

MYELOID NEOPLASIA

Perturbation of fetal hematopoiesis in a mouse model of Down syndrome's transient myeloproliferative disorder

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Key Points

- Transient expansion of fetal megakaryocyte progenitors in ERG/Gata1s mouse is biologically similar to Down syndrome TMD.
- The N-terminal domain of GATA1 and the downregulation of ERG expression are essential for normal fetal erythropoiesis.

Children with Down syndrome develop a unique congenital clonal megakaryocytic proliferation disorder (transient myeloproliferative disorder [TMD]). It is caused by an expansion of fetal megakaryocyte-erythroid progenitors (MEPs) triggered by trisomy of chromosome 21 and is further enhanced by the somatic acquisition of a mutation in GATA1. These mutations result in the expression of a short-isoform GATA1s lacking the N-terminal domain. To examine the hypothesis that the Hsa21 ETS transcription factor ERG cooperates with GATA1s in this process, we generated double-transgenic mice expressing hERG and Gata1s. We show that increased expression of ERG by itself is sufficient to induce expansion of MEPs in fetal livers. Gata1s expression synergizes with ERG in enhancing the expansion of fetal MEPs and megakaryocytic precursors, resulting in hepatic fibrosis, transient postnatal thrombocytosis, anemia, a gene expression profile that is similar to that of human TMD and progression to progenitor myeloid leukemia by 3 months of age. This ERG/Gata1s transgenic mouse model also uncovers an essential role for the N terminus of Gata1 in erythropoiesis and the antagonistic role of ERG in fetal erythroid differentiation and survival. The human relevance of this finding is underscored by the recent discovery of similar mutations in GATA1 in patients with Diamond-Blackfan anemia. (*Blood*. 2013;122(6):988-998)

Introduction

Neonates with Down syndrome (DS) have hematologic abnormalities resulting from inappropriate fetal hematopoiesis,¹ characterized by expansion of fetal liver (FL) megakaryocyte-erythroid progenitors (MEPs) in second-trimester fetuses.^{2,3} In ~5% of children with DS, this expansion is dramatically enhanced by an acquired mutation in the X-chromosome transcription factor *GATA1*, resulting in a congenital transient clonal myeloproliferative disorder (TMD).⁴⁻⁶

GATA1 regulates the megakaryocytic-erythroid lineages.^{7,8} Inherited mutations in the N-terminal Zinc finger domains cause X-linked dyserythropoietic anemia and thrombocytopenia.⁹ The *GATA1* mutations found in DS-TMD cause the expression of a shorter truncated isoform (GATA1s) lacking the N-terminal transactivation domain. This protein is normally coexpressed with full-length *GATA1* due to an alternative translation initiation site or alternative splicing.¹⁰ Mice expressing only Gata1s exhibit transient early fetal megakaryocytic hyperproliferation without perinatal abnormalities.¹¹ This hyperproliferative was recently attributed to removal of inhibitory activity of the Gata1 N-terminal domain on E2F.¹²

The unique TMD of DS suggests a specific collaboration between the expansion of fetal MEPs caused by trisomy 21 and the differentiation arrest and hyperproliferation conferred by Gata1s.¹³ Several

chromosome 21 gene products have been suggested to cooperate with GATA1s in the generation of DS-TMD.^{2,6,12,14-18} Because a trisomy causes overexpression of many genes,¹⁹ it is possible that cooperation between several genes on the trisomic chromosome generates the hematopoietic phenotype.

The role of the chromosome 21 ETS transcription factor ERG²⁰ in hematopoiesis²¹ and leukemia²² places it as a potential regulator of MEP expansion in DS fetuses and a collaborator with GATA1s in TMD. *ERG* is expressed in megakaryocytic and erythroid progenitors and is downregulated upon their differentiation.⁶ ERG is essential for maintenance of fetal hematopoietic stem cells (HSCs).^{21,23} It is one of a heptad of transcription factors, bound in combinatorial interactions to HSC enhancers.²⁴ Developmental cooperation between ERG and GATA1 has been suggested by demonstrating their cobinding to regulatory elements of key hematopoietic genes such as *Scl/Tal1*.²⁴ ERG is also a known oncogene involved in chromosomal translocations in leukemia, Ewing sarcoma, and prostate cancer.^{25,26} We demonstrated in vitro and in vivo that ERG is a megakaryocytic oncogene that cooperates with Gata1s in transforming mouse megakaryocytic progenitors.^{6,16,17} Further support to the hematopoietic role of ERG was recently given by an experiment in which reversing *ERG* trisomy to functional

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disomy in the Ts65Dn mouse model of DS corrected the myeloproliferative syndrome observed in these adult mice.²⁷ Yet, unlike human DS fetuses, no prenatal hematopoietic abnormalities were reported in Ts65Dn mice.

To study the putative fetal developmental hematopoietic cooperation between ERG and Gata1s, we generated double transgenic mice expressing hERG and Gata1s. We show that similar to trisomy 21 in DS, increased expression of ERG by itself is sufficient to induce megakaryopoiesis and expansion of MEPs in FLs. We also demonstrate that expression of Gata1s together with ERG further enhances and prolongs hyperproliferation of fetal MEP and megakaryocytic progenitors, generating a human TMD-like gene expression profile and liver pathology. Strikingly, most male transgenic ERG/Gata1s did not survive beyond embryonic day 12.5 due to severe anemia, revealing the necessity of the N terminus of Gata1 and of downregulation of ERG for fetal erythropoiesis.

Materials and methods

Generation of double *ERG/Gata1s* transgenic mouse

The *ERG* transgene was generated as described previously.²⁸ In brief, the human *ERG3* hematopoietic isoform was cloned into the HS21/45-*vav* vector replacing hCD4.²⁹ Double transgenic mice expressing both ERG and Gata1s were generated by crossing Tg*ERG* males with homozygous *Gata1s* knock-in females.¹¹ Animal studies were approved by the institutional animal care and use committee of the Chaim Sheba Medical Center at Tel Hashomer.

Isolation and immunostaining of FL, hematopoietic stem, and progenitor cells

FL single-cell suspensions were washed in phosphate-buffered saline (PBS) with 0.05M EDTA, 0.1% bovine serum albumin. Lin progenitors were isolated using the lineage cell depletion kit (Miltenyi Biotec). Cells were stained with phycoerythrin-Cy7-conjugated C-kit, fluorescein isothiocyanate-conjugated Sca1, allophycocyanin (APC)-conjugated FCγR, and phycoerythrin-conjugated CD34 (eBioscience). Erythroid FL cells were stained with fluorescein isothiocyanate-conjugated Ter119 and APC-conjugated CD71 (eBioscience). For apoptosis assays, cells were stained with APC-conjugated annexin V (Enzo Life Sciences), washed with PBS, 1% fetal bovine serum, resuspended with propidium iodide (5 μg/mL), and were analyzed by flow cytometry (FACSCalibur; BD Biosciences). Flow cytometry data were analyzed by Flowjo software.

Colony-forming assay

FL single cells were cultured in methylcellulose (Stem Cell Technologies). For megakaryocytic colonies, cells were plated in Methocult 3231 supplemented with 50 ng/mL thrombopoietin (TPO) (PeproTech) and scored 5 to 7 days later. For erythroid colonies, cells were plated in Methocult 3334 containing 3 U/mL erythropoietin (EPO) and colonies scored 7 to 10 days later.

RNA preparation

Total cellular RNA was prepared using TRIzol (Life Technologies). Complementary DNA was generated using the Verso cDNA kit (Thermo Scientific). Real-time quantitative polymerase chain reaction (PCR) was done using SYBR Green and a Prism 7900HT Fast Sequence Detection System (Applied Biosystems).

Gene expression profiling

Experiments were performed using Affymetrix Mouse gene 1.0 ST oligonucleotide arrays (Affymetrix) with total RNA from each FL sample (GSE46481) (supplemental Methods, available on the *Blood* website).

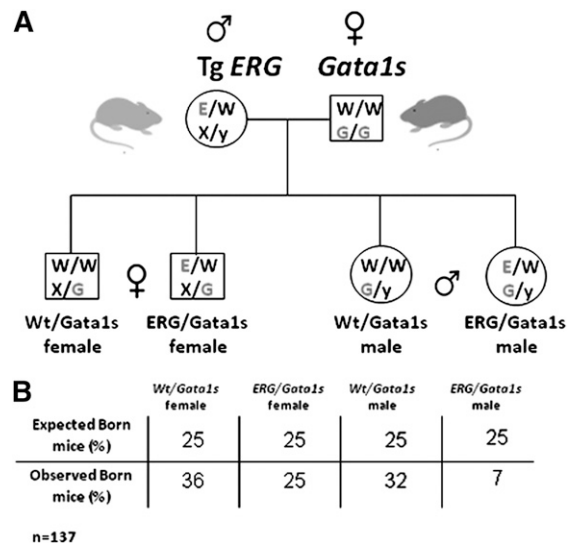


Figure 1. Generation of *ERG/Gata1s* mice. (A) Tg*ERG* males were mated with *Gata1s* knock-in homozygous females to generate animals that express the hERG transgene and the short isoform of GATA1, GATA1s. As GATA1 is located on the X chromosome; male offspring from this cross are all hemizygous and females are all heterozygous for GATA1s. Half of the animals carry the *ERG* transgene. (B) A table representing the observed vs the expected born mice from the different genotypes. Statistical significance was tested using the Fisher exact test ($P < .01$ for *ERG/Gata1s* males and $P = .09$ for *ERG/Gata1s* females; $n(\text{total}) = 137$) and the χ^2 test ($P = .0047$).

G1ME and FL cell infection and western blots

G1ME cells³⁰ and FL cells were infected with MIGR1-GATA1, MIGR1-GATA1s, or vector alone as described.³¹ Two days after infection, RNA was extracted and cell lysates were generated and the proteins were separated by gel electrophoresis. Anti-GATA-1 (sc-1234), HA-tag (sc-7392), FOG-1 (sc-9361), GATA-2 (sc-9008), and HSC70 (sc-7298) antibodies were purchased from Santa Cruz Biotechnology. The anti-Myb antibody (05-175) was purchased from Millipore.

Benzidine staining and cytopins

FL cells were stained with benzidine as described.³² Cytopins were generated by centrifuging 5×10^5 cells at 600 rpm for 10 minutes on glass slides and then Giemsa staining (Sigma-Aldrich).

Reticulin staining

FL tissues were fixed in 4% neutral-buffered formalin and paraffin-embedded. Reticulin staining was carried out using conventional techniques.

Results

Fetal expansion of megakaryocytic-erythroid and megakaryocytic progenitors in an *ERG/Gata1s* mouse model

We generated transgenic mice expressing the human *ERG3* hematopoietic isoform⁶ regulated by the *vav* promoter that is expressed in all hematopoietic lineages starting at embryonic day 9.5 (E9.5).³³ Four transgenic lines were generated with a similar phenotype. *ERG* expression was detected in yolk sacs from E10.5 and in FLs (supplemental Figure 1). The level of Tg*ERG* expression was comparable to the endogenous ERG expression levels in human cord blood CD34⁺ cells and in acute megakaryocytic leukemia (AMKL) cell lines (supplemental Figure 1). To model the fetal hematopoietic events of DS, Tg*ERG* male mice were crossed with homozygous *Gata1s* knock-in females.¹¹ This cross generates 4 genotypes

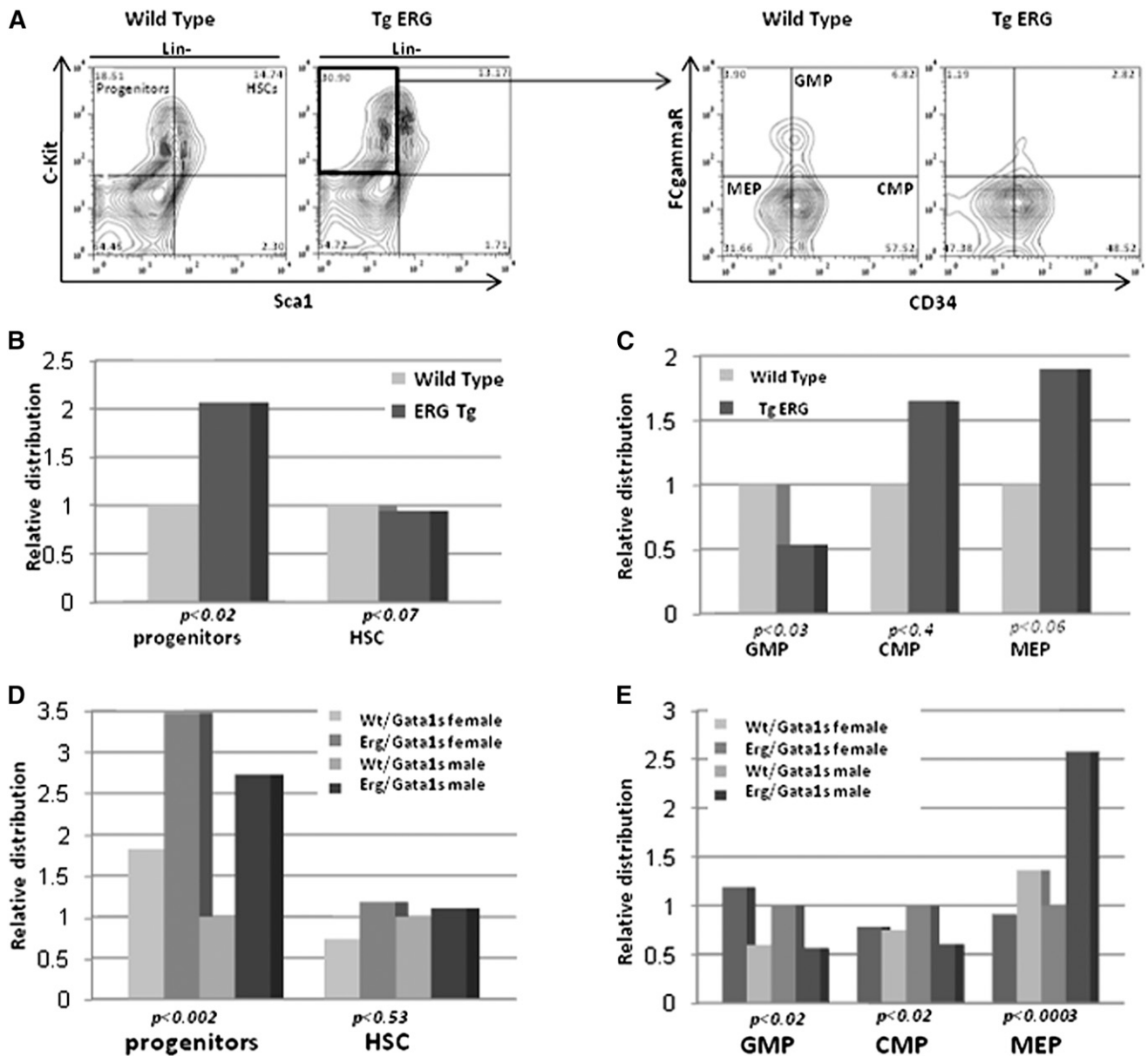


Figure 2. Expansion of MEPs in TgERG embryo FLs is enhanced by *Gata1s*. (A) Representative flow cytometric analysis showing immunostaining of lineage-negative (Lin^-) cells. HSCs are c-Kit^+ and Sca1^+ and HPCs are c-Kit^+ and Sca1^- . HPCs were subclassified into GMPs ($\text{Fc}\gamma\text{R}^+$ and CD34^+), CMPs ($\text{Fc}\gamma\text{R}^-$ and CD34^+), and MEPs ($\text{Fc}\gamma\text{R}^-$ and CD34^-), respectively. (B) Relative progenitor and stem cell populations in wild-type and TgERG E14.5 FLs. (C) Distribution of myeloid progenitors in wild-type and TgERG E14.5 FLs. (D) Relative progenitor and stem cell populations in *Wt/Gata1s* and *ERG/Gata1s* E14.5 FLs. (E) Distribution of myeloid progenitors in *Wt/Gata1s* and *ERG/Gata1s* E14.5 FLs. The bar graphs represent the average of at least 3 independent experiments with $n > 10$ embryos for each genotype. Statistical significant differences (t test for pairs and ANOVA for groups) are detailed in the figure. ANOVA, analysis of variance.

(Figure 1): females are heterozygous and males are hemizygous for *Gata1s* while 50% of the animals express the *hERG* transgene.

In humans, constitutional trisomy 21 causes a marked expansion of fetal MEPs preceding occurrence of the *GATA1s* mutation.^{2,3} We therefore examined whether increased ERG expression affects fetal hematopoietic progenitors. Lin^- cells isolated from E14.5 FLs were immunostained for c-Kit and Sca1 to distinguish between HSCs (Lin^- , Sca1^+ , c-Kit^+) and hematopoietic progenitor cells (HPCs) (Lin^- , Sca1^- , c-Kit^+).³⁴ HPCs but not HSCs were significantly expanded in FLs from TgERG embryos compared with Wt littermates (Figure 2A-B). *ERG* overexpression in FLs of E14.5 embryos caused a nearly twofold expansion of MEPs ($\text{Fc}\gamma\text{R}^-$, CD34^-)³⁴ (Figure 2A,C) compared with Wt embryos and a smaller increase in common myeloid progenitors (CMPs) ($\text{Fc}\gamma\text{R}^-$, CD34^+).

MEP expansion was on the expense of granulocyte/monocyte progenitors (GMPs) ($\text{Fc}\gamma\text{R}^+$, CD34^+) as a significant decrease in these progenitors was observed in TgERG embryos. Hence, increased expression of ERG during fetal hematopoiesis resulted in significant increase in MEPs, similar to the expansion observed in DS fetuses.^{2,3}

To study the consequences of ERG and *Gata1s* interactions on expansion and distribution of E14.5 FL progenitors, these immunostainings were repeated on Lin^- cells isolated from E14.5 FLs of *Wt/Gata1s* and *ERG/Gata1s* fetuses. Similar to the TgERG phenotype, *ERG/Gata1s* FLs exhibited extensive expansion of MEPs (Figure 2D-E). The magnitude of the expansion was higher in *ERG/Gata1s* fetuses than those expressing *ERG* alone (compare the scales in Figure 2, B-C to D-E; $P < .05$ for progenitors and

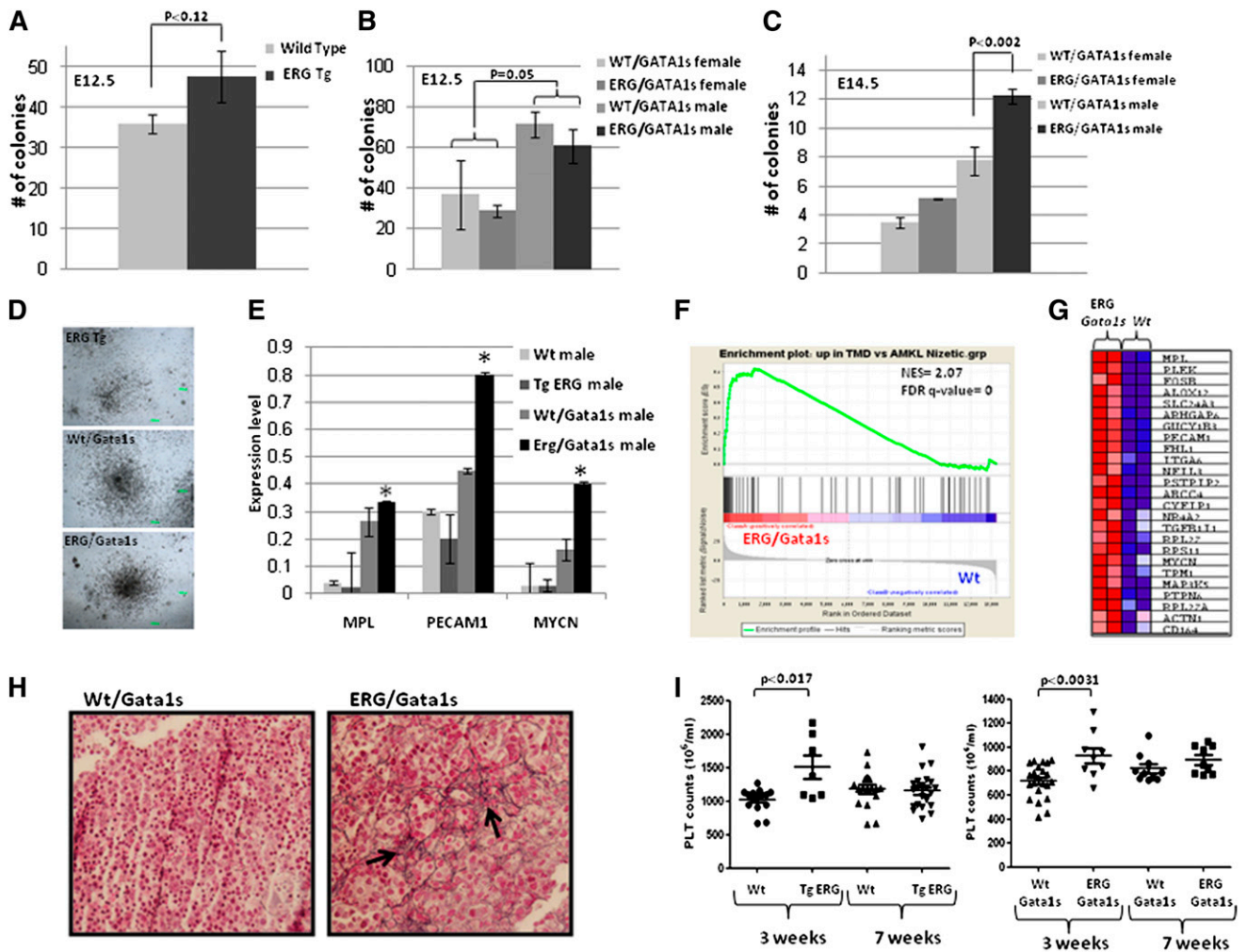


Figure 3. ERG synergizes with Gata1s to promote transient megakaryocytic proliferation presenting a human TMD expression profile. (A-C) FL cells were isolated from E12.5 (A-B) and E14.5 (C) wild-type, TgERG embryos and males and females of *Wt/Gata1s* and *ERG/Gata1s* embryos. The cells were plated on methylcellulose supplemented with TPO to promote the growth of megakaryocytic colonies (CFU-MK). MK colonies were counted 5 to 7 days following plating. Each bar graph represents the average of at least 3 independent experiments. (D) Representative figure of megakaryocytic colonies from (top) TgERG, (middle) *Wt/Gata1s*, and (bottom) *ERG/Gata1s* FLs. (E) Real-time PCR of common human TMD genes (11). *Statistical significance was tested using the *t* test ($P = .028$ for *Mpl*; $P = .009$ for *Pecam1*; and $P = .0$ for *Mycn*). (F) Gene set enrichment analysis (GSEA) using gene expression of E14.5 *ERG/Gata1s* FL cells (GSE46481) compared with the respective Wt control shows significant enrichment of genes that were upregulated in human TMD (GSE4119). NES and FDR q values are shown. (G) Heat map showing the core enrichment genes from the GSEA presented in panel F. (H) Hepatic fibrosis in *ERG/Gata1s* male FL. Reticulin staining (arrowheads) of (left) *Wt/Gata1s* and (right) *ERG/Gata1s* FL tissues (magnification, × 400). (I) Transient thrombocytosis in TgERG and *ERG/Gata1s* mice. Platelet counts were retrieved from male and female TgERG and *ERG/Gata1s* and their *Wt* and *Wt/Gata1s* littermates at 3 and 7 weeks of age; $n =$ at least 6 for each group. A significant difference was found between TgERG and *Wt* and between *ERG/Gata1s* and *Wt/Gata1s* (*t* test: $P < .017$, $P < .0031$, respectively). FDR, false detection rate; NES, normalized enrichment score.

$P < .06$ for MEPs), demonstrating an additive effect of the 2 transcription factors.

Both ERG and GATA1s are implicated in megakaryopoiesis.^{6,7,16,17,21} As TMD is a perinatal megakaryocytic proliferation syndrome, we next examined their effect on fetal megakaryopoiesis. FL cells were isolated from TgERG and *Wt* littermates and from *Wt/Gata1s* and *ERG/Gata1s* mice, and cultured in methylcellulose supplemented with TPO for growth of megakaryocytic colonies (colony forming unit megakaryocyte [CFU-MK]) (Figure 3A-D). Consistent with previous reports, we observed increased formation of CFU-MK colonies in E12.5 FL cells expressing *Gata1s*, as males (which are hemizygous for *Gata1s*) formed twice the number of colonies compared with females that express the *Gata1* Wt isoform (Figure 3B). At E12.5, the presence of the *ERG* transgene led to a slight increase in MK colonies on a *Gata1* wt background (Figure 3A) and caused no further enhancement of the effect of *Gata1s* (Figure 3B). This may result from the low level of *ERG* expression at E12.5

(supplemental Figure 1). In contrast, at E14.5, *ERG* significantly enhanced the number and size of *Gata1s* CFU-MK (Figure 3C-D). These observations indicate a synergistic effect of the 2 proteins in promoting fetal megakaryocytic proliferation.

The transient prenatal proliferation of megakaryoblasts in DS newborns represents a later stage of the progenitor expansion observed in DS FL.^{3,35} Therefore, we hypothesized that expanded fetal progenitors in *ERG/Gata1s* mice may have similar biological properties to human DS-TMD. To test this hypothesis, we profiled gene expression (supplemental Figure 2A) of FL RNA derived from E12.5 and E14.5 *Wt*, TgERG, *Gata1s*, and *ERG/Gata1s* pooled embryos. E14.5 FL cells from *ERG/Gata1s* mice demonstrated significantly increased expression of megakaryocytic genes (supplemental Table 1), including the *Mpl* gene and *Pecam1* in addition to *Mycn*, that were previously associated with DS-TMD³⁶ (Figure 3E). GSEA demonstrated significant enrichment of the *ERG/Gata1s* gene signature in DS-AMKL compared with

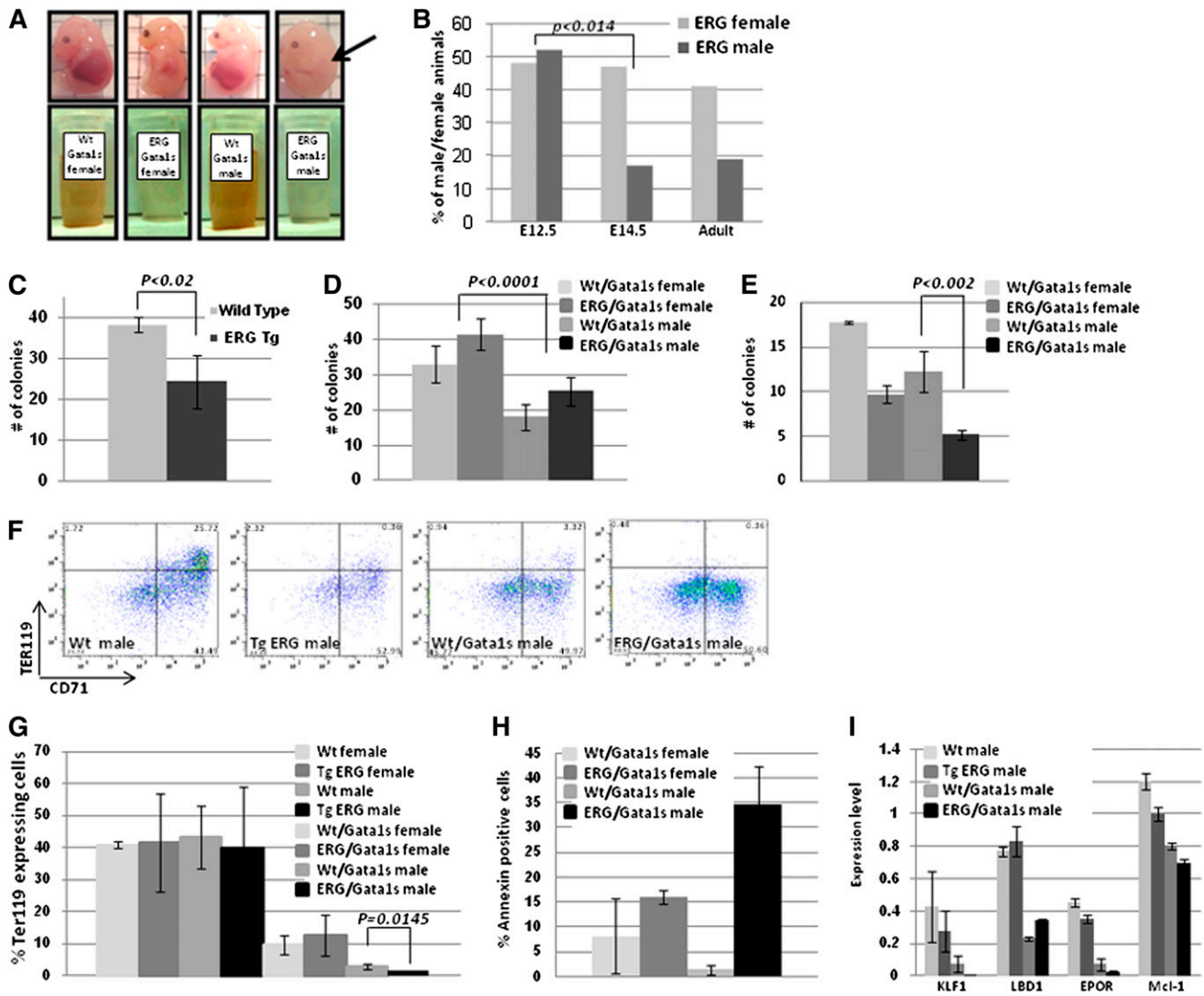


Figure 4. Requirement of the N terminus of GATA1 in fetal erythropoiesis. (A) Representative pictures of E14.5 embryos. The FL of *ERG/Gata1s* males is smaller and paler (arrow). A total of 1×10^7 FL cells were resuspended in PBS. The light color of the *ERG/Gata1s* FL cells points to the decrease number in mature erythrocytes in those embryos. (B) *ERG/Gata1s* males die between E12.5 and E14.5. Females and males generated from crossing heterozygous TgERG males with homozygous *Gata1s* KI females were tested for the presence of *ERG* transgene on E12.5, E14.5, and adults ($n = 85, 98$, and 137 for E12.5, E14.5, and adults, respectively). A significant difference in the TgERG male population was found between E12.5 and E14.5 (t test: $P < .014$). (C-E) *ERG* and *Gata1s* inhibit erythroid colony formation. FL cells were isolated from (C) E12.5 TgERG embryos and Wt littermates and (D-E) E12.5 and E14.5 embryos of *ERG/Gata1s* embryos and *Wt/Gata1s* littermates and were plated on methylcellulose supplemented with EPO to promote growth of erythroid colonies (BFU-E). Erythroid colonies (BFU-E) were counted 7 to 10 days after plating. Each bar graph represents the average of at least 3 independent experiments. Statistical significance was tested using the t test. (F) Representative figures of flow cytometric analysis of male E12.5 FL cells using the erythroid markers Ter119 and CD71. (G) Expression of *Gata1s* impairs erythropoiesis. Expression of Ter119 erythroid marker as measured by flow cytometry analysis in FL cells generated from E12.5 females and males of Wt, TgERG, *Gata1s*, and *ERG/Gata1s* animals. (H) Increase apoptosis in *ERG/Gata1s* males. Annexin V levels were measured by flow cytometry in Ter119-positive FL cells for the detection of apoptosis. FL cells were isolated from E12.5 Wt, TgERG, Wt/*Gata1s*, and *ERG/Gata1s* males. Significant increase in apoptosis in *ERG/Gata1s* males compared with Wt/*Gata1s* males was measured using the t test ($P = .017$). (I) Decreased expression of erythroid and antiapoptotic genes in *ERG/Gata1s* males. Expression level of the different genes was obtained from the Affymetrix mouse gene 1.0 ST chip array and confirmed by real-time PCR on at least 2 additional samples for each genotype.

non-DS-AMKL¹⁴ (supplemental Figure 2B) and in TMD³⁶ vs DS-AMKL (Figure 3F-G), which was higher than the enrichment observed with *Gata1s* alone (supplemental Figure 2C).

Hepatic fibrosis is a major complication of human DS-TMD.³⁷ Accordingly, liver fibrosis was demonstrated by reticulin stain in *ERG/Gata1s* FLs (Figure 3H). Postnatally, the transient feature of this fetal megakaryocytic progenitor expansion is evident by a transient increase in platelet counts in 3-week-old TgERG and *ERG/Gata1s* mice that was reduced to normal levels by 7 weeks (Figure 3I). Together, these phenotypic and genomic studies suggest that the coexpression of *ERG* and *Gata1s* causes a prenatal syndrome that is biologically similar to the TMD associated with DS.

Constitutive *ERG* expression uncovers a vital role for the N-terminal domain of GATA1 in fetal erythropoiesis

Genotyping offspring born to TgERG males and *Gata1s* homozygous females (Figure 1A) revealed a dramatic decrease in *ERG/Gata1s* live males (7%, expected 25%, $P < .01$) (Figure 1B). Gross examination of FLs at E14.5 revealed that the *ERG/Gata1s* males suffer from severe anemia; *ERG/GATA1s* FLs were considerably smaller than littermate FLs (Figure 4A arrow) and exhibited a paler cellular suspension (Figure 4A). Genotyping confirmed early embryonic death of *ERG/Gata1s* males between E12.5 and E14.5 (Figure 4B).

We therefore studied early fetal erythropoiesis in the transgenic embryos. E12.5 and E14.5 FL cells were isolated from females and males of *Wt*, *TgERG*, *Wt/Gata1s*, and *ERG/Gata1s* embryos and cultured in methylcellulose supplemented with EPO to promote erythroid colonies (burst-forming unit-erythroid [BFU-E]). In E12.5 *Gata1s* FLs, regardless of ERG expression, the number of erythroid colonies (BFU-E) was significantly lower in male FL cells, that are hemizygous for *Gata1s* (Figure 4D). The number of erythroid colonies (BFU-E) that were formed with E12.5 *TgERG* FLs was lower compared with *Wt* FL (Figure 4C). In E14.5, *Gata1s* and constitutive ERG expression synergized to inhibit BFU-E formation (Figure 4E). There was a significant drop in BFU-E colony number in FLs that express both *Gata1s* and *ERG* compared with the other genotypes.

To further study the effect of the 2 transcription factors on erythropoiesis, E12.5 FL cells were immunostained for expression of the erythroid marker Ter119 (Figure 4F-G). *Gata1s* expressing FLs had significantly reduced Ter119-expressing cells compared with their level in *Wt* FLs. The decrease in Ter119 cells was larger in hemizygous males (lacking a *Wt* *Gata1* protein). Although the presence of *ERG* with a *Wt* *Gata1* background had no effect on Ter119 cells, it added to the effect of *Gata1s*, leading to an almost complete absence of erythroid cells at E12.5 *ERG/Gata1s* males with only 1.4% of Ter119-expressing cells compared with 2.8% in *Wt/Gata1s* males ($P = .0145$) (Figure 4G). Furthermore, about one-third of Ter119 cells in *ERG/Gata1s* male FLs were apoptotic compared with 2% apoptosis found in the absence of the *ERG* transgene ($P = .017$) (Figure 4H). These phenotypes were reflected in gene expression profiles of E12.5 FL cells displaying a reduction in expression of erythroid genes (supplemental Tables 2-3), key erythroid transcription factors (Klf1, Ldb1), Epo receptor, and the antiapoptotic gene *Mcl1* (Figure 4I).

Together, these observations demonstrate that the lack of the N terminus of *Gata1* coupled with the constitutive expression of *ERG* block fetal erythropoiesis, resulting in severe anemia below the survival threshold for most *ERG/Gata1s* male embryos.

ERG synergizes with GATA1s to attenuate terminal erythroid differentiation

We next studied erythropoiesis in embryos surviving the initial block in erythropoiesis. Similar to E12.5 embryos, in E14.5 FL cells the generation of BFU colonies and the number of Ter119-positive cells were lower in *Gata1s* mice and were further reduced by the expression of *ERG* (Figure 4E, supplemental Figure 3A). These observations were accompanied by a decrease in the expression of erythroid genes (supplemental Figure 3B).

The combined expression of TER119 and CD71 can discriminate early and late differentiated erythroid cells (Figure 5A). The ratio between early (TER119⁺/CD71⁺) and late (TER119⁺/CD71⁻) erythroid cells was significantly higher in E14.5 *ERG/Gata1s* male FL cells (Figure 5B). Benzidine staining that measures hemoglobin carrying cells³² also showed that male *ERG/Gata1s* FL cells contain less differentiated erythrocytes as <10% of the cells were positive for benzidine staining compared with 20% observed in *ERG/Gata1s* FL female cells ($P = .0004$) (Figure 5C-D). Accordingly, cytopins show that *ERG/Gata1s* male FL cells contain mainly immature proerythroblasts (Figure 5E). Furthermore, the expression of the hemoglobin major β -chain, Hbb-b1, was reduced by 10-fold in the FLs of *ERG*- and *Gata1s*-expressing animals compared with *Wt* FLs (Figure 5F). The block in fetal erythroid differentiation was manifested by transient reduction in red blood

cell and hemoglobin levels in peripheral blood of 3-week-old *TgERG* and *ERG/Gata1s* mice compared with *Wt* and *Wt/Gata1s* mice (supplemental Figure 4). Consistently, GSEA analysis using gene expression of human erythroid cells at different stages of differentiation³⁸ shows enrichment of *Wt/Gata1s* and, more significantly, the *ERG/Gata1s* E14.5 FL gene signature in early erythroid cells (Figure 6A-B). Taken together, these results show that constitutive expression of ERG throughout erythroid differentiation and expression of *Gata1s* blocks terminal erythroid maturation.

Altered expression of the early erythroid genes GATA2 and Myb in ERG/Gata1s FL cells

Using the GSEA described in the previous paragraph for *ERG/Gata1s* FL (Figure 6A right panel), we identified *Gata2* among the top 20 core enrichment genes (Figure 6B). *Gata2* expression levels, as measured by expression array and confirmed by real-time PCR on separate embryos, was elevated in *ERG/Gata1s* E14.5 FLs (Figure 6C). *GATA2* expression is directly activated by ERG and repressed by GATA1.^{23,39} In G1ME cells,³⁰ *Gata2* expression was reduced by an average of threefold upon expression of *Gata1* compared with *Gata1s*-expressing G1ME cells (Figure 6D-E). In E14.5 *Wt/Gata1s* FL cells transduced with GATA1, *GATA2* expression was reduced by an average of 1.7-fold compared with its expression in E14.5 FL cells that expressed only GATA1s (Figure 6F). Both in G1ME and in the FL cells, *GATA2* expression was significantly reduced when GATA1 was expressed, despite the much higher expression levels of GATA1s (Figure 6D-F). This implies that repression by GATA1s is less efficient than repression by GATA1 and that even small amount of GATA1 is sufficient to repress *GATA2*.

Another potential cause of the persistent high levels of *Gata2* and the fetal anemia of *ERG/Gata1s* FL cells is the repression of *c-myb* by ERG. *c-myb* suppresses *GATA2* during erythroid differentiation.³⁸ Low levels of *c-myb* cause anemia and enhance megakaryopoiesis.^{40,41} ERG binds to *c-myb* promoter²⁴ and represses *c-myb* expression in transduced primary FL hematopoietic cells regardless of GATA1 expression.¹⁷ Consistent with this data, we observed a fourfold reduction in *c-myb* expression in *TgERG* FL cells (Figure 6C). Together, these results suggest that persistent expression of *Gata2* and suppressed expression of *c-myb* contribute to the block in erythroid differentiation in *ERG/Gata1s* FLs.

Discussion

We present here a detailed analysis of the developmental interactions between ERG and *Gata1s* during fetal hematopoiesis. The collaboration between these 2 mutated genes results in marked suppression of the generation of fetal erythroid cells, their differentiation and their survival, coupled with expansion of megakaryoid and megakaryocytic progenitors similar to those observed in DS-TMD.

DS-TMD is characterized by the transient expansion of fetal megakaryoblasts that continue in the first 3 months of life. Although often asymptomatic, some of the patients develop liver failure caused by liver fibrosis secondary to the infiltration of pathological fetal megakaryoblasts. Up to 30% of the DS neonates recovering from the TMD develop AMKL before the age of 4 years. The syndrome we observed in *ERG/Gata1s* transgenic mice is similar in the phenotype and gene expression of the expanded fetal cells, its transient nature, its association with liver fibrosis, and the

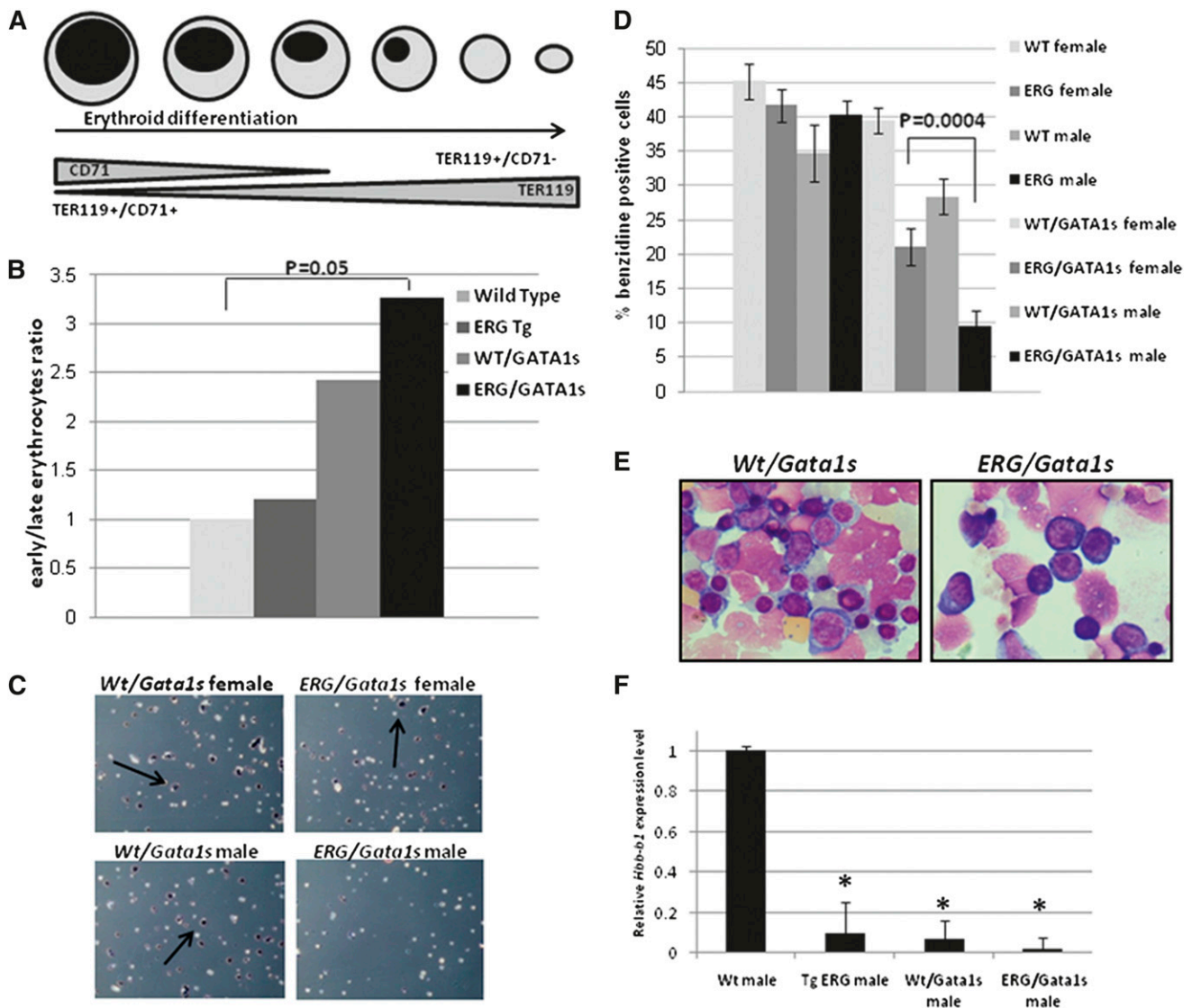


Figure 5. Erythroid differentiation arrest in *ERG/GATA1s* mice. (A) Schematic representation of erythroid differentiation indicating early erythrocytes as $Ter119^+/CD71^+$ and late differentiated erythrocytes as $Ter119^+/CD71^-$. (B) Increase in early erythroblast population compared with late differentiated erythrocytes in *ERG/Gata1s* males. FL cells were isolated from E14.5 Wild-type, TgERG, *Wt/Gata1s*, and *ERG/Gata1s* male embryos and stained with Ter119 and CD71. Statistical significance between *Wt* embryos and the remaining genotypes was measured using the *t* test. (C-D) Reduced benzidine-stained cells in *ERG/Gata1s* males. Representative images of benzidine-stained FL cells (C) and a graph averaging the ratio of benzidine-positive cells in FLs isolated from E14.5 *Wt*, TgERG, *Wt/Gata1s*, and *ERG/Gata1s* male and female embryos (D). The bar graph represents the average of at least 3 experiments. (E) Giemsa stain of cytopins of E14.5 FL cells show a decrease in maturing erythroblasts and increase in immature proerythroblasts in *ERG/Gata1s* males compared with *Wt/Gata1s* males. (F) Expression of hemoglobin. Adult hemoglobin β major chain (*Hbb-b1*) expression was measured by real-time PCR in E14.5 FL cells from *Wt*, TgERG, *Wt/Gata1s*, and *ERG/Gata1s* males. *Significant difference from *Wt* hemoglobin expression was measured using the *t* test ($P = .00037$ for TgERG; $P < .000001$ for *Wt/Gata1s*; and $P < .00001$ for *ERG/Gata1s*).

progression to megaerythroid progenitor leukemia (supplemental Figure 5). Yet, it differs in the timing of the TMD which is prenatal in the mice and perinatal in humans.

The current model for DS-TMD assumes 2 cooperating processes.¹³ First trisomy 21 causes expansion of fetal MEPs. Then, GATA1s, created by a somatic mutation in *GATA1*, blocks their terminal megakaryocytic differentiation and enhances their proliferation, leading to the perinatal TMD. Here we show that increased expression of a single chromosome 21 gene, ERG, can cooperate with Gata1s to create a TMD-like fetal hematopoietic state in a mouse model.

ERG was recently demonstrated to be required for maintenance of the FL HSCs.²³ We show here for the first time that constitutive hematopoietic expression of human ERG causes expansion of FL MEPs in a mouse model with no other genetic aberrations related

to DS. Although Gata1s enhanced early fetal megakaryopoiesis, as previously reported,¹¹ it did not affect the percentage of MEPs. These results support previous observations in human FLs^{2,3,35} that the expansion of fetal MEPs is caused by trisomy 21 preceding the “second hit” of the mutations in *GATA1*.

Constitutive transgenic expression of hERG, to a similar level observed in normal human hematopoietic precursors (supplemental Figure 1) cooperated with Gata1s to further expand the same fetal megakaryocytic precursor observed in human TMD as demonstrated by gene expression profiling. However, in humans, it is likely that the same effect is achieved by cooperation of several Hsa21 genes, such as ERG, ETS2,^{17,42} DYRK1A¹⁸ or miR-125-b2.¹² Yet, consistent with our results, Ng et al²⁷ reported that the addition of 1 copy of Erg was both necessary and sufficient for the postnatal myeloproliferation in a mouse model of DS.

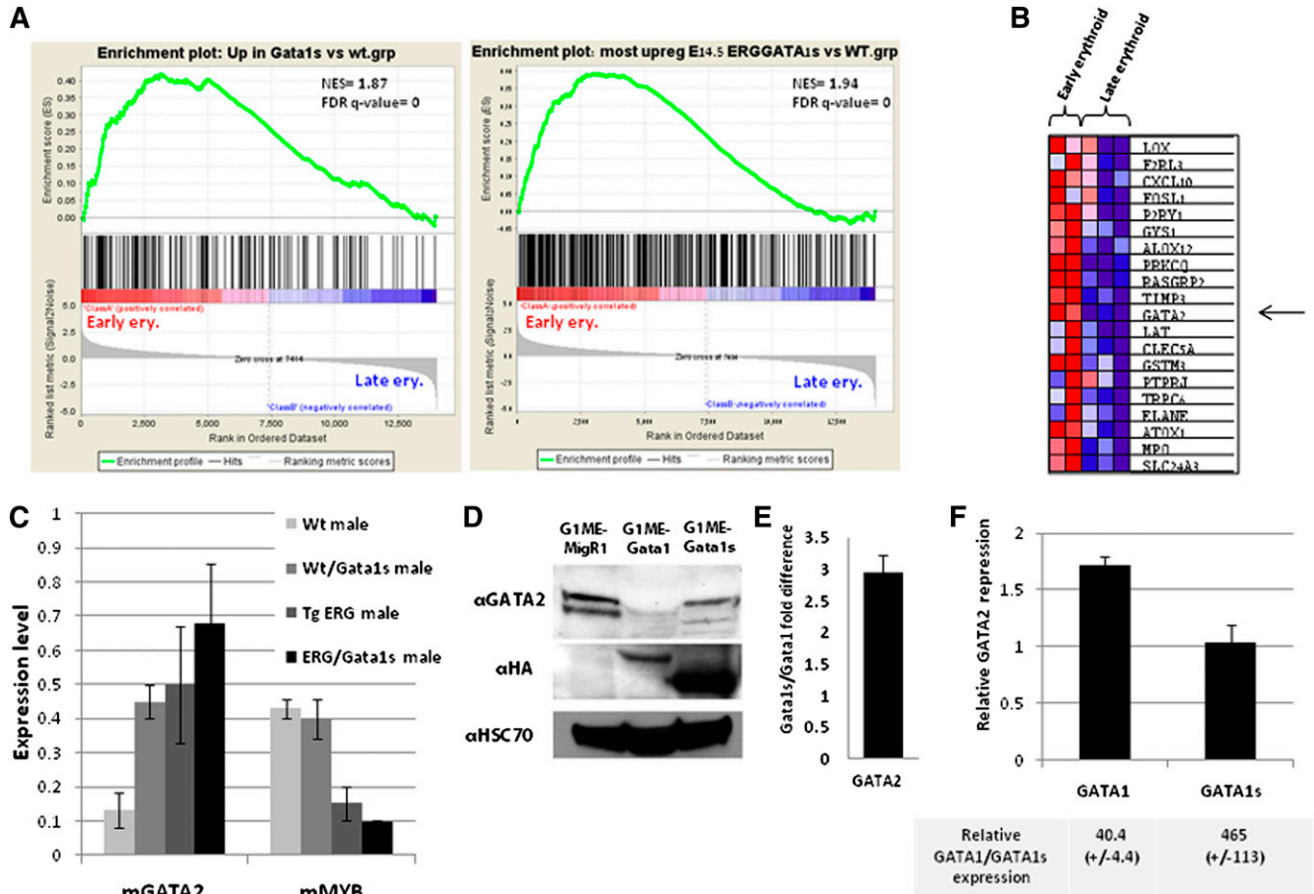


Figure 6. ERG/Gata1s FL cells present an early erythroid expression profile. (A) GSEA using gene expression of human erythroid cells at different stages of differentiation³⁸ shows enrichment of *Wt/Gata1s* and *ERG/GATA1s* E14.5 FL gene signature (GSE46481) in early erythroid cells. (B) Heat map showing the top 20 core enrichment genes from the GSEA presented in panel A, right. (C) Expression level of *Gata2* and *Myb* genes as obtained from the mouse gene 1.0 ST chip array and confirmed by real-time PCR on at least 2 additional samples for each genotype. (D) Representative western blot from G1ME cells transduced with MigR1, MIGR1-GATA1, and MIGR1-GATA1s using the indicated antibodies. (E) Average difference in GATA2 expression from 3 independent G1ME experiments presented in panel D. (F) An average repression of GATA2 mRNA by real-time PCR in E14.5 GATA1s KI FL cells infected with MIGR1-GATA1 and MIGR1-GATA1s relative to MigR1-infected cells. Relative GATA1 and GATA1s RNA expression levels are indicated below (n = 3).

The gene expression profile of *Gata1s/ERG* fetal cells was similar to both human TMD and DS AMKL, consistent with the development of DS leukemias from the same cells causing TMD. Specifically, we saw increased expression of *Mycn* in FLs of the double transgene *ERG/Gata1s* (Figure 3E). *Mycn* is highly expressed in DS-TMD³⁶ and was suggested to participate in the elimination of *Gata1*-mediated cell-cycle arrest via *Cdkn2c*.²⁹ Therefore, elevated *Mycn* levels may contribute to the hyperproliferation phenotype by repressing *Cdkn2c*, similar to what has been previously shown for *cMyc*.²⁹ Indeed, we see about a twofold decrease in *Cdkn2c* expression in *Gata1s* mice compared with animals expressing *Wt Gata1*, with the highest repression observed in *ERG/Gata1s* male FLs (supplemental Figure 6).

GATA1 transcription factor plays a key role in differentiation and maturation of erythroid cells by regulating numerous erythroid genes.⁴³⁻⁴⁵ However, the role of the N-terminal “transactivation” domain in erythropoiesis is unclear. The initial report of the *Gata1s* “knock in” mouse, used in our study, reported reduced fetal E12.5 erythropoiesis without any detailed analysis.¹¹

We show here that the expression of ERG dramatically enhances the fetal erythropoiesis defect caused by the elimination of the N terminus of *Gata1*. Embryos that express both *ERG* and *Gata1s* suffer from severe anemia as demonstrated by low cellularity in

their FLs (Figure 4A) and almost complete absence of erythroid cells (Figure 4G), leading to death of *ERG/Gata1s* males at embryonic day E13.5 (Figure 4B). Our detailed analysis reveals a block in all stages of erythropoiesis and decreased numbers and survival of erythroid progenitors coupled with arrest in erythroid differentiation. The erythroid phenomenon is not observed in children with DS because the GATA mutation in DS is somatic, occurring only in the TMD cells and not in the germline as in the *Gata1s* mouse.

ERG expression is normally decreased with erythroid differentiation⁶ (supplemental Figure 7). However, in this transgenic mouse model, ERG constitutive expression is controlled by the hematopoietic *vav* promoter. We demonstrate here that the differential regulation of ERG expression along erythroid differentiation is critical, and that lack of its normal downregulation causes a block in final erythroid maturation (Figure 7).

Consistent with these findings, we observed significant enrichment in early erythroid genes in E14.5 *ERG/Gata1s* FLs (Figure 6A right panel). *Gata2* which was among the top core enrichment genes (Figure 6B), is a key regulator of early erythropoiesis, and promotes the survival and proliferation of immature cells.⁴⁶ *Gata2* downregulation is required for erythroid differentiation.⁴⁷ In K562 cells overexpressing *Gata2*, a reduced growth rate and inhibition of erythroid differentiation was observed. More significantly, primary

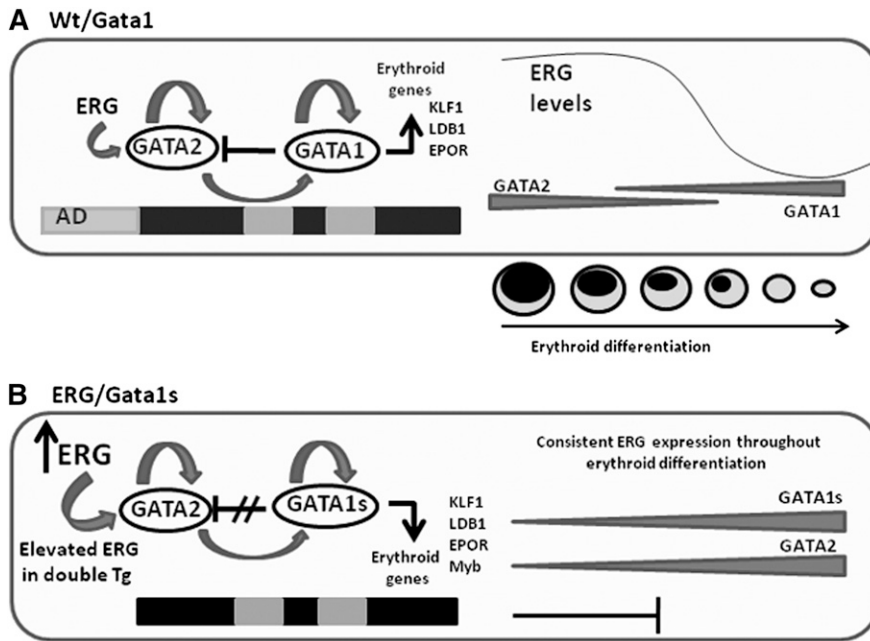


Figure 7. Schematic representation of the main events leading to impaired fetal erythropoiesis. (A) Gata2 which is expressed in erythroid progenitors, positively regulates Gata1 and its own expression.³⁹ Gata1 also positively regulates its own expression but represses Gata2. Progression in erythroid differentiation becomes possible due to the Gata1/Gata2 switch on the promoter of erythroid genes resulting in their activation. Gata2 repression is an essential step in erythroid differentiation and is executed by several factors including Gata1 and the decline in ERG expression. (B) In the *ERG/Gata1s* mice, several factors block erythroid differentiation. In the absence of the N-terminal domain of Gata1, Gata1s fails to repress Gata2, erythroid genes are not activated, and differentiation is blocked. Furthermore, the Gata2 repressor Myb is down-regulated by ERG and Gata1s. Because Myb functions as a Gata2 repressor, decreased Myb levels result in an increase in Gata2 expression. In the *ERG* transgenic model, the *ERG* expression level fails to decline with erythroid differentiation, thus further maintaining Gata2 expression and preventing erythroid cells to fully differentiate to erythrocytes.

adult erythroid progenitor cells that highly expressed Gata2 showed reduced growth, decrease in the number and proportion of mature erythroid cells, and reduced hemoglobin A and F levels in a dose-dependent manner with increasing amounts of GATA2 expression, suggesting that the blockage in erythroid maturation is due to increased expression of GATA2.⁴⁷

The regulation of erythroid gene expression and erythroid differentiation is governed by the interplay between GATA1 and GATA2, which share a common DNA-binding motif.³⁹ GATA2 directly activates GATA1 expression in early erythroid progenitors, and positively regulates its own expression, whereas GATA1 accelerates its own expression and represses GATA2,³⁹ allowing occupancy of regulated erythroid genes by GATA1 during erythroid maturation³⁹ (Figure 7). The absence of the transactivation domain in Gata1s alleviates the repression of Gata2 by Gata1; Gata2 levels were higher in Gata1s FL cells compared with those that express wt Gata1 (Figure 6C). Moreover, induction of GATA1 but not GATA1s in G1ME cells and in GATA1s FLs results in GATA2 repression (Figure 6D-F). These results indicate a specific role for the N-terminal domain of Gata1 in Gata2 transcriptional regulation and thus in regulating the transcriptional network in early erythropoiesis.

As shown by chromatin immunoprecipitation studies, Gata2 is a direct target of ERG, which positively regulates its expression in fetal megaerythroid progenitors.²³ The constitutive expression of vav-driven transgenic hERG throughout erythropoiesis may further explain the consistent high levels of Gata2.

The upregulation of Gata2 in *ERG/Gata1s* transgenic FL cells may also be explained by the repression of c-myb in these mice. c-Myb is expressed in early erythroid differentiated cells and is downregulated with differentiation by Gata1 in association with the transcription co-repressor FOG1.⁴⁸ It was proposed that c-Myb may also function as a Gata2 repressor because Gata2 expression levels were increased by fivefold in c-Myb^{KD/KD} E14.5 FL cells, inducing an immature erythroid phenotype.⁴⁰ Furthermore, ERG is a direct repressor of c-myb as previously shown by ChIP-Seq and gene expression studies.^{17,24} Thus, we suggest that persistent expression of Gata2 coupled with the repression of c-myb play a

central role in the fetal erythropoiesis defects observed in *ERG/Gata1s* embryos.

In summary, we present here an *ERG/Gata1s* double transgenic mouse model of TMD. We show that moderate increased expression of ERG was sufficient to generate expansion of the MEP compartment in FLs and that expression of Gata1s, together with ERG, enhances this expansion. We also demonstrated the requirement of ERG downregulation and of the N-terminal domain of Gata1 for embryonic and fetal erythropoiesis. The relevance of these findings to human diseases is highlighted by a previous case report of anemia in humans with GATA1s germline mutation⁴⁹ and the recent discovery of GATA1s mutations in some patients with the congenital Diamond-Blackfan anemia.⁵⁰

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Authorship

Contribution: Y.B. designed and performed experiments, analyzed data, and wrote the paper; L.G. performed experiments, analyzed

data, and helped write the paper; T.M.C., B.G., and G.S. performed experiments; I.M. provided research assistance; J.J.-H. performed array experiment and analysis; G.R. provided guidance; J.D.C. assisted in manuscript preparation; and S.I. designed the study, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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