frontiers in Oncology

REVIEW
published: 11 March 2021
doi: 10.3389/fonc.2021.636633

Molecular Mechanisms of the Genetic Predisposition to Acute Megakaryoblastic Leukemia in Infants With Down Syndrome

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Individuals with Down syndrome are genetically predisposed to developing acute megakaryoblastic leukemia. This myeloid leukemia associated with Down syndrome (ML–DS) demonstrates a model of step-wise leukemogenesis with perturbed hematopoiesis already presenting *in utero*, facilitating the acquisition of additional driver mutations such as truncating *GATA1* variants, which are pathognomonic to the disease. Consequently, the affected individuals suffer from a transient abnormal myelopoiesis (TAM)—a pre-leukemic state preceding the progression to ML–DS. In our review, we focus on the molecular mechanisms of the different steps of clonal evolution in Down syndrome leukemogenesis, and aim to provide a comprehensive view on the complex interplay between gene dosage imbalances, *GATA1* mutations and somatic mutations affecting JAK-STAT signaling, the cohesin complex and epigenetic regulators.

Keywords: ML–DS, transient myeloproliferative disorder of Down syndrome, TAM, genetic predisposition, Trisomy 21 (Down syndrome), acute myeloid leukemia, acute megakaryoblastic leukemia

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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: *01 December 2020*
Accepted: *12 January 2021*
Published: *11 March 2021*

Citation:
Grimm J, Heckl D and Klusmann JH (2021) Molecular Mechanisms of the Genetic Predisposition to Acute Megakaryoblastic Leukemia in Infants With Down Syndrome. Front. Oncol. 11:636633. doi: 10.3389/fonc.2021.636633

BACKGROUND: LEUKEMIC PREDISPOSITION IN DOWN SYNDROME

Trisomy 21 (T21), which results in the development of Down syndrome (DS), is the most frequent numeric chromosomal aberration with an incidence of approximately one case in 1,000 births (1). Besides many complications resulting from T21—such as craniofacial dysmorphia, cognitive deficits, and congenital heart defects—DS individuals are known to have a 150-fold increased risk of suffering from myeloid leukemia within their first years of life (2). In contrast, the risk of developing solid malignancies is significantly decreased in DS individuals (3, 4), arguing against DS being a general cancer predisposition.

Myeloid leukemia associated with DS (ML–DS) phenotypically reflects acute megakaryoblastic leukemia (AMKL) observed in patients without DS. However, unlike non-DS-AMKL, patients with ML–DS harbor an excellent prognosis (5).

ML–DS displays a model of step-wise leukemogenesis. T21 already perturbs hematopoiesis *in utero*, causing pronounced megakaryocytic and erythroid lineage commitment and proliferation (6–10). As early as during fetal liver hematopoiesis, mutations in the hematopoietic master regulator

GATA1 are acquired, leading to the exclusive expression of a N-terminal truncated isoform (GATA1s) and loss of the full length transcription factor (11–20). *GATA1*s mutations are indispensable for ML–DS leukemogenesis, as they are found in almost all ML–DS individuals (12). The consequence of the *GATA1*s mutations are uncontrolled expansion of fetal megakaryocytic cells and perturbed terminal erythroid differentiation (6, 21–23). This leads to a disease called transient abnormal hematopoiesis (TAM), which is usually diagnosed within the first week after birth and occurs in about 10–30% of DS individuals (24, 25). TAM is a pre-leukemic state as the course of disease is usually self-limiting within the first months of life (25). However, TAM clones can persist, acquire additional somatic driver mutations, and finally give rise to ML–DS.

To date, it is not fully understood why some patients with TAM progress to ML–DS and others do not. However, large sequencing studies of TAM and ML–DS samples shed new light on the molecular landscape of ML–DS (26–28) and give insight into the transformative character of many somatic mutations (27). On the other hand, the role of "third hit" mutations that also occur in TAM patients who do not develop ML–DS needs to be established (27). In addition, the molecular mechanisms of T21-driven genetic predisposition to myeloid leukemia have been extensively studied, but still need further characterization.

In this review article, we summarize what we have learned from studies on the molecular background of T21-driven genetic predisposition to myeloid leukemia and from analyzing the consecutive steps during DS leukemogenesis, and how this has increased our knowledge of the pathogenesis of leukemia beyond ML–DS. We also include the most recent insights into the molecular landscape of ML–DS and outline what are the remaining open questions to fully understand the role of T21 in leukemia.

IMPACT OF T21 ON HEMATOPOIESIS

Previous studies have shown that T21 severely affects hematopoiesis *in utero*, even in the absence of additional mutations (*e.g. GATA1* mutations). Due to difficulties in the accessibility of primary material, induced pluripotent stem cells (iPSCs) have been used to model hematopoiesis during embryogenesis and fetal development. In an iPSC model of primitive hematopoiesis derived from yolk sac progenitors, T21 and euploid controls formed comparable proportions of hematopoietic progenitors. However, the T21 cells were biased towards erythropoiesis, producing erythroblasts and normoblasts at higher percentages, while neutrophils were reduced compared to euploid samples (29). This iPSC model—mirroring primitive hematopoiesis—did not show increased megakaryopoiesis, suggesting that embryonic hematopoiesis is unlikely to be the origin of ML–DS development (29). In contrast, other studies investigating the impact of T21 on definitive fetal liver hematopoiesis demonstrated enhanced megakaryopoiesis in addition to the increased erythroid differentiation of T21 iPSC and primary T21 fetal liver hematopoietic stem cells (HSCs) (6–10). This pronounced megakaryocyte–erythroid differentiation was accompanied by an increased frequency and clonogenicity, not only of HSCs but also of megakaryocytic–erythroid progenitors (MEPs) (8, 9, 23). As a result of the enlarged MEP compartment during fetal liver hematopoiesis, the proportion of common myeloid progenitors (CMP) and granulocytic-monocytic progenitors (GMP) was reduced (7, 10). Additionally, germline T21 led to a differentiation block in B cell development (9).

The observed differences during distinct steps of *in utero* hematopoiesis point toward a developmental stage-specific effect of T21 on hematopoietic stem and progenitor cells (HSPCs), which might be even increased by the shift of hematopoiesis to the fetal liver and a consequent change of the microenvironment.

Comparable to the observations in human primary material, murine Down syndrome models consistently display perturbed hematopoiesis with expansion of HSPCs and the megakaryocytic department (30–32). Ts65Dn mice—a model with partial trisomy of murine chromosome 16 which contains about two third of the homologues on the human chromosome 21—even develop a myeloproliferative disease with dysplastic megakaryopoiesis (33). However, this phenotype is only observed in aged mice, and the relevance of these findings for our understanding of ML–DS—a fetal disease—remains unclear.

MOLECULAR MECHANISMS OF PERTURBED HEMATOPOIESIS IN T21 INDIVIDUALS

The molecular basis of T21-driven perturbation of hematopoiesis has been intensively studied, but remains to be fully understood. Early genotype–phenotype correlations—especially of cases with partial T21—suggested that a circumscribed region on chromosome 21 is essential for the majority of DS phenotypes, which resulted in the concept of a Down syndrome critical region (DSCR) (34–38). Initially the DSCR was mapped to the bands 21q22.2–21q22.3 including ~6 Mb and 25–50 genes (35–38). However, with an increasing number of studies, it became clear that there might be different critical regions on chromosome 21 for distinct phenotypes, rather than one region being responsible for all phenotypes (39–42). Concerning ML–DS, an around 4 Mb segment was identified that seems to be essential for T21-driven leukemogenesis (**Figure 1**). This segment comprises ~20 genes including *RUNX1*, *ERG*, and *ETS2* (**Figure 1**), which play a pivotal role in hematopoietic differentiation (23, 42). To further understand the molecular mechanisms of T21 altered hematopoiesis, multiple studies analyzed the expression of those and other genes in T21 HSPCs. These investigations consistently demonstrated only slight increases in gene expression not exceeding a two-fold upregulation, which is at least partially explained by increased gene dosages resulting from T21 (7–9, 29).

This supports the concept of only mildly elevated expression of a plurality of genes having extensive effects on downstream targets and regulatory circuits and thereby cooperating to

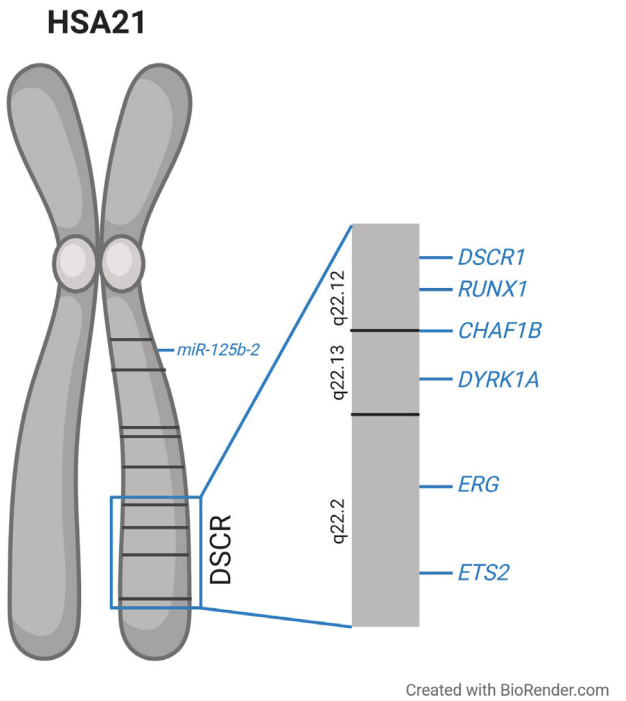

FIGURE 1 | Schematic overview of human chromosome 21 (HSA21) and the proposed Down syndrome critical region (DSCR), along with the location of the genes discussed in this article which are suggested to be involved in the dysregulated hematopoiesis observed in individuals with trisomy 21.

perturb hematopoiesis in individuals with T21. In the following, we focus on specific genes on chromosome 21, which seem to play pivotal roles in the pathogenesis of T21-driven leukemogenesis.

RUNX Family Transcription Factor 1

RUNX1 is an indispensable transcription factor for hematopoiesis, and forms the core binding factor transcription complex together with its subunit CBFβ (43). Complete *Runx1* deficiency is incompatible with life, since *Runx1*$^{-/-}$ murine embryos die around E12.5 in the absence of fetal liver hematopoiesis (44, 45). There are at least three different RUNX1 isoforms resulting from alternative splicing: RUNX1a, RUNX1b, and RUNX1c. While RUNX1b and RUNX1c contain the DNA binding Runt homologous domain and the transactivation domain, RUNX1a lacks the latter (46). Previously, imbalance of the *RUNX1* isoforms was implicated in leukemogenesis, with RUNX1a exhibiting a pro-leukemic effect *in vivo* (47).

Additionally, *RUNX1* is a translocation partner contributing to numerous fusion oncogenes in AML, of which *RUNX1-ETO* resulting from t(8;21) is the most common, presenting in 12% of cases with *de novo* AML (48).

RUNX1 seems to be essential for lineage programming, as its upregulation precedes megakaryocytic differentiation, while it becomes downregulated upon erythroid lineage commitment (49). In this context, RUNX1 cooperates with GATA1 in the promoter activation of megakaryocytic genes through direct protein-protein interaction (49).

It was shown that perturbation of hematopoiesis in T21 individuals is at least partially executed *via* upregulation of *RUNX1* expression. In a T21 iPSC model, increased *RUNX1* gene dosage caused an expansion of the HSPC pool, especially in early fetal hematopoiesis (23). In contrast, in the Ts65Dn murine DS model, restoring disomy of the *Runx1* locus reduced the number of megakaryocytic colonies but did not completely abrogate the myeloproliferative disease observed in elderly mice, pointing towards the cooperation of multiple genes in DS leukemogenesis (33).

ETS Transcription Factor ERG and ETS Proto-Oncogene 2

The ETS transcription factor ERG is a proto-oncogene that is essential for HSC maintenance and megakaryocytic differentiation (50–54). Along with its transcription factor family member ETS2, *ERG* was shown to be upregulated in AML with complex karyotypes involving chromosome 21 and in patients with AMKL with or without DS (55, 56). Overexpression of *ERG* as well as *ETS2* caused a switch from erythroid to megakaryocytic differentiation in K562 cells (50, 56). In the regulation of megakaryopoiesis, ETS transcription factors might cooperate with GATA1, since many genes essential for megakaryopoiesis harbor GATA along with ETS binding motifs in their promoters (57, 58). Ectopic expression of *ERG* or *ETS2* together with *GATA1* knock-down induced immortalization of fetal liver cells, as demonstrated in serial replating assays (53).

In vivo, increased expression of *ERG* during fetal hematopoiesis led to an expansion of MEPs while GMPs were reduced, comparable to changes observed in T21 individuals (59). Ectopic expression of *ERG* in *Gata1* mutated cells further amplified megakaryocytic differentiation while terminal erythroid differentiation was blocked. Additionally, these *ERG*/*Gata1s* mice demonstrated liver fibrosis and postnatal transient expansion of megakaryocytic progenitor cells, demonstrating that interaction between increased *ERG* expression and *Gata1s* is sufficient to cause a disease with key features of TAM in a murine model (59).

Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A

DYRK1A was shown to be a key regulator of calcineurin/NFAT signaling, which is involved in many developmental processes, such as organogenesis, neuronal growth and T cell function (60–63). Moreover, *Nfatc2*$^{-/-}$ and *Nfatc4*$^{-/-}$ double knockout mice develop typical craniofacial features comparable to the changes observed in human DS (64).

Upon cellular Ca^{2+} intake, calcineurin is activated leading to the dephosphorylation of NFATc proteins. Consequently, NFATc is transported into the nucleus where it activates transcription together with other binding partners (65). NFATc is exported to the cytoplasm upon rephosphorylation, which is executed by

glycogen synthase kinase 3 (GSK3) and DYRK1A (60, 64, 66, 67). Hence, *DYRK1A* and *DSCR1* encode inhibitors of calcineurin/NFAT signaling and are both located in the DSCR on chromosome 21 (**Figure 1**). It was shown that a 1.5-fold increase of *DYRK1A* and *DSCR1* expression drastically reduced calcineurin/NFAT pathway activity (64, 68).

Increased *Dyrk1a* gene dosage was previously linked to megakaryocytic leukemogenesis in the Ts1Rhr Down syndrome mouse model (32). Overexpression of *Dyrk1a* in the bone marrow of these mice led to megakaryocytic expansion, which was even more pronounced in the presence of the *Gata1s* mutation. In addition, inhibition of the calcineurin/NFAT pathway by treatment with ciclosporin A in T21 and euploid samples suggested that increased *Dyrk1a* expression causes megakaryocytic expansion at least partially by downregulating the calcineurin/NFAT signaling (32).

Chromatin Assembly Factor 1 Subunit B

CHAF1B encodes the subunit of the chromatin assembly factor 1 complex (CAF1), which is essential for nucleosome assembly during S phase (69, 70). It is located in the DSCR on chromosome 21 (**Figure 1**) and was shown to be overexpressed in ML–DS compared to non-DS-AMKL (32). Additionally, *CHAF1B* overexpression promotes murine megakaryopoiesis (32). In *KMT2A*-rearranged AML, *CHAF1B* overexpression induces a differentiation block and promotes HSPC proliferation (71). Taken together these findings suggest that increased *CHAF1B* gene dosage due to T21 might contribute to the megakaryocytic differentiation block observed in TAM and ML–DS.

miR-125b-2

MicroRNAs are 21 to 23 nucleotide long non-coding RNAs, which execute post-transcriptional regulation of gene expression by binding to the 3′UTR of their target mRNA and leading to mRNA degradation (72). *MiR-125b-2* is encoded on chromosome 21 (**Figure 1**) and its overexpression in MEPs and megakaryocytic progenitors was shown to enhance self-renewal capacity and proliferation (73). When overexpressed in HSPCs, *miR-125b-2* caused a myeloid differentiation block. The expansion of megakaryocytic cells induced by *miR-125b-2* was even more pronounced in *Gata1s* fetal liver cells, pointing towards synergistic properties in DS leukemogenesis (73). It remains open, whether the other members of the *miR-99a~125b-2* tricistron (*miR-99a* and *let-7c*) on chromosome 21 further enhance or inhibit the oncogenic effects of *miR-125b* in concert with GATA1s (74).

DEVELOPMENT OF TAM: THE ROLE OF GATA1S IN DS LEUKEMOGENESIS

The origin of TAM *in utero* marks the second step in DS leukemogenesis. Acquiring a *GATA1* mutation—leading to the loss of full length GATA1 expression—in T21 fetal HSPCs is both sufficient and essential for TAM pathogenesis.

Natural History of *GATA1s* Mutations in TAM and ML–DS

Exclusive translation of the short isoform of GATA1 is found in over 90% of TAM and ML–DS cases (12). Thus, we can infer that *GATA1s* mutations occur very early during leukemogenesis, most likely during fetal hematopoiesis (11, 15, 17, 18). To date, it is not clear whether the presence of certain *GATA1s* mutations increases the risk of progression to ML–DS. While Alford et al. showed that the type of *GATA1s* mutation is not predictive for transformation from TAM to ML-DS (12), Kanezaki et al. demonstrated a correlation between the mutation type and *GATA1*s expression levels and that low *GATA1s* expression in TAM patients is significantly associated with a higher risk of progression to ML-DS (75). In most cases, the *GATA1s* mutation identified in the TAM sample is also detectable after progression to ML–DS (13, 16), suggesting clonal evolution from TAM to ML-DS. This is even the case if the *GATA1s* clone was not detectable during complete remission. However, new *GATA1*s clones can also arise and contribute to the dominant clone in ML-DS (26). Hence, the dominant *GATA1s* clone can differ between TAM and ML–DS, indicating that evolution from minor TAM clones is a mechanism of ML–DS development (11, 26). *GATA1s* mutations are hardly found in euploid individuals who develop AMKL, underlining the specificity for DS leukemogenesis (11, 76).

GATA1 in Normal Hematopoiesis

Altogether there are six GATA genes, all of which encode for DNA binding proteins that play a pivotal role in transcriptional regulation (77, 78). The six members of the GATA family all harbor two zinc fingers as their common structure. While the C-terminal zinc finger binds DNA *via* recognition of the GATA motif, the N-terminal zinc finger interacts with important cofactors such as FOG1 (79–82).

GATA1 is located on the X chromosome and encodes an essential transcription factor for hematopoiesis—especially for the erythroid and megakaryocytic lineages, but also for the development of eosinophil and basophil granulocytes and mast cells (83).

To ensure proper megakaryocytic and erythroid differentiation, the tight transcriptional regulation of *GATA1* and its family member *GATA2* is crucial, and is also referred to as the "GATA switch". While the expression of *GATA2* is mandatory for the self-renewal capacity of HSPCs, high GATA1 levels are needed for the transition to MEPs and the subsequent differentiation of the megakaryocyte–erythroid lineage (84, 85). This switch between GATA transcription factor expression is realized through the direct transcriptional regulation of GATA1 by GATA2 and *vice versa*, as well as through epigenetic mechanisms, such as DNA methylation (85, 86). It was already shown that, as a consequence of the loss of full length *Gata1*, the "GATA switch" is impaired, causing perturbation of erythropoiesis (87).

Loss of GATA1 expression leads to an erythroid differentiation block and apoptosis of erythroid precursors (23, 88–93). *GATA1* knock-out in murine embryonic stem cells results in embryonic lethality between days 10.5 and 11.5 due

to anemia (88). Consistently, *GATA1* knock-out drastically impairs megakaryocytic maturation, resulting in reduced platelet counts. However, immature megakaryocytes undergo excessive proliferation (27, 94, 95).

These findings underline the pivotal role of full length GATA1 for megakaryocyte and erythroid differentiation.

Functional Consequences of *GATA1* Mutations on Hematopoiesis

In line with the crucial role of GATA1 in physiological hematopoiesis, germline *GATA1* mutations are associated with hereditary thrombocytopenia, dyserythropoietic anemia, and Diamond-Blackfan anemia. However, the majority of germline *GATA1* mutations do not increase the probability of developing leukemia in the absence of T21 (96–100). To date, there is only one published case of a newborn who was diagnosed with TAM at birth, who had an N-terminal *GATA1* mutation but no T21 or any copy-number alterations conformable with T21. However, the identified *GATA1* mutation was a large deletion resulting in the loss of the entire N-terminal zinc finger and parts of the transactivation domain of the transcription factor (101).

In contrast, *GATA1* mutations associated with TAM and ML–DS are typically small insertions or deletions or point mutations in exon 2, which lead to the introduction of a premature stop codon or loss of the adjacent splice site (11–20). As a consequence, only the short isoform GATA1s (~ 40 kD)—which lacks the first 83 amino acids, including the N-terminal transactivation domain (12–17, 19)—is translated from a start codon in exon 3. As a result, GATA1s contains both zinc finger domains, but possesses reduced transactivation potential compared to the full length protein (19).

Additionally, GATA1s shows perturbed binding and activation of important erythroid genes, while its transcriptional activation of megakaryocytic and myeloid target genes is comparable to full length GATA1 (6). Other studies suggest that altered gene expression in the presence of GATA1s might also result from the loss of transcriptional repression at certain GATA1 target genes (102, 103). In general, GATA1s induced changes in transcriptional regulation might be caused by disturbed binding with co-factors, such as RB1 and E2F (104–106). Of note, in fetal liver cells, the expression of the *GATA1* V205G mutant, which is unable to interact with FOG1, did not lead to megakaryocytic hyperproliferation but prevented cells from undergoing terminal differentiation, while GATA1s rescued the megakaryocytic differentiation block in GATA1 deficient cells but sustained uncontrolled expansion (107).

In addition, changes in gene regulation by GATA1s and the resulting hematopoietic alterations also seem to be developmental stage-specific, comparable to perturbations of hematopoiesis caused by T21. In iPSC models of early hematopoiesis derived from yolk sac progenitors and fetal hematopoiesis, GATA1s caused impaired erythropoiesis, even in the presence of T21, thus overriding the pronounced erythroid differentiation caused by T21 (6, 22). On the contrary, GATA1s enhanced the proliferation of dysplastic megakaryocytes—a phenotype which is independent from, but which becomes accelerated in, a T21 background during fetal hematopoiesis (21–23). When *GATA1* mutations were introduced into neonatal HSPCs using a CRISPR-Cas9-system, increased proliferation of erythroid precursors was observed. However, the accumulation of immature erythroid cells was only transient and applying the same method to adult HSPC caused only mildly increased proliferation of the erythroid lineage (21). These results, obtained from *in vitro* studies using primary human material, are in line with data from *GATA1s* knock-in mice demonstrating transient reduction of erythropoiesis and aberrant hyperproliferation of megakaryocytic progenitors during fetal hematopoiesis, but normal hematopoietic differentiation in adult mice (103).

Two recent studies in murine embryonic stem cells (ES) and human T21 iPSCs narrowed down the search for the cellular origin of TAM to a population of immature megakaryocytic progenitors characterized by high CD41 expression (108, 109). During step-wise hematopoiesis *in vitro*, these cells showed delayed and aberrant megakaryocytic differentiation, reduced erythroid differentiation and gave rise to an increased number of myeloid cells upon *GATA1s* expression (108, 109).

IGF Signaling as Mechanisms of Developmental-Stage Specific Effects of *GATA1s* Mutations

Perturbation of hematopoiesis caused by *GATA1s* mutations as well as T21 show strong dependency on the stage of development. The fact that *GATA1s* knock-in mice display normal hematopoiesis in adult life (103) along with the self-limiting course of TAM in the majority of patients (25) suggests an important role of the fetal liver microenvironment, since hematopoiesis is shifted from the fetal liver to the bone marrow after birth. It was previously shown that fetal liver stromal cells secrete a variety of cofactors supporting the expansion of HSCs, *e.g.* IGF2 (110, 111).

In contrast to equivalent adult cells, fetal megakaryocytic progenitors depend on the IGF/IGFR1/mTOR pathway for proliferation and differentiation, which is constantly active in the fetal liver microenvironment (104). In the presence of continuous IGF/IGFR1 signaling, megakaryocytic expansion needs to be tightly controlled, which is at least partially realized by regulation of the E2F transcription factor. While E2F is activated by the IGF/IGFR1/mTOR cascade, direct interaction with GATA1 inhibits E2F and consequently its downstream targets, *e.g. MYC* (104, 105). However, GATA1s shows reduced binding to the E2F factors and the inhibitory RB1 protein, resulting in an overactivation of E2F target genes and uncontrolled expansion of megakaryocytic progenitors (104). Consistently, another study demonstrated insufficient repression of the E2F transcription network and MYC as reasons for increased proliferation of eosinophil precursors after ectopic *GATA1s* expression in fetal HSPC (112).

These data suggest that the hyperproliferative phenotype in the presence of *GATA1s* mutations results from the overactivity of pro-proliferative genes as a consequence of ineffective suppression of the E2F transcription factor, and deregulated

IGF signaling, which might be even further pronounced in a T21 genetic background and fetal liver microenvironment.

Synergy Between T21 and GATA1s in TAM Pathogenesis

Given that a *GATA1s* mutation in the T21 genetic background is mandatory for the development of TAM, cooperative effects between both aberrations have to be assumed. As previously discussed, T21 causes an expansion of MEPs during fetal liver hematopoiesis (8, 9, 23). This enlarged pool of cells with increased proliferative capacity might be especially susceptible to the acquisition of *GATA1s* mutations. GATA1s leads to hyperproliferation of the megakaryocytic lineage – an effect that is increased in the presence of T21, as previously shown (21–23). Thus, the hypothesis of a positive selection for randomly emerging *GATA1s* mutations in the megakaryocyte–erythroid compartment during T21 fetal liver hematopoiesis seems rational.

Further supporting the idea of cooperation between T21 and GATA1s, it was shown that GATA1s expression is elevated in T21 iPSCs compared to euploid cells (23). In the T21 background, increased gene dosage of *RUNX1*, *ERG*, and *ETS2* upregulate GATA1s expression, which itself further enhances transcription of *RUNX1*, *ERG*, and *ETS2* (23, 87). Besides a direct interaction between *Gata1s* and *Runx1*, another mechanism of increased *Runx1* expression in *Gata1s* erythroid cells is the reduction of the repressive H3K27me3 mark and higher chromatin accessibility at the *Runx1* locus (87). As a consequence of this, gene levels rise two to three-fold compared to euploid cells, leading to the hyperproliferation of aberrant megakaryocytic cells (23).

GATA1 was implicated along with RUNX1, ERG, FLI-1, TAL1, LYL1, and LMO2 to be part of a heptad of transcription factors which cooperatively control gene transcription via DNA and protein-protein interaction upon differentiation of HSPCs (113). Increased gene dosages of *RUNX1*, *ERG*, and *ETS2* together with exclusive GATA1s expression might lead to disruption of this regulatory network, resulting in the pronounced megakaryocytic and impaired erythroid differentiation observed in TAM.

However, further studies are needed to completely understand the difficult interplay between gene dosage changes due to T21 and the disruption of regulatory circuits, and to determine how these alterations translate into leukemogenesis (**Figure 2**).

CLINICAL CHARACTERISTICS AND MANAGEMENT OF TAM

About 10% of neonates with DS experience TAM—characterized by the clonal proliferation of myeloid blasts with a megakaryoblastic or erythroblastic phenotype, which are detected in peripheral blood (24, 114, 115). Morphologically, TAM blasts cannot be distinguished from ML-DS blasts. Typically, the abundance of TAM blasts in the peripheral blood is not accompanied by a high bone marrow infiltration (116, 117).

TAM is usually diagnosed within the first week after birth, underlining that the disease originates *in utero*. Although stringent diagnostic criteria for TAM do not exist, the diagnosis is usually confirmed by the presence of typical TAM blasts in peripheral blood and the presence of T21 and a *GATA1s* mutation (11, 18). Still, defining TAM is complicated by the fact that the percentage of TAM blasts in the peripheral blood highly varies during the course of the disease, and that individuals without clinical signs of TAM might also harbor *GATA1s* mutations and thus be at risk for developing ML–DS (11).

The clinical presentation ranges widely, from asymptomatic children to fatal cases resulting in early death due to organ complications. The early death rate in TAM ranges between 11 and 23% (118–120). Typical clinical signs are leukocytosis, anemia, thrombocytopenia, and hepatosplenomegaly as an indicator of liver infiltration and fibrosis (25, 116, 119, 120). In severe cases, the progressive infiltration can cause liver failure accompanied by coagulopathy (121). TAM can also lead to hydrops fetalis and cause the miscarriage of DS fetuses (122–127).

In the majority of TAM patients, the course of disease is self-limiting. However, intervention is needed for individuals with severe TAM-related clinical symptoms, which carry a high risk of causing early death. Since the hyperproliferative TAM blasts are very susceptible to cytarabine, various studies investigated the use of cytarabine in high risk TAM patients (25, 116, 128). Although the application of cytarabine might increase survival rates in TAM patients with critical disease, the dosing scheme and indications for chemotherapy differ between study groups (25, 116, 128, 129).

With about 13–33%, a high portion of TAM patients progress to ML–DS, usually before they reach the age of four years (2, 116, 118, 130). Unfortunately, measurable residual disease (MRD)-monitored low-dose cytarabine treatment of TAM patients was not able to reduce this high progression rate (25, 129).

FROM TAM TO ML–DS: CLONAL EVOLUTION IN DS LEUKEMOGENESIS

Once progressed from TAM to ML–DS, the disease course is no longer self-limiting, and all patients need intensive chemotherapy to achieve long-term survival (5).

Evolution from TAM to ML–DS seems to depend on the acquisition of additional mutations in persistent *GATA1* mutant cells. Somatic mutations in ML–DS patients most frequently affect cohesin complex genes, JAK family kinases, and epigenetic regulators, but mutations frequently observed in AML, such as *FLT3* or *TP53* mutations, can also be found (**Table 1**) (26–28). While TAM samples harbor on average 0.4 mutations in addition to the *GATA1s* mutation, ML–DS samples had 1.6 detectable variants per sample (26). Although at a low frequency, some TAM patients were shown to harbor somatic variants in addition to the *GATA1s* mutation. However, "third

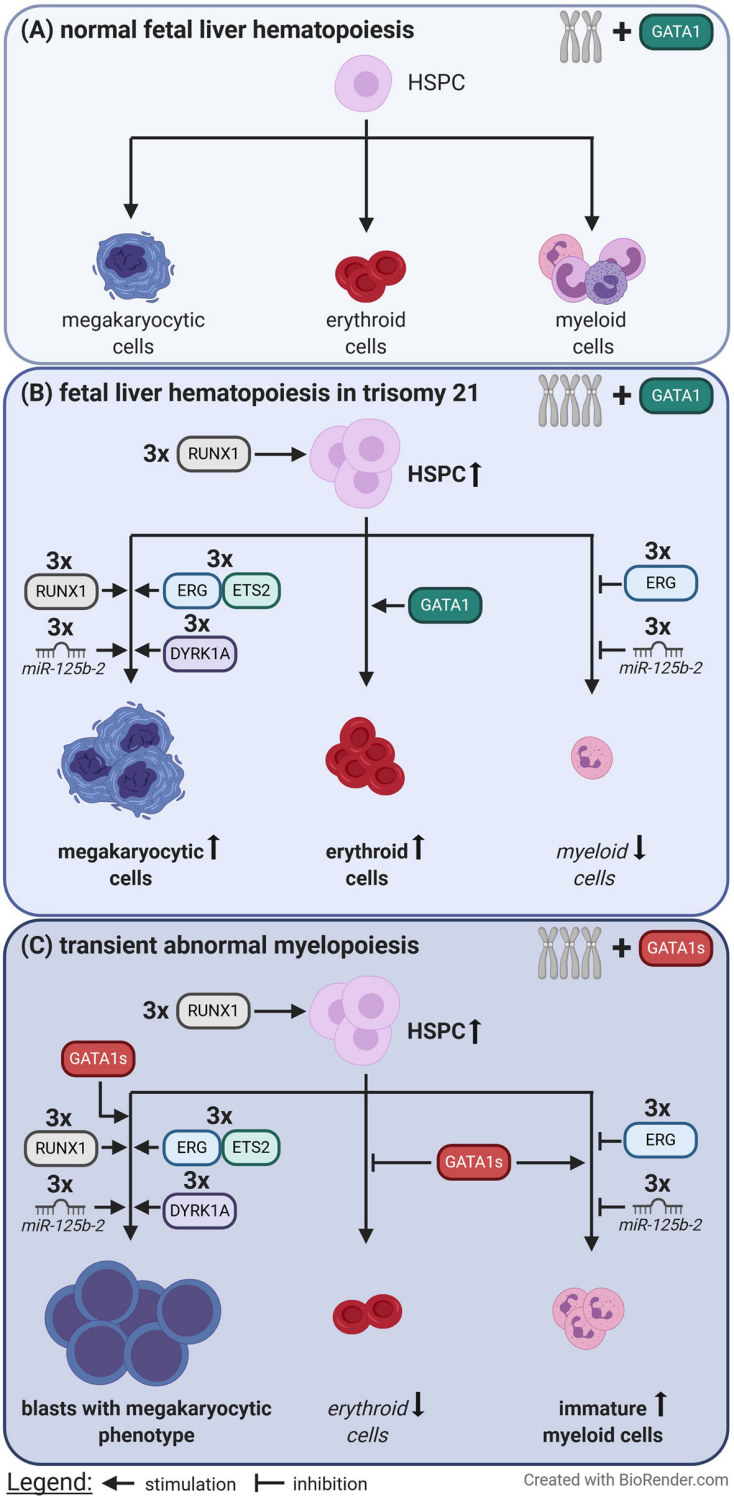

FIGURE 2 | Perturbation of fetal liver hematopoiesis caused by trisomy 21 and loss of full length GATA1. **(A)** In euploid individuals the balanced gene dosages of chromosome 21 and the presence of full length GATA1 contribute to normal fetal liver hematopoiesis. **(B)** In contrast trisomy 21 causes expansion of hematopoietic stem and progenitor cells (HSPC), megakaryocytic and erythroid cells via increased gene dosages. **(C)** When an additional *GATA1* mutation, which leads to the expression of only the short isoform of GATA1 (GATA1s), is acquired immortalized megakaryocytic blasts rapidly expand at the expense of erythropoiesis. This is known as transient abnormal myelopoiesis which typically originates during fetal liver hematopoiesis.

TABLE 1 | Summary of somatic mutations identified in ML–DS samples in addition to the mandatory *GATA1s* mutation.

	Genes mutated	Frequency of mutation in different studies (%)	References
cohesin complex	*CTCF*	16/141 (11.3); 10/49 (20.4)	(26, 28)
	NIPBL	5/141 (3.5); 3/49 (6.1)	(26, 28)
	RAD21	16/141 (11.3); 11/49 (22.4)	(26, 28)
	SMC1A	9/141 (6.4); 2/49 (4.1)	(26, 28)
	SMC3	1/141 (0.7); 1/7 (14.3); 1/49 (2.0)	(26–28)
	STAG2	19/141 (13.5); 9/49 (18.4)	(26, 28)
epigenetic regulators	*ASXL1*	1/49 (2.0)	(28)
	BCOR	2/141 (1.4); 2/49 (4.1)	(26, 28)
	DNMT3A	1/49 (2.0)	(28)
	EED	1/141 (0.7)	(26)
	EP300	1/141 (0.7)	(26)
	EZH2	10/141 (7.1); 1/7 (14.3); 16/49 (32.7)	(26–28)
	KANSL1	17/141 (12.1); 3/49 (6.1)	(26, 28)
	KDM6A	1/141 (0.7)	(26)
	KMT2C	1/141 (0.7)	(26)
	NAT6	1/141 (0.7)	(26)
	SUZ12	9/141 (6.4); 1/49 (2.0)	(26, 28)
	TET2	2/141 (1.4)	(26)
tyrosine kinases	*GNB1*	1/141 (0.7)	(26)
	JAK1	6/141 (4.3); 1/7 (14.3); 2/49 (4.1)	(26–28)
	JAK2	14/141 (9.9); 4/49 (8.2); 1/7 (14.3)	(26, 28, 131)
	JAK3	19/141 (13.5); 6/49 (12.2); 2/13 (15.4); 1/3 (33.3); 1/7 (14.3); 1/14 (7.1)	(26, 28, 131–134)
	KIT	2/141 (1.4)	(26)
	MPL	10/141 (7.1); 3/49 (6.1)	(26, 28)
	PTEN	1/141 (0.7)	(26)
	PTPRD	1/141 (0.7)	(26)
	SH2B3	4/141 (2.8); 4/49 (8.2)	(26, 28)
RAS	*KRAS*	7/141 (5.0); 4/49 (8.2)	(26, 28)
	NF1	4/141 (2.8)	(26)
	NRAS	6/141 (4.3); 4/49 (8.2)	(26, 28)
	PTPN11	1/49 (2.0)	(28)
transcription factors	*CREBBP*	1/141 (0.7)	(26)
	FLT3	1/7 (14.3); 2/7 (28.6)	(27, 131)
	MYC	1/141 (0.7)	(26)
	RUNX1	3/141 (2.1)	(26)
	TP53	5/141 (3.5); 3/49 (6.1); 2/13 (15.4)	(26, 28, 132)
	WT1	1/141 (0.7); 2/49 (4.1)	(26, 28)
others	*CSF2RB*	7/141 (5.0)	(26)
	DCAF7	1/141 (0.7); 2/49 (4.1)	(26, 28)
	DLEC1	1/7 (14.3)	(27)
	DHX29	1/7 (14.3)	(27)
	PI3KC2A	1/7 (14.3)	(27)
	POLE	1/7 (14.3)	(27)
	SF3B1	3/141 (2.1)	(26)
	SRSF2	12/141 (8.5); 1/49 (2.0)	(26, 28)
chromosomal aberrations	del(5q)	3/141 (2.1); 1/7 (14.3)	(26, 27)
	tetrasomy 14	1/7 (14.3)	(27)
	tetrasomy 21	1/7 (14.3)	(27)
	i(7q)	1/7 (14.3)	(27)
	submicroscopic del(8q)	1/7 (14.3)	(27)
	submicroscopic del(6q)	1/7 (14.3)	(27)
	trisomy 8	1/7 (14.3)	(131)
	inv (9)(p11;q12)	1/7 (14.3)	(131)
	complex karyotype	1/7 (14.3)	(131)

hit" TAM mutations were not necessarily associated with progression to ML–DS (27).

Mutations in Cohesin Complex Genes

The cohesin complex is essential for the controlled course of mitosis, as it holds the replicated chromosomes together during metaphase. Still, in recent years, the role of the cohesin complex in transcriptional regulation has been recognized, as it organizes higher order chromatin structure. It was shown that the cohesin complex brings together enhancer and promoter regions by forming DNA loops (135). In this process, CTCF is known to cooperate with the cohesin complex to form DNA loops within topologically associated domains (130, 135).

Mutations in the four main components of the cohesin complex—SMC1, SMC2, RAD21, STAG2—are frequently found in ML–DS patients, but also in other myeloid neoplasms and solid cancers (26, 28). Cohesin complex mutations are mutually exclusive and in the majority of cases are loss of function mutations (136, 137). Additionally, recurrent *CTCF* mutations were identified, which are unique to the molecular landscape of ML–DS (28). In a murine GATA1s model using CRISPR-Cas9 to recreate the clonal evolution from TAM to ML–DS, cohesin complex and *Ctcf* loss-of-function mutations were significantly underrepresented compared to human ML–DS samples (26). Since this model lacked the presence of T21, these data might underline the importance of a T21 genetic background for the oncogenic effect of cohesin complex mutations. Moreover, species-specific functions of the cohesin complex during hematopoiesis cannot be excluded—an alternative explanation that requires further investigations. Previously, cohesin complex mutations were demonstrated to block differentiation in human HSPCs while increasing their self-renewal capacity, in line with data from a murine model (138, 139). Consistently, loss of *rad21* in zebrafish causes impaired hematopoiesis during embryonic development by preventing the expression of *runx1* (140). Deletion of *Smc3* resulted in severe pancytopenia and 100% mortality in mice (141). Of note, haploinsufficiency of *Smc3* led to a proliferative advantage over *Smc3* wild-type bone marrow cells and cooperated with *Flt3*-ITD in AML progression (141).

In addition to transcriptional regulation through the looping of DNA, changes in chromatin accessibility were observed in cohesin mutant and knock-down models (138, 139, 141). In contrast to a global reduction of chromatin accessibility, ERG, RUNX1, and GATA2 motifs displayed increased accessibility in cohesin mutant cells, suggesting that these transcription factors—which are also implicated in DS leukemogenesis—largely contribute to an enhanced transcriptional stemness program observed in cohesin mutant HSPCs (138).

Mutations in JAK-STAT-Signaling Pathways

Activating mutations in the tyrosine kinases JAK1, JAK2, and JAK3 were previously identified in AMKL in individuals with or without DS (26, 131–133, 142, 143). However, some variants seem to be exclusive to ML-DS (134). Interestingly, activating mutations were only identified in ML-DS, while the significance of JAK mutations in TAM samples was unknown or the variant caused loss of function, suggesting that aberrant activation of JAK-STAT signaling is essential for leukemic transformation in ML–DS (26).

Recently, a new hotspot mutation in *CSF2RB* was identified in ML-DS samples (26). *CSF2RB* encodes the common β chain of various cytokine receptors, which activate downstream JAK-STAT and other pathways. The $CSF2RB^{A455D}$ variant is predicted to lead to a constitutively active cytokine receptor due to aberrant dimerization of the transmembrane domains of two β chains or an α and a β chain—a hypothesis which is supported by the cytokine independent growth of TF1 cells harboring the $CSF2RB^{A455D}$ mutant (26). When the $CSF2RB^{A455D}$ mutant was expressed in HSPCs, a differentiation block in terminal megakaryopoiesis along with an expansion of immature erythroid cells was observed (26). These changes were reversed upon treatment with the JAK inhibitor Ruxolitinib, suggesting that the oncogenic potential of the $CSF2RB^{A455D}$ variant manifests in aberrant JAK-STAT-signaling. Further work showed that $CSF2RB^{A455D}$ activates TPOR, to drive pathogenic TPOR signaling in ML–DS (144).

Mutations in Epigenetic Modifiers and Altered DNA Methylation

ML–DS samples were shown to harbor a genome-wide pattern of hypomethylation discriminating them from non-DS AMKL, which in comparison displayed hypermethylation at the analyzed differentially methylated regions (145). Thus, this global hypomethylation seems to be driven by T21. Upon acquisition of *GATA1s* mutations certain genetic regions gain aberrant hypermethylation compared to T21-$GATA1^{wild\text{-}type}$ fetal liver cells and pathway analyses revealed that gene networks involved in cell cycle, cell signaling and proliferation were especially affected by this local hypermethylation implying functional relevance of these differentially methylated regions (145).

The importance of epigenetic changes for leukemic transformation in individuals with TAM is also underlined by the frequent identification of mutations in epigenetic modifiers, such as *EZH2*, *KANSL1*, and *SUZ12* (26, 28). However, the spectrum of mutated epigenetic regulators in ML–DS largely differs from the genes that are frequently mutated in adult AML, such as *DNMT3A*, *IDH1*, *IDH2*, and *TET2*.

Role of Non-Coding RNAs in the Development of ML–DS

MicroRNAs play pivotal roles as post-transcriptional regulators in leukemogenesis. We already discussed the impact of *miR-125b-2*, which is located on chromosome 21 and promotes megakaryocytic expansion upon increased gene dosage in T21 individuals (73).

Another microRNA implicated in DS leukemogenesis is *miR-486*, which is encoded within its host gene *ANK1* on chromosome 8. *ANK1* is a known target gene of GATA1 and *miR-486* levels were directly correlated with *GATA1s* expression in ML-DS samples (146). Overexpression of *miR-486* alone failed to transform fetal liver cells, but increased self-renewal capacity when expressed together with *GATA1s* (146). The oncogenic potential of *miR-486* might be exerted through activation of the PI3K-AKT pathway.

The importance of non-coding RNAs in DS leukemogenesis was also recently underlined by a large sequencing study analyzing samples of normal hematopoietic cells and different AML subgroups, such as ML–DS (147). It was shown that ML-DS samples harbor a non-coding RNA signature with similarities to healthy HSCs, characterized by the down-regulation of non-coding RNAs associated with differentiation (147). The lncRNAs *MONC* and *MIR100HG* are the host genes of the *miR-99a~125b-2* tricistron and its homolog on chromosome 11, respectively and were also implicated in AMKL and ML–DS pathogenesis, as knock-down of both lncRNAs resulted in reduced proliferation in corresponding leukemic cell lines (148). Interestingly, overexpression of a spliced form of *MONC* in HSPCs caused an erythroid lineage bias and expansion of immature erythroid cells independent from the *miR-99a~125b-2* tricistron (148).

Chromosomal Aberrations as Drivers for ML-DS Progression

In addition to the discussed point and indel mutations, the acquisition of structural chromosomal aberrations was also observed in ML–DS samples. These range from small submicroscopic deletions to tetrasomy of whole chromosomes (27). For instance, partial deletions of two regions of chromosome 5, which are usually not affected in other hematopoietic malignancies such as 5q-myelodysplastic syndrome, were found and resulted in deletion of the tumor suppressor APC (27).

As opposed to ML–DS samples, copy number alterations are hardly observed at the TAM stage, pointing to the transformative character of these genomic changes (27, 149). However, another study involving serial transplantations of primary TAM samples suggested that structural chromosomal changes may be already present in very small TAM subclones and expand due to positive selection upon progression to ML–DS (149).

CONCLUDING REMARKS

T21 predisposes individuals to the development of ML–DS, with pre-leukemic TAM already originating *in utero*. ML–DS displays a step-wise model of leukemogenesis offering the unique opportunity to investigate clonal evolution in myeloid leukemias.

As discussed in this review, the first step in DS leukemogenesis is the disruption of hematopoietic transcription factor networks resulting from the increased gene dosages of some members of these circuits due to T21, consequently leading to megakaryocytic

expansion (**Figure 2**). The acquisition of truncating *GATA1* mutations in this susceptible, highly proliferating cell population during fetal liver hematopoiesis marks the second step in DS leukemogenesis (**Figure 2**). Upon exclusive GATA1s expression, dysplastic megakaryocytic cells undergo uncontrolled expansion, accompanied by disrupted erythroid differentiation. Again, this aberrant proliferation facilitates the acquisition and positive selection of clones with additional somatic mutations, ultimately paving the way to progression to ML–DS.

Strikingly, recent studies in pediatric non-DS-AMKL also pointed towards developmental stage-specific effects of fusion genes essential to this entity (150) and suggested a fetal origin of the disease (150–152) similar to ML–DS.

In DS leukemogenesis the developmental stage-specific effects of T21 and *GATA1s* mutations and the impact of the fetal liver microenvironment, both contribute to the self-limiting nature of TAM in the majority of patients. However, further work needs to be done to better characterize the molecular synergy between T21 and GATA1s in driving ML-DS development, and to unravel the transformative features of additional somatic mutations, as not all TAM individuals with "third hit" mutations progress to ML–DS. The insights gained from such studies will shed light onto the mechanisms of genetic predisposition to cancer development, which can also be extrapolated to other entities, making ML–DS a valuable model of leukemogenesis.

AUTHOR CONTRIBUTIONS

JG drafted the manuscript. DH and JHK revised the content and approved the manuscript for publication. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding to JHK from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement #714226). JHK is a recipient of the St. Baldrick's Robert J. Arceci Innovation Award. DH is supported by the German Cancer Aid (#111743).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.