturation block associated with upregulation of erythroid differentiation-associated genes on the mRNA and global proteome level. Despite very similar Gata1 mRNA and protein levels, only Nsd1−/− erythroblasts expressing wildtype Nsd1 showed significantly increased binding of Gata1 to many known target genes. These observations suggested that the catalytic activity of NSD1 is an essential permissive factor for proper transactivation of GATA1 targets for productive terminal erythroid maturation. Importantly, knockdown of NSD1 mRNA significantly altered the clonogenic growth of human CD34+ HSC leading to accumulation of immature erythroid progenitor cells strongly suggesting that independent of the species, NSD1 activity controls terminal erythroid maturation.

Hematopoietic expression of an inducible H3K36M oncohistone transgene

H3K36 is target of multiple aberrations in human cancer including mutations miswriting the marks or aberrant expression of the respective HMT, but also by mutations of the. Originally identified in the histone 3.3. (H3.3.) variant in chondroblastoma, H3 lysine (K) to methionine (M) mutations were later also found in H3.1 in several human cancers (also known as oncohistones) including pediatric soft-tissue sarcomas and some head and neck squamous cell carcinoma. The H3K36M mutant protein seems to sequester the SETD2 HMT resulting in globally reduced H3K36me3 marks but also inhibits other HMTs active on H3K36 such as NSD1 and NSD2 resulting in reduced H3K36me1/2. To address the impact of a H3K36M mutation in primary cells in vivo, Hochedlinger and colleagues established ES cells and mice with Doxycycline (DOX)-induced overexpression of a H3K36M transgene integrated in the Col1A1 gene locus. Adult iH3K36M transgenic mice developed symptoms of disease after 4–7 weeks on DOX. In addition to nonhematopoietic aberrations (testicular atrophy, lack of Paneth cells in the intestine), the mice developed thymic atrophy, splenomegaly, and reduced BM cellularity. They presented with anemia, thrombocytopenia, and increased white blood counts with erythroid progenitors in the periphery mimicking acute erythroleukemia. Gene expression profiling revealed an increased expression of regulators of the erythroid lineage and downregulation of genes known to control HSC and/or myelopoiesis. Notably, the H3K36me3 mark was depleted in downregulated genes of which some showed increased H3K27me3 marks at promoters. These observations suggested that the catalytic activity of NSD1 controls terminal erythroid differentiation.

Emerging molecular mechanism of erythroleukemia

Is GATA1 a core player in the pathogenesis of human erythroleukemia?

GATA1 is a master regulator of normal erythropoiesis acting in transcriptionally active complexes with TAL1, LMO2, LDB1, RUNX1, ETO-, and ETS-family proteins. GATA1 undergoes multiple posttranslational modifications including phosphorylation, acetylation, sumoylation, and ubiquitination that in part control its transcriptional activity. Mutational and transcriptional analysis of primary human AEL cells supports the idea that several alterations may converge on the functional alteration of GATA1 through various mechanisms. First, some transcriptionally active proteins of the GATA1 complexes, including GATA1 itself, are targeted by mutations or part of fusion genes in AEL (eg, GATA1s, NFIA-ETO2, or MYB-GATA1). However, these alterations are rare and therefore do not account for the erythroid phenotype of the majority of AEL. Second, mouse models have shown that altering the expression of several factors, including ectopic expression of ERG, SPI1, or FLI1 or reduced expression of GATA1 can induce AEL phenotypes, indicating that additional mechanisms converging on GATA1 lead to the development of erythroid leukemia. In the line of these observations in mice, we found that aberrantly high expression of several proteins that impact GATA1 function are recurrent alterations in human primary AEL cells. This includes high expression of factors like ERG, CBF2T3, or SKI that functionally antagonize GATA1-dependent differentiation.

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Table 3. Unexpected AEL mouse model

| Year | Gene | Model | Phenotype | Major Findings | Surface markers on leukemic cells | Reference |
|------|------|-------|-----------|---------------|-----------------------------------|-----------|
| 2008 | Nsd1−/− | Constitutive gene knockout | Hete−/− fetal liver hematopoietic cell transplant, 7% erythroleukemia, some lymphoma, 11% erythroleukemia upon co-deletion of Tp53 | Anemia, splenomegaly, infiltration by erythroblasts | | |
| 2019 | iH3K36M | Dox-inducible (rtTA, Rosa26), transgene in Col1A1 locus | Lethal hematologic disorder after median latency of 50 d | Anemia, thrombocytopenia, splenomegaly, accumulation of erythroid progenitors. Relative BM hypocellularity | Increase in colony forming unit-erythroid progenitors and CD71+ proerythroblasts, expressing low levels of c-Kit. | |
| 2020 | Nsd1−/− | Targeted gene knockout (Vav-iCre; Nsd1fl/fl) | Pure erythroleukemia-like disease, transplantable, 100% penetrance | | | |

AEL = acute erythroleukemia; BM = bone marrow.
in G1E cells. Notably, ectopic expression of these transcription factors in mouse erythroblasts resulted in their immortalization associated with decreased chromatin accessibility at GATA1 binding sites.21 In the 2 AEL cohorts for which transcriptome data are available, the subset of AEL samples presenting alterations of expression of either transcription factors or signaling intermediates impacting GATA1 activity represents up to 25% of cases.21 Notably, aberrantly high expression levels of ERG or ETO2 were also reported in another independent AEL cohort resulting from bona fide genomic amplifications including ERG and ETO2 genes.116,117,133 Overall, these findings strongly support a functional convergence on aberrant GATA1 activity in human AEL either through direct genetic alterations (e.g., in regulatory regions to be identified) or as a result of an epigenetic drift as outlined in the following section.

Mechanistically, aberrant GATA1 activity may result in at least 2 cellular consequences. First, inhibition of GATA1 activity may derive from distinct mechanisms involving aberrant maintenance of GATA1 transcriptional repressor (e.g., ETS-associated or ETO2 transcriptional complexes), GATA1 destabilization at the protein levels through alterations of GATA1 posttranslational modifications or aberrant protein-protein interaction, or by alterations affecting GATA1 chromatin binding. These mechanisms would contribute to prevent erythroid progenitor differentiation progress toward fully mature erythroid cells. Second, GATA1 activity could be aberrantly activated or maintained, including through constitutive activation of signaling factors (e.g., through JAK2141,147 or high EPO expression), leading to the abnormal commitment of early immature progenitors toward the erythroid lineage. This idea is supported by the recent model combining bi-allelic Cebpα and Gata2 mutations.101 Hereby, Cebpα and Gata2 mutations synergize by increasing erythroid transcription factor (Gata1, Klf1, and Zmp1) expression and erythroid chromatin access, respectively, thereby installing ectopic erythroid potential. In addition, while bi-allelic Cebpα mutation resulted in increased erythroid transcription factors expression, it also led to increased expression of several erythroid repressors including Gata2, Erg, or Cbfa2α1. These findings collectively suggest that a combination of these 2 antagonistic mechanisms on GATA1 activity in the same progenitor could explain both, the erythroid bias and the blockage of differentiation. The degree of erythroid commitment may depend on both the place of the targeted progenitor in the hematopoietic hierarchy and the cooperating mutations. Of note, GATA1 is also mutated in myeloid leukemia of Down syndrome (ML-DS) that frequently present with erythroid cell marker expression, and is generally considered at the frontier between megakaryoblastic and erythroid leukemia.132 While numerous studies have addressed GATA1 function, it is likely that further analyses are required to fully understand how the activity of GATA1 is fine-tuned and how these alterations contribute to erythroid transformation.

**Impaired TP53 activity and malignant erythropoiesis**

TP53 regulates HSPC quiescence and self-renewal; thus, impaired function of TP53 promotes HSPC proliferation that likely leads to additional DNA damage and hematopoietic malignancies.129 Although TP53 mutations represent by far the most frequent mutated genes in AEL and particularly in AEL, no model clearly demonstrated yet a link with the erythroid phenotype. Impaired erythropoiesis in MDS carrying a deletion on 5q has been linked to activation of TP53 upon inactivation of the ribosomal protein small subunits (RPS)-14 or -19.151 More recently, activation of TP53 during ribosomal biogenesis have been proposed to regulated normal erythroid differentiation.116 Most TP53 alterations identified in AEL and other AML subtypes are missense mutations in the DNA binding domain but their functional consequences (i.e., inactivating, gain-of-function or dominant negative) remain a matter of debate. Functional studies in mice suggested that TP53 mutations are drivers of clonal hematopoiesis.137 Several TP53 DNA-binding domain mutations have been reported to disrupt the structure and activity of the protein, allowing neomorphic interactions with several tumor suppressive factors, including TP73 that abrogate its function.138 In addition, such potentially TP53-gain-of-function mutants appear to be stabilized by binding to HSP90 leading to the inactivation of MDM2 and CHIP E3 ligase-mediated degradation.139 Of importance, these molecular mechanisms could be pharmacologically blocked by small-molecules interfering with TP73 and HSP90.140,141 However, more recent functional studies suggested that the most prevalent AML-associated TP53 mutations act in a dominant-negative manner rather than gain-of-function.141 TP53 interferes with the activity of multiple transcription factors. For example, some TP53 mutants have been shown to interact with the ETs1 transcription factor leading to increased expression of multidrug-resistance 1 (MDR1) associated with a poor outcome in AML.142 Experimental evidence linked TP53 to GATA1 activity in normal erythropoiesis. TP53 and GATA1 may interact, through their transactivation domain and DNA-binding domain, respectively, leading to mutual inhibition of their transcriptional activities.143

TP53-DNA-binding domain mutations were shown to interact with epigenetic factors, including EZH2, KMT2A, KMT2D, and KAT6A. Notably, TP53 mutants bind to and enhance EZH2 chromatin association, resulting in an increased level of H3K27me3 at essential regulator of HSC function and differentiation.143 We recently found that the PEL-associated NFIA-ETO2 fusion gene functionally cooperated with one of the most prevalent AEL-associated TP53R248Q mutation most likely also through functional interference with the PRC2 complex.112 TP53-DNA-binding domain mutations may also interact and enhance the activity of the methyltransferase KMT2A/2D and the acetyl transferase KAT6A leading to increase genome-wide methylation and acetylation.144 Consistently, KMT2A/2D and KAT6A are upregulated in mutant TP53 AML and are often mutated in AML, including AEL.15,145 While additional work is necessary to dissect the link between TP53 mutation and epigenetic gene regulation, it could open the avenue for novel therapeutic avenues. If TP53 mutants indeed enhance KMT2A activity through KMT2A partners such as MEN1, TP53 mutant leukemia including AEL may benefit from the recent development of highly potent and selective small molecules blocking the functionally critical interaction of KMT2A and Menin.146

In MDS, TP53 mutations are generally associated with high-risk disease, rapid transformation to AML, therapy resistance, and poor outcome. Studying a large MDS patient cohort, Papemanouil and colleagues recently found that two-thirds of the patients had multiple hits indicating biallelic targeting which was predictive for leukemic transformation and early death. Interestingly, monoallelic patients die not differ from TP53 wildtype patients in outcomes and therapy response which would not really support a dominant-negative activity of these mutations (at least in the context of MDS).147 However, in at least 80% of TP53-mutated AML patients, more than 1 genetic alteration is present, reflecting the requirement for different oncogenic cooperation mechanisms.113 Indeed, TP53 mutations were shown to cooperate with multiple cellular signaling pathways. Loss of TP53 activity has been shown to cooperate with the KRASG12D activating mutation, inducing an aggressive AML in mice and with NRASG12D to promote megakaryocytic-erythroid progenitor (MEP) transformation leading to AML.148,149 The RAS signaling pathway is target of recurrent
mutations in AEL.\textsuperscript{19,21} Similarly, as outlined before, mouse models have shown that genetic TP53 inactivation cooperates with several mutations, such as loss of function alterations of CEBPA or BCOR or constitutively active mutated tyrosine kinases such as JAK2\textsuperscript{Y617F} or NTRK1\textsuperscript{I1498R} to induce erythroleukemia in mice.\textsuperscript{19,23,100–103,114} Hence, treatment of xenografted AEL patient cells carrying either JAK2 or EPOR amplification with a JAK2 inhibitor significantly suppressed cell growth and prolonged overall survival.\textsuperscript{136} Together, these observations strongly suggest that TP53 mutations are essential players not only in AEL development but also crucial for the maintenance of the disease. However, it remains to be elucidated how TP53 mutations functionally interfere with transcriptional control of erythroid differentiation.

**Aberrant chromatin organization and erythroleukemia**

AEL mouse models as well as sequencing of human samples strongly suggest that several proteins that control chromatin architecture by DNA methylation or histone modification play a central role in erythroid malignancies. DNA methylation is regulated by several factors including TET2, DNMT3A/B, or H3K36me1.\textsuperscript{101} While DNMT3A/B is involved in de novo methylation by transferring a methyl group S-adenosyl-L-methionine to carbon position 5 of the nucleotide cytosine (5-mC), TET2 controls demethylation through the oxidation of 5-mC in 5-hmC. Mutational inactivation of these factors is recurrently observed in patients with MDS and AML resulting in increased HSC self-renewal and a decline in the output of differentiated progeny, thus predisposing to leukemic transformation in mice.\textsuperscript{131} Inactivation of Tet2 or Dnmt3a in HSC resulted in increased myeloid and decreased erythroid gene expression signature resulting in aberrant accumulation of erythroid progenitors in mice. Notably, DNMT3A and TET2 were suggested to regulate hematopoietic differentiation by controlling accessible binding sites for distinct transcription factors including GATA1.\textsuperscript{152–158} In addition, very recent work has shown that precise DNA methylation patterning can control binding and regulation of GATA1 activity.\textsuperscript{159} Interestingly, a novel signaling pathway has been characterized that links TET2 activation to JAK2-mediated phosphorylation resulting not only in increased cytosine hydroxymethylation and genome-wide loss of cytosine methylation but also enhanced interaction with the erythroid transcription factor KLF1.\textsuperscript{160} Interestingly, combined TET2 and DNMT3A inactivation, as frequently found in AEL, was also reported to increase of KLF1 and EPOR expression.\textsuperscript{161} As outlined above, genetic inactivation of Dnmt3A in mice lacking the transcriptional coregulator Bcor shifted the phenotype from macrocytic anemia to an erythroleukemia-like disease.\textsuperscript{159} Based on these observations, we speculate that the erythroid phenotype in some AEL cases is based on aberrant DNA methylation that impairs the erythroid transcriptional program. Aberrant DNA methylation is therapeutically targeted by cytidine analogs (eg, 5-Azacytidine or Decitabine) that incorporate DNA instead of deoxycytidine, covalently bind the enzyme and lead to DNMT degradation. Although a rather low specificity, positive clinical effects resulted into FDA approval to treat MDS and AML patients.\textsuperscript{162} Notably, earlier case reports of positive therapeutic responses to azacitidine were supported in a larger retrospective study including over 80 AEL patients treated with hypomethylating agents.\textsuperscript{163} Although a deeper molecular understanding of these effects would be important, it appears that similar to other AML forms HMAs as single agents are not curative for AEL.

Unexpected erythroleukemia models emerged from the inactivation or mutations of several chromatin-associated factors. Inactivation of the histone H3K36me1/2 methyl-transferase NSD1 leads to uncontrolled accumulation of erythroid progenitor cells resulting in a fully penetrant erythroleukemia-like disease in mice. Molecular analyses revealed a decrease of GATA1 target genes expression without significant expression changes in known GATA1-repressors suggesting that NSD1 is an essential epigenetic modulator of GATA1 target genes.\textsuperscript{117} Of importance, NSD1 has been identified being mutated in some cases of pediatric AEL either through an NUP98-NSD1 fusion or a NSD1 loss-of-function mutation found in a single patient.\textsuperscript{19} Notably, the NSD1 gene is located on the long arm of chromosome 5, a region that is most frequently target of cytogenetic alteration in human erythroleukemia.\textsuperscript{5,10} As outlined above, inducible hematopoietic overexpression of an H3K36me3 transgene resulting in reduced H3K36me1/2 methylation induced a very similar erythroid phenotype in mice as observed upon NSD1 inactivation. Molecular characterization of H3K36me3 overexpressing HSPC revealed an expression of an aberrant erythroid signature, but the putative relation to GATA1 target activation remains unknown.\textsuperscript{116} Extensive biochemical in vitro experiments revealed that NSD1-mediated H3K36me2 marks are required for the recruitment of DNMT3A and maintenance of DNA methylation.\textsuperscript{164} Genetic ablation of Nad1 and its paralog Nsd2 in murine cells resulted in a redistribution of DNMT3A to H3K36me3-modified gene bodies and a reduction in the methylation of intergenic regions. Notably, both, blood samples from individual with SOTOS overgrowth syndrome (carrying germ-line NSD1 loss of function mutations) as well as NSD1-mutant cancer cells exhibited hypomethylation of intergenic DNA. This suggests that reduced H3K36 methylation connects human cancers and developmental overgrowth through aberrant intergenic CpG methylation.

Another chromatin modifier that has also been recently found to interact with GATA1 and other components of the GATA1-transcriptional complex is the KDM5A histone H3K4me demethylase.\textsuperscript{165} Notably, NUP98-KDM5A fusions have been found in pediatric AMKL and AEL patients.\textsuperscript{19,35} Although the ectopic expression of this fusion in human cord blood HSPC lead to immortalization and multilineage leukemia when
injected in mice its impact on the chromatin and on GATA1 activity remains unknown.166

Chromatin organization is also orchestrated by the cohesin protein family, frequently mutated in myeloid malignancies including AEL.19,21,130,167 Mutations in cohesin-encoding genes are strongly associated with GATA1 mutations in ML-D5.168,169 Overall, cohesin mutations are associated with a global decrease in chromatin accessibility, but a relative enhancement of chromatin accessibility at binding sequences for some master hematopoietic stem-cell transcription factors such as GATA2, RUNX1, and ERG involved in myeloid transformation.170 Importantly, cohesin deficiency severely impaired erythroid differentiation of a multipotent cell line and enhanced self-renewal programs.171 These observations suggest that altered cohesion function may support erythroid transformation; however, their role for induction or maintenance of AEL remains to be elucidated. Collectively, these data support the idea that erythroid differentiation is tightly linked to chromatin organization and that aberrant expression or mutations of the regulatory proteins may lead to erythroid malignancies. While relatively few direct erythroid regulatory genes have been identified mutated in AEL, aberrant activity of epigenomic factors that control erythroid differentiation on the chromatin level are therefore valuable candidates to explain the erythroid phenotype of the disease.

Taken together, insights from multiple mouse models as well as the epigenomic landscape recently defined by deep sequencing and transcriptomics studies suggest that AEL reflects a disease continuum between MDS and AML that is characterized by a unique erythroid identity. While the molecular links between genetic/transcriptomic/epigenetic AEL alterations network remains to be elucidated (Figure 3), several therapeutic opportunities can be envisioned. Interference with the most prevalent genetic lesions like TP53 DNA binding mutations but also inhibition of classical signaling pathways, including the JAK/STAT pathway supports growth and survival, appears of primary interest and will require further preclinical assessments. In addition to these signaling nodes that are drivers of several myeloid malignancies, AEL is characterized by different degrees of aberrant erythroid maturation. Recent insights from rationale and unexpected mouse models indicate that the erythroid identity in a significant fraction of AEL is based on the impaired activity of transcriptional master regulators like GATA1. Therapeutic restoring of the GATA1 activity could lead to terminal differentiation of aberrantly accumulated erythroblasts, which may resolve PEL and reduce the cellular burden of AEL. However, more research is needed to dissect the critical molecular mechanisms to translate this strategy into clinically effective therapies.

Disclosures

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