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Integrating Enhancer Mechanisms to Establish a Hierarchical Blood Development Program

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SUMMARY

Hematopoietic development requires the transcription factor GATA-2, and GATA-2 mutations cause diverse pathologies including leukemia. GATA-2-regulated enhancers regulate *Gata2* expression in hematopoietic stem/progenitor cells and control hematopoiesis. The +9.5 kb enhancer activates transcription in endothelium and hematopoietic stem cells (HSCs), and its deletion abrogates HSC generation. The -77 kb enhancer activates transcription in myeloid progenitors, and its deletion impairs differentiation. Since +9.5^{-/-} embryos are HSC-deficient, it was unclear whether the +9.5 functions in progenitors or if GATA-2 expression in progenitors solely requires -77. We further dissected the mechanisms using -77/+9.5 compound heterozygous (CH) mice. The embryonic lethal CH mutation depleted megakaryocyte-erythrocyte progenitors (MEPs). While the +9.5 suffices for HSC generation, the -77 and +9.5 must reside on one allele to induce MEPs. The -77 generated Burst Forming Unit-Erythroid through induction of GATA-1 and

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AUTHOR CONTRIBUTIONS

C.M., K.D.J. and E.H.B. conceived all studies and designed experiments. Experiments were conducted by C.M., K.D.J., X.G., K.R.K. and S.M. E.A.R. conducted Giemsa staining. Computational/statistical analysis was conducted by IMO. The manuscript was drafted by C.M., K.D.J. and E.H.B. and revised and approved by all authors.

ACCESSION NUMBERS

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other GATA-2 targets. The enhancer circuits controlled signaling pathways that orchestrate a GATA factor-dependent blood development program.

Keywords

GATA-2; Enhancer; Hematopoiesis; Erythroid; Myeloid; Progenitor; Mouse model

INTRODUCTION

Genetic networks governing complex developmental processes are commonly established and maintained by master regulatory transcription factors (Orkin and Zon, 2008). When stem and progenitor cells differentiate into blood cells, GATA-2 serves this function (Tsai et al., 1994). GATA-2 also controls non-hematopoietic processes, including pregnancy (Rubel et al., 2016), neurogenesis (Zhou et al., 2000), renal function (Yu et al., 2014) and prostate cancer (Vidal et al., 2015). *Gata2* nullizygous mouse embryos are severely anemic and die at ~E10.5 (Tsai et al., 1994). Studies with *Gata2*^{+/-} mice revealed that GATA-2 ensures steady-state HSC levels in adults (Ling et al., 2004; Rodrigues et al., 2005). Targeted deletion of an intronic *Gata2* enhancer 9.5 kb downstream of the transcription start site (+9.5) abrogated HSC genesis in the aorta-gonad-mesonephros (AGM) region, yielding ~E14 lethality (Gao et al., 2013; Johnson et al., 2012). Analysis of conditional *Gata2* knockout mice yielded similar results (de Pater et al., 2013; Lim et al., 2012). In addition to regulating HSC genesis and function, GATA-2 confers myeloid progenitor differentiation (Johnson et al., 2015) and Granulocyte Macrophage Progenitor (GMP) function (Rodrigues et al., 2008). Myeloid progenitors lacking the *Gata2* enhancer 77 kb upstream of the start site (-77) generate macrophages, but not erythroid or granulocytic cells, *ex vivo* (Johnson et al., 2015).

Human heterozygous *GATA2* mutations cause primary immunodeficiency, myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and vascular/lymphatic dysfunction (Dickinson et al., 2011; Hahn et al., 2011; Hsu et al., 2011; Ostergaard et al., 2011), reinforcing the importance of GATA-2 mechanisms derived from mouse models (Gao et al., 2013; Johnson et al., 2012; Johnson et al., 2015; Ling et al., 2004; Rodrigues et al., 2005; Tsai et al., 1994). Mutations in the DNA binding C-terminal zinc finger inhibit binding (Hahn et al., 2011), while +9.5 enhancer mutations reduce *GATA2* expression (Hsu et al., 2013; Johnson et al., 2012). Mutations also occur in the N-terminal zinc finger that is not required for DNA binding (Ping et al., 2017). GATA-2 promotes AML cell proliferation (Katsumura et al., 2016) and mediates leukemogenic activity in a mouse model involving Tet2 deficiency and mutant Flt3(ITD) expression (Shih et al., 2015). Leukemogenic cohesin mutants increase GATA-2 chromatin occupancy in human HSPCs, and GATA-2 is implicated in the cohesin mutant-induced differentiation block (Mazumdar et al., 2015).

The results described above indicate that GATA-2 levels/activity must be stringently regulated to ensure normal hematopoiesis. Considering the low frequency of GATA motif (WGATAR) occupancy in cells (<0.1%), based on genome-wide chromatin occupancy analysis (Fujiwara et al., 2009; Kang et al., 2012), and differential GATA-2 occupancy in distinct systems (Beck et al., 2013; DeVilbiss et al., 2014; Dore et al., 2012; Fujiwara et al.,

2009; Li et al., 2011; Wilson et al., 2010; Wu et al., 2014b), it is instructive to compare mechanisms governing GATA-2 expression/function in different cell types. A major mechanism determining GATA-2 levels involves the +9.5 and -77 enhancers (Gao et al., 2013; Grass et al., 2006; Johnson et al., 2012; Johnson et al., 2015). Although GATA-2 occupies both enhancers, only the +9.5 triggers HSC genesis in the mouse embryo. Transcriptomes from *Gata2* enhancer knockout mice (Gao et al., 2013; Johnson et al., 2012; Johnson et al., 2015), *Gata2* conditional knockout mice (de Pater et al., 2013) and GATA-2-knockdown endothelial cells (Linnemann et al., 2011) suggest GATA-2-regulated genetic networks differ considerably.

To compare GATA-2 expression and function in different contexts, we developed a compound heterozygous (CH) model bearing +9.5 and -77 enhancer mutations on different *Gata2* alleles. Dissecting mechanisms in this system and in -77^{-/-} erythroid precursors revealed that while the +9.5 suffices to trigger HSC genesis, both enhancers must reside on a single allele to induce megakaryocyte erythrocyte progenitors (MEPs). GATA-2-dependent transcriptomes in myelo-erythroid progenitors and erythroid precursors differed greatly. A critical -77 enhancer function involved establishing circuits required for BFU-E activity, the vital erythroid precursor that ensures steady-state erythropoiesis and red blood cell regeneration in anemic stress (Koury, 2014).

RESULTS

Genetic Interactions Between *Gata2* Enhancers Reveal a Mechanism Underlying a Hierarchical Blood Development Program

Whereas the +9.5 enhancer confers *Gata2* expression in hemogenic endothelium and HSPCs (Gao et al., 2013; Johnson et al., 2012), the -77 enhancer increases *Gata2* expression in myeloid progenitors, but not HSCs (Johnson et al., 2015). However, both enhancers contain conserved GATA motifs, which can be GATA-2-occupied. To test whether the distinct mechanisms establish unique GATA-2-regulated enhancer activities *in vivo*, we compared enhancer functions in distinct sectors of the hematopoietic hierarchy. As the +9.5 enhancer deletion abrogates HSC genesis in the mouse embryo and is lethal at ~E14 (Gao et al., 2013; Johnson et al., 2012), it was unclear whether it functions in myeloid progenitors (Figure 1A). The -77 enhancer deletion is lethal after E15.5 (Johnson et al., 2015). +9.5^{+/-} and -77^{+/-} mouse strains are viable, and adult steady-state hematopoiesis is not impaired (Johnson et al., 2012; Johnson et al., 2015). We tested whether downregulating GATA-2 in endothelium and HSCs (+9.5^{+/-}) and myeloid progenitors (-77^{+/-}) concomitantly impacts hematopoiesis in a way predictable by phenotypes of individual enhancer homozygous mutations or uniquely.

We considered the following models: (i) +9.5 might also function in myeloid progenitors, and compound heterozygous (CH) mice would phenocopy -77^{-/-} mice. (ii) +9.5 and -77 might function in mutually exclusive contexts, and CH mice would resemble individual enhancer heterozygous mice. (iii) CH mutations might compromise hematopoiesis in a way not predictable from individual enhancer mutations. To test these models, CH mice, bearing a mutant +9.5 enhancer on one allele and a mutant -77 on the other allele, were produced by mating +9.5^{+/-} and -77^{+/-} mice to yield WT, +9.5^{+/-}, -77^{+/-} and CH progeny. Unlike

+9.5^{+/-} and -77^{+/-} genotypes, no CH mice were alive at weaning (Figure 1B). The CH embryos were alive at E13.5, but most died by E14.5. *Gata2* is expressed in endothelial cells (Linnemann et al., 2011; Wilson et al., 1990) and confers vascular integrity (Johnson et al., 2012; Lim et al., 2012). +9.5^{-/-} but not -77^{-/-}, embryos, exhibit hemorrhage (Johnson et al., 2012; Johnson et al., 2015). CH fetal liver size was reduced, and hemorrhaging was apparent (Figure 1C). HSC genesis in the +9.5^{-/-} AGM is defective, as HSC-containing clusters within the AGM are absent, the mutant AGM lacks long-term repopulating HSCs in transplant assays (Gao et al., 2013) and the fetal liver does not acquire long-term repopulating HSCs (Johnson et al., 2012). -77^{-/-} embryos produce normal HSC levels in the AGM (Johnson et al., 2015). A homozygous +9.5 mutation ablates HSC-containing c-Kit⁺ cells residing in clusters that emerge in the AGM (Gao et al., 2013). By contrast, c-Kit⁺ cell quantitation with E10.5 +9.5^{+/-} and CH embryos revealed 29% ($p = 0.010$) and 43% ($p < 0.0001$) decreases vs. WT embryos respectively (Figure 1D). As the +9.5^{+/-} and CH results did not differ significantly, the CH mutation did not exacerbate the partial inhibitory effect of +9.5 allele loss on c-Kit⁺ cell emergence in the AGM.

+9.5^{-/-} and -77^{-/-} embryos are anemic with reduced fetal liver size and a deficiency of Ter119⁺ cells (Johnson et al., 2012; Johnson et al., 2015). At E13.5, CH liver cellularity was 4.4-fold lower than WT littermates (Figure 2A). +9.5^{+/-} and -77^{+/-} fetal livers were comparable to WT. Unlike the dearth of HSCs in +9.5^{-/-} fetal livers (Johnson et al., 2012), HSCs in -77^{-/-} fetal liver are higher than WT (Johnson et al., 2015). As the -77 homozygous mutation did not reduce HSCs or impact *Gata2* expression in Lineage⁻ Sca1⁺c-Kit⁺ (LSK) cells, it appears that the -77 enhancer does not function in HSCs. However, *Gata2* expression was not quantified in -77^{-/-} HSCs (LSK CD150⁺CD48⁻), and if the -77 enhancer contributed modestly to *Gata2* expression in HSCs, deleting the -77 enhancer from one allele and the +9.5 enhancer from the other, might impact HSC levels. Flow cytometric analysis of fetal liver HSCs and MPPs (Figure S1) revealed a 2.9-fold reduction in +9.5^{+/-} HSCs ($p = 0.003$) and a 2.5-fold increase in MPPs ($p = 2 \times 10^{-6}$) versus WT littermates (Figure 2B). CH and +9.5^{+/-} embryos contained indistinguishable HSC and MPP numbers, despite the 4.1-fold difference in CH liver cellularity ($p < 2 \times 10^{-17}$). The failure of the -77 enhancer deletion to exacerbate HSC loss caused by +9.5 deletion from the other allele (Figs. 1D and 2B) provides further evidence that the -77 is dispensable in HSCs.

Reduced GATA-2 expression in -77^{-/-} myeloid progenitors caused a several-fold decrease in MEPs and biased CMP and GMP activities to differentiate predominantly into macrophages, but did not affect HSCs (Johnson et al., 2015). As CH embryos have modestly reduced HSC numbers, based on flow cytometry of fetal liver (2.9 fold) and AGM confocal imaging (41%), we considered how this might impact myeloid progenitors. Flow cytometric analysis of Lin⁻Sca1⁻Kit⁺ myeloid progenitors (Figure S2) revealed +9.5^{+/-} fetal livers had 2-fold fewer progenitors, which involved similar reductions of CMP (2.1-fold; $p = 1 \times 10^{-6}$), GMP (2.6-fold; $p = 0.0007$) and MEP (1.9-fold; $p = 0.002$) (Figure 2C). Myeloid progenitors were 6.6-fold lower in CH vs. WT embryos ($p = 1.8 \times 10^{-11}$), with the difference involving disproportionate losses of GMP (8.6-fold; $p = 3.7 \times 10^{-6}$) and MEP (379-fold; $p = 3.2 \times 10^{-9}$). The 2.2-fold CMP decrease in CH ($p = 1.2 \times 10^{-6}$), resembled +9.5^{+/-} embryos. Despite the similar CMP numbers, *Gata2* mRNA levels in +9.5^{+/-} CMPs did not differ significantly from that of WT or 77^{+/-} CMPs. *Gata2* mRNA was 9.6-fold

lower ($p = 0.008$) in CH CMPs (Figure 2D) and 5.3 fold lower in $77^{-/-}$ CMPs than WT (Johnson Sci. Adv 2015).

Consistent with myeloid progenitor reductions and MEP depletion, CH fetal livers lacked BFU-E and CFU-GEMM (Figure 2E). Colonies containing granulocytes and/or macrophages (enumerated as CFU-GM) were reduced significantly. Giemsa staining of cells within CFU-GM colonies after 8 days of culture revealed abundant macrophages and neutrophils in CH colonies, with a deficiency of the myeloid precursors common in WT, $77^{+/-}$ and $9.5^{+/-}$ colonies (Figure 2F). The genetic interaction between $+9.5$ and -77 enhancer alleles in myeloid progenitors, but not HSCs, revealed a novel mechanism in which one *Gata2* allele must harbor both enhancers to induce MEPs, the critical red blood cell and platelet precursor (Akashi et al., 2000). Both enhancers function in progenitors to induce MEPs. These results highlight the uniqueness of the CH model, relative to other *Gata2* enhancer mutants, *Gata2* conditional knockout and *Gata2* whole body knockout models, vis-à-vis phenotypes and utility for dissecting mechanisms underlying GATA-2 function and hematopoiesis.

Gata2* -77 Enhancer-dependent Erythroid Maturation *In Vivo* and *Ex Vivo

GATA-2 is expressed in erythroid precursors (Leonard et al., 1993; Weiss et al., 1994), and as GATA-1 expression rises, it represses *Gata2* transcription through a GATA switch mechanism (Bresnick et al., 2010; Grass et al., 2003). However, the consequences of premature GATA-2 loss on erythroid precursor function are unclear. Unanswered questions include whether mechanisms mediating GATA-1 upregulation are sustained, whether GATA-2-deficient erythroid precursors precociously differentiate or die and if GATA-2-deficient erythroid precursors acquire an ectopic fate change?

As CH embryos lacked MEPs, the CH model could not be used to ascertain the impact of reduced GATA-2 expression on MEP-derived erythroid precursors. We used the $-77^{-/-}$ model characterized by reduced, but detectable, MEPs to address this issue. $-77^{-/-}$ E13.5 fetal livers have reduced percentages of R1 (CD71^{med}Ter119⁻) and R2 (CD71^{high}Ter119⁻) erythroid precursors, suggesting defective erythroid precursor genesis, proliferation and/or survival (Johnson et al., 2015). The prior study did not establish whether reduced erythroid precursors reflected impaired upstream progenitors or an erythroid cell-intrinsic -77 enhancer requirement. $-77^{-/-}$ E14.5 fetal livers were pale and smaller than WT (Figure 3A). $-77^{-/-}$ fetal liver cellularity was reduced 7.2-fold (Figure 3B), with major decreases in basophilic and orthochromatic erythroblasts, as well as reticulocytes, despite normal numbers of nucleated primitive red cells (EryP) (Figure 3C). Flow cytometric analysis of erythroid maturation revealed an increased percentage of R1 cells in $-77^{-/-}$ fetal livers (from 4.2 to 26%, $p = 0.0001$) (Figure 3D). Though the percentage of $-77^{-/-}$ R1 cells decreased at E13.5 (Johnson et al., 2015), at E14.5, the proportion of $-77^{-/-}$ R1 cells increased concomitant with decreased R3 cells (Figure 3D). When adjusted for liver size, there was little to no difference in the number of E14.5 R1 cells per $-77^{-/-}$ liver vs. WT littermates ($p = 0.31$). By contrast, $-77^{-/-}$ R2-R5 cells declined sharply (R2, 8.2-fold, $p = 0.004$; R3, 14-fold, $p < 10^{-5}$; R4, 9.7-fold, $p = 0.002$; R5, 14-fold, $p = 0.087$), indicating a maturation defect (Figure 3D). Annexin VDRAQ7 flow cytometric analysis (Figure S3) revealed $-77^{-/-}$

R1, R2 and R3 fetal liver cell populations contained early, but not late, apoptotic cells (Figure 3E). Most of the cells (~90%) were live and morphologically normal. The profound maturation defect provided rationale for dissecting how the -77 enhancer controls the erythroid maturation program.

To test for intrinsic maturation and/or survival defects, lineage-depleted fetal liver progenitors were cultured for 72 hours in expansion medium containing Stem Cell Factor (SCF) and Erythropoietin (Epo). Flow cytometric analysis of CD71 and Ter119 revealed a maturation blockade for the mutants. After 72 hours *ex vivo* culture, WT cells distributed largely in R2 (24%) and R3 (55%) populations (proerythroblasts and basophilic/polychromatic erythroblasts, respectively), with small percentages in R1 (8%) and R4 (2.6%) (Figure 4A). By contrast, 32% of -77^{-/-} cells persisted in the R1 population, with reduced R3 (24%) and R4 (0.8%) cells, recapitulating the maturation blockade detected with uncultured livers (Figure 4A). The expanded cells were cultured in high Epo-containing medium (6 U/ml) to promote erythroid maturation. Resembling the expansion culture, -77^{-/-} cells had reduced R3 versus WT (-77^{-/-}, 36%; -77^{+/-}, 59%, $p < 0.05$), and R1 cells accumulated in the 77^{-/-} population vs. WT (9 and 3.6%, respectively, $p = 0.043$) (Figure 4B). CD71⁻Ter119⁻ (non-erythroid) cells persisted in the -77^{-/-} population (22 and 3.4%, respectively, $p < 10^{-5}$). Giemsa staining and morphological analysis indicated that -77^{-/-} cultured cells contained a higher percentage of proerythroblasts (WT, 13%; -77^{-/-}, 41%, $p = 0.003$) concomitant with reduced more mature basophilic/polychromatophilic (59 versus 38%) and orthochromatic (14 versus 4.6%, $p = 0.047$) erythroblasts (Figure 4C). Thus, the erythroid maturation blockade involves an intrinsic defect in -77^{-/-} precursors that cannot be rescued with high Epo.

Generating the Vital Erythroid Precursor Burst Forming Unit-Erythroid

Considering the large decline of R2–R5 cells in -77^{-/-} fetal livers, we analyzed mechanisms underlying the maturation blockade by comparing transcriptomes of FACS-isolated 77^{-/-} and WT R1 cells from E14.5 livers (Figure 5A). Monocytes (Mac1⁺Gr1⁻), granulocytes (Mac1⁺Gr1⁺), B cells (B220⁺) and T cells (CD4/CD8⁺) were very low in both populations. Erythroid precursors comprised 80–85% of R1 population cells (Figure 5B). *Gata2* mRNA levels were reduced 5.7-fold ($p < 10^{-5}$) in -77^{-/-} R1 cells (Figure 5C). RNA-seq was conducted with three -77^{-/-} or WT R1 samples, and transcriptomes were compared with DESeq2 (Love et al., 2014). This analysis revealed 2805 and 2519 upregulated and downregulated ($p < 0.05$) genes, respectively, in -77^{-/-} R1 cells (Figure 5D). Gene ontology analysis of the downregulated (> 2 fold) genes yielded categories relevant to erythropoiesis, including heme biosynthesis and erythrocyte differentiation (Figure 5E). The erythrocyte differentiation category included genes encoding proteins with critical erythroid functions, such as GATA-1, KLF1, c-Kit (*Kit*), and FOG-1 (*Zfpml*). qRT-PCR confirmed strongly reduced *Gata1* and *Zfpml* expression in -77^{-/-} R1 cells. -77^{-/-} R1 cells lacked GATA-1 protein (Figure 5F).

The GATA-2 target gene *Kit* (Jing et al., 2008; Munugalavadla et al., 2005) encodes the c-Kit receptor tyrosine kinase that controls HSPC and erythroid precursor proliferation, differentiation and survival (Chabot et al., 1988; Hewitt et al., 2015; Munugalavadla and

Kapur, 2005; Wojchowski et al., 2010). c-Kit is activated by Stem Cell Factor (SCF) (Huang et al., 1990), a critical determinant of hematopoiesis (Ding and Morrison, 2013). *Kit* mRNA was modestly downregulated 1.5-fold, and cell surface c-Kit was 3.4-fold ($p = 0.009$) reduced in E14.5 $-77^{-/-}$ R1 cells (Figure 5G). We tested whether -77 -dependent *Kit* expression in erythroid precursors establishes developmental signaling that is limiting in $-77^{-/-}$ cells using a phospho-flow cytometry assay (Hewitt et al., 2015; McIver et al., 2016). $-77^{-/-}$ and WT fetal liver Ter119⁺ cells were serum-starved, treated with SCF or vehicle and p-Akt was quantified. While SCF increased p-Akt 2.3-fold ($p = 0.003$) in WT cells, $-77^{-/-}$ cells were non-responsive (Figure 5H). Basal p-Akt levels were equivalent in unstimulated WT and $-77^{-/-}$ cells. These results support a model in which loss of a single enhancer (-77) creates severe defects in erythroid precursors involving defective signaling and transcriptional circuitry required for erythroid maturation.

Though prior studies suggested that GATA-2 transcriptionally induces GATA-1 expression (Bresnick et al., 2010), the GATA-2 contribution to GATA-1 expression *in vivo* is unclear. Since -77 increases GATA-1 expression in erythroid precursors, we assumed it would control a large GATA-1 target gene ensemble. While GATA-1 represses certain GATA-2-activated genes, e.g. *Kit*, GATA-1 might sustain expression of others. Thus, $-77^{-/-}$ transcriptomic defects might reflect defects in genes regulated exclusively by GATA-2, by GATA-1 and/or jointly by GATA-1 and GATA-2.

To assess the contribution of distinct regulatory modes to the $-77^{-/-}$ erythroid precursor transcriptomic defects, we compared the R1 cell RNA-seq data with GATA-1-regulated genes in G1E-ER-GATA-1 cells. The G1E-ER-GATA-1 data was derived from RNA-seq analysis 48 h post-ER-GATA-1 activation (Tanimura et al., 2016) and microarray analysis 24 h after ER-GATA-1 activation (DeVilbiss et al., 2013; Pope and Bresnick, 2013) (Datasets 1 and 2, respectively) (Figure 6A). In R1 cells, -77 loss decreased *Gata2* expression, which reduced *Gata1* expression. In G1E-ER-GATA-1 cells, genetic complementation with β -estradiol-activated ER-GATA-1 (Gregory et al., 1999) represses *Gata2* transcription (Grass et al., 2003). Of the 2519 genes downregulated in $-77^{-/-}$ R1 cells, representing GATA-2 and/or GATA-1-activated genes, 731 were GATA-1-activated in G1E-ER-GATA-1 cells. 1376 genes downregulated in R1 cells were GATA-1-repressed, and therefore are GATA-2-activated/GATA-1-repressed. In combined GATA-2 and GATA-1 deficiency, e.g. in $-77^{-/-}$ R1 cells, these genes are not highly expressed. Of the remaining 412 genes, 253 are expressed in G1E-ER-GATA-1 cells but not ER-GATA-1-regulated. These 253 genes are candidate direct GATA-2-activated genes, without a GATA-2 requirement for maintenance, since their expression is sustained in G1E-ER-GATA-1 cells in which GATA-2 transcription is repressed by GATA-1. After GATA-1 represses *Gata2* transcription in G1E-ER-GATA-1 cells, GATA-1 might sustain their expression. We quantified mRNAs in G1E-ER-GATA-1 cells before and after ER-GATA-1 induction. While ER-GATA-1 repressed the established GATA-2 target gene *Kit* 47-fold ($p < 0.0001$), *Ryk*, *Acyp1*, *Mknk2* and *Prnp* expression were not significantly affected (Figure 6A), demonstrating the existence of GATA-2 target genes not regulated by GATA-1 in the complementation assay.

We described deregulated expression of 133 genes in E13.5 CMPs from $-77^{-/-}$ fetal livers (Johnson et al., 2015); 104 were downregulated. Comparison of the 253 R1 cell and 104

CMP genes yielded an overlap of 13 (Figure 6B). qRT-PCR analysis confirmed *Grb10* and *Tgfbr3* expression were reduced in $-77^{-/-}$ R1 cells (Figure 6B). The signaling adapter GRB10 is implicated in promoting HSC self-renewal and regeneration (Yan et al., 2016). *Tgfbr3*, which encodes TGF β receptor type III (Wang et al., 1991), is a marker of late BFU-Es (Gao et al., 2016). GATA-2 occupied GATA-2-activated genes *Ryk*, *Acyp1*, *Mknk2*, *Prnp*, *Grb10* and *Tgfbr3* in hematopoietic or erythroid progenitors (Figure 6C and Figure S5).

Ryk encodes an atypical Wnt ligand receptor (Green et al., 2014). Wnt5a induction of HSC quiescence (Nemeth et al., 2007) and protection against myeloablative injury (Povinelli et al., 2015) is blocked by Ryk inhibition (Povinelli and Nemeth, 2014). HSC self-renewal is reduced in *Ryk* $^{-/-}$ mice (Famili et al., 2016). However, Ryk function in erythroid precursors has not been reported. Colony assays were conducted with lineage-depleted WT fetal liver cells using two distinct shRyk-expressing retroviruses to downregulate Ryk. The shRNAs reduced *Ryk* mRNA by 75% (shRyk 927) and 67% (shRyk 1617) and decreased BFU-E and CFU-GM by 60–62% and 70–75%, respectively (Figure 6D). Thus, Ryk is one component of the GATA-2-regulated network that generates BFU-E and CFU-GM.

In E13.5 $-77^{-/-}$ fetal livers, BFU-Es were 11-fold lower, and R1 cells were 4-fold lower than WT (Johnson et al., 2015). However, BFU-Es were readily detectable in $-77^{-/-}$ fetal livers. It was not clear if deficient $-77^{-/-}$ BFU-E activity reflects defective R1 cells or impaired function of progenitors to generate BFU-E. We quantified the differentiation potential of purified $-77^{-/-}$ R1 cells. Two days after plating identical cell numbers, CFU-Es decreased 20-fold ($p < 0.0001$) with $-77^{-/-}$ cells (Figure 6E). By Day 8, $-77^{-/-}$ BFU-Es were 10-fold lower ($p < 0.0001$), which was comparable to our prior result with total fetal liver cells. These results provide evidence for an intrinsic defect in R1 cells that impairs their BFU-E-generating capacity. The R1 cells generated little to no CFU-GM and CFU-GEMM colonies (Figure 6E). The -77 enhancer is therefore a crucial determinant of BFU-E and its progeny, CFU-E (Figure 7), and $-77^{-/-}$ erythroid precursors did not undergo a fate switch to generate myeloid progeny.

The gene signatures of early and late BFU-Es have been described, and comparison of the two transcriptomes revealed high *Tgfbr3* as a hallmark of late BFU-Es (Gao et al., 2016). As BFU-Es and *Tgfbr3* expression are reduced in $-77^{-/-}$ R1 cells, we compared WT and $-77^{-/-}$ R1 cell transcriptomes with those of early and late BFU-Es. This analysis revealed $-77^{-/-}$ R1 cells exhibit an early BFU-E signature (Figure 6F). The WT R1 cell signature significantly correlated with the late BFU-E signature ($r = 0.62$, $p < 0.0001$) and negatively correlated with the early BFU-E signature ($r = -0.34$, $p = 0.005$). The $-77^{-/-}$ R1 signature positively correlated with the early BFU-E signature ($r = 0.73$, $p < 0.0001$) and negatively correlated with the late BFU-E signature ($r = -0.42$, $p = 0.0004$). The transcriptome alterations arising from -77 enhancer deletion indicate the -77 enhancer and GATA-2 are vital determinants of early erythroid precursor maturation and the genesis of BFU-E activity.

DISCUSSION

Recurring *GATA2* mutations in cancer, immunological and vascular/lymphatic disorders highlight critical GATA-2 functions in human biology and pathology (Dickinson et al., 2014; Katsumura et al., 2017; Spinner et al., 2014). Since *GATA2* mutations impair DNA binding (Hahn et al., 2011; Katsumura et al., 2016; Katsumura et al., 2014) or reduce GATA-2 expression (Hsu et al., 2013; Johnson et al., 2012), and elevated *GATA2* expression can correlate with poor prognosis of AML (Vicente et al., 2012), strict control of GATA-2 levels/activity ensures normal hematopoiesis. Many *GATA2* pathology phenotypes are predictable from concepts derived from mouse models (Gao et al., 2013; Johnson et al., 2012; Johnson et al., 2015; Ling et al., 2004; Rodrigues et al., 2005; Tsai et al., 1994). *Gata2* enhancer mutants represent unique systems to unravel how GATA-2 expression is maintained within a critical physiological window and how altered GATA-2 expression impacts HSPC genesis/function. The +9.5 enhancer mutant revealed GATA-2 activity to trigger HSC generation in the AGM and to confer vascular integrity (Gao et al., 2013; Johnson et al., 2012). The -77 enhancer mutant revealed GATA-2 activity to confer myeloid progenitor differentiation into diverse progeny (Johnson et al., 2015). The -1.8 enhancer mutant revealed its requirement to maintain, but not establish, *Gata2* repression during erythroid maturation (Snow et al., 2010). The -2.8 (Snow et al., 2011) and -3.9 (Sanalkumar et al., 2014) mutants revealed how *cis*-elements predicted to be important, based on conserved factor binding motifs, factor occupancy, and enhancer activity in artificial assays, may not be critical *in vivo*.

Herein, we described a *Gata2* enhancer CH mutant model with one allele lacking the +9.5 enhancer and the other lacking the -77, which is embryonic lethal at ~E14.5. This unique model and the -77^{-/-} model led to the discovery of mechanisms responsible for establishing two vital hematopoietic progenitor populations - MEP and BFU-E (Figure 7). The mechanisms were not predictable from those dictating HSC emergence (Gao et al., 2013). The *Gata2* enhancers had both functional similarities and differences *in vivo*. As mutation or disruption via chromosomal rearrangement of both enhancers are implicated in human MDS and AML, and other pathologies (Groschel et al., 2014; Hsu et al., 2013; Johnson et al., 2012; Katsumura et al., 2017; Yamazaki et al., 2014), insights from this model will inform human enhanceropathies.

While the +9.5, but not the -77, triggers HSC genesis in the AGM, MEP production requires both enhancers on a single allele to increase *Gata2* transcription. The simultaneous mutation of a single copy of each enhancer in *trans* abrogates *Gata2* expression and MEP production. MEPs are implicated as myeloid leukemia-initiating cells (Dang et al., 2017; Zhang et al., 2017). Considering GATA-2 function in distinct sectors of the hematopoietic hierarchy, and its requirement for MEPs, it is attractive to consider how alterations in GATA-2-regulated circuits influence MEP genesis and function. Such alterations may involve mutations that reduce GATA-2 activity or expression or those impacting expression/activity of constituents of the GATA-2-regulated genetic network.

In addition to the enhancer-dependent MEP genesis mechanism, -77 deletion depleted BFU-Es. Major progress has been made in identifying BFU-E self-renewal regulators, including glucocorticoid receptor, peroxisome proliferation-activated receptor- α and the RNA binding

protein ZFP36L2 (Flygare et al., 2011; Gao et al., 2016; Lee et al., 2015). Despite little to no alterations in expression of mRNAs encoding these factors in $-77^{-/-}$ R1 cells (1.5 fold), these mutant cells lacked BFU-E activity.

The -77 enhancer induced expression of GATA-1, a large ensemble of erythroid genes and previously undescribed GATA-2 targets. The latter genes included *Ryk*, *Tgfb3* and *Grb10*, encoding hematopoietic signaling components (Famili et al., 2016; Gao et al., 2016; Povinelli and Nemeth, 2014; Povinelli et al., 2015; Randrianarison-Huetz et al., 2010; Yan et al., 2016), and other factors not implicated in red cell biology. Based on the large reduction in *Ryk* expression in $-77^{-/-}$ R1 cells and reduced CFU activity upon *Ryk* knockdown, *Ryk* signaling may constitute an important determinant of erythroid precursor cell maturation and/or function. The -77 targets reveal a new dimension of GATA-2 function to control erythroid precursor genesis/function that could not be inferred from GATA-2 mechanisms in other systems.

A prior transcriptomic analysis identified early and late BFU-E signatures (Gao et al., 2016). Although it is assumed that early BFU-Es progress to a late BFU-E state, no mutants that ablate or enhance this process have been described. As defective BFU-E maturation/function may underlie pathologies, such as Epo-resistant anemias (Gao et al., 2016; Lee et al., 2015), the mechanisms are of great interest. The $-77^{-/-}$ R1 cell transcriptome resembled early BFU-E, suggesting the -77 controls BFU-E maturation, and the mutant cells lacked BFU-E activity. The diverse constituents of the -77 -regulated genetic network include new targets for manipulation of erythroid precursors, e.g. to stimulate red blood cell regeneration.

In summary, CH and $-77^{-/-}$ models revealed mechanisms governing the production of vital hematopoietic progenitors. Why do the -77 and $+9.5$ enhancers need to reside on the same allele to generate myeloid progenitors, while the $+9.5$ has a unique function in HSC genesis? The $+9.5$ enhancer may exist in an active state in hemogenic endothelium and in HSCs, while the -77 enhancer complex has not yet been commissioned. The $+9.5$ active state would persist as myeloid progenitors develop, and the -77 active state would be acquired. In a myeloid progenitor with diminished developmental potential in comparison to HSCs, presumably, the distinct epigenomic environments create new obstacles and opportunities for gene control. The requirement for $+9.5$ and -77 enhancers on the same allele points to a model in which *Gata2* transcription has a dual enhancer requirement to establish and/or maintain the active state. Why is the developmental stage- and cell type-specific dual enhancer mechanism required for generating a progenitor, but not a stem cell? It will be instructive to identify the shared and the developmental stage- and cell type-specific, components mediating the unique enhancer activities.

EXPERIMENTAL PROCEDURES

Mouse Crosses and Timed Matings

$-77^{+/-};+9.5^{+/-}$ CH embryos were generated by mating $+9.5^{+/-}$ and $-77^{+/-}$ mice. Pregnant females were euthanized with CO₂, and the uterus was removed into IMDM (Gibco) containing 10% FBS (Gemini). Embryo viability was scored by the presence of a beating heart and were photographed with an Olympus Szx16 stereomicroscope. Animal

experiments were performed with the ethical approval of the Association for the Assessment and Accreditation of Laboratory Animal Care at UW-Madison.

Flow Cytometry

Fetal liver cells from E13.5 or E14.5 embryos were dissociated and resuspended in PBS with 2% FBS and passed through 25 μ m cell strainers to obtain single-cell suspensions. Detailed protocols can be found in the Supplemental Experimental Procedures.

Colony Forming Unit Assays

Dissociated cells from E13.5 or E14.5 fetal livers were plated in duplicate in Methocult M3434 complete media (StemCell Technologies) at 2×10^4 cells per 35 mm plate or 2000 sorted R1 cells per 35 mm plate. Plates were incubated for 7–8 days, and colonies were enumerated. For Giemsa staining, cells were isolated from the plates using PBS containing 2% FBS, centrifuged for 10 min, resuspended in PBS containing 25% FBS and centrifuged (CytoSpin4, ThermoFisher Scientific) for 10 min at 500 rpm onto slides.

Gene Expression Analysis

RNA was purified from fetal livers or sorted cells using TRIzol (Invitrogen). cDNA was synthesized by Moloney murine leukemia virus reverse transcription (RT). Real-time PCR was conducted with SYBR green master mix. Detailed protocols can be found in the Supplemental Experimental Procedures.

Primary Cell Isolation and Culture

Erythroid precursors were enriched from E14.5 fetal livers with EasySep negative selection Mouse Hematopoietic Progenitor Enrichment Kit (StemCell Technologies). Detailed protocols can be found in the Supplemental Experimental Procedures.

Statistics

For quantification of cells, colonies, or mRNA, the results are presented as mean \pm SEM. Determination of significance was conducted using the 2-tailed unpaired Student's *t* test. A *p* value <0.05 was considered significant. Single cell RNA-Seq data from a prior report (Gao et al., 2016) were averaged for each of the 68 signature genes for each of the *Gata1*^{hi} and *Gata1*^{lo} cells. RNA-Seq data from our R1 cell expression profiles were averaged for the $-77^{+/+}$ and $-77^{-/-}$ samples. Pairwise correlation was computed and plotted using `cor.test()` and the `pairs()` function in R (<http://www.r-project.org>) statistical computing environment (Wu et al., 2014a).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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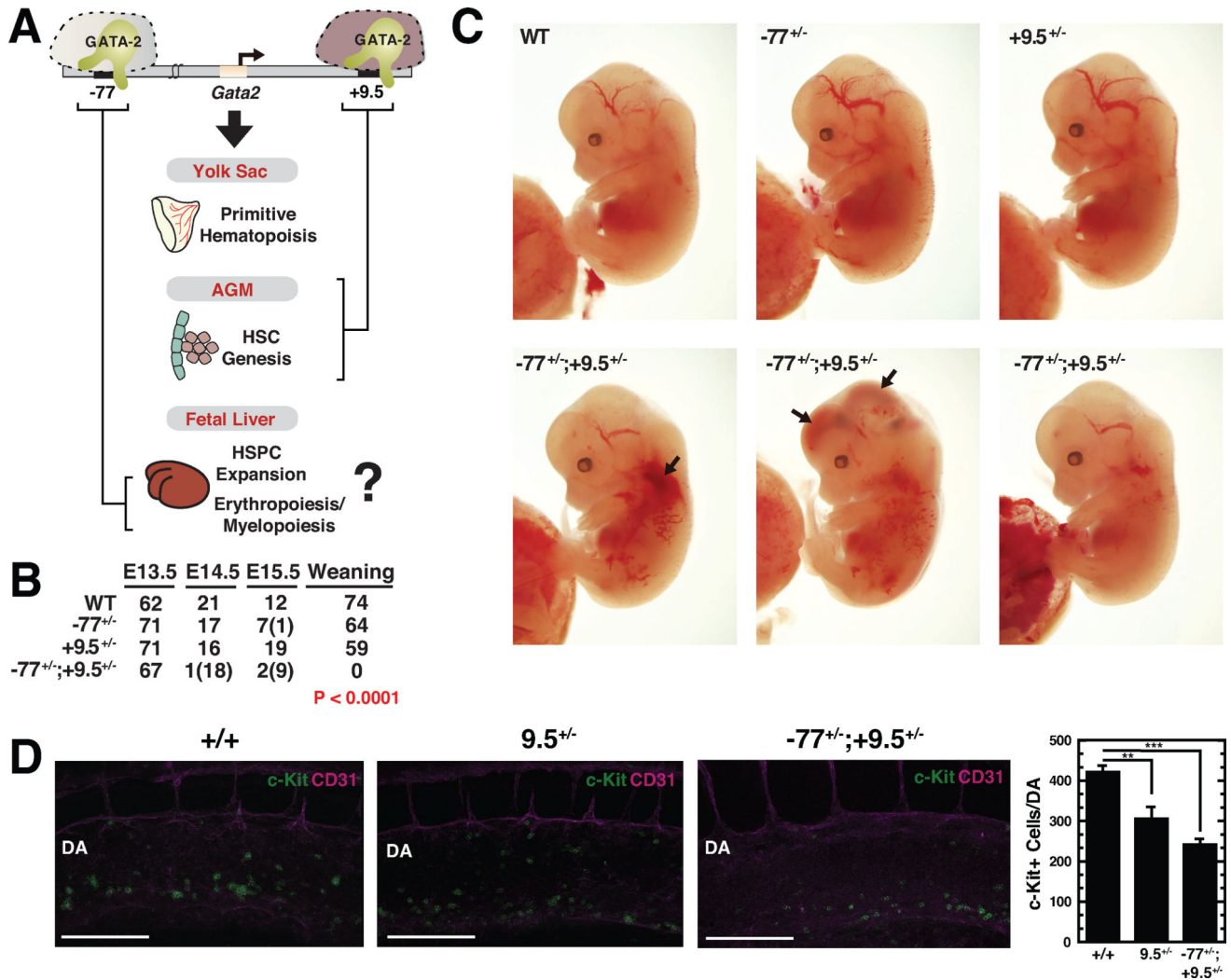


Figure 1. Unique Constellation of Hematopoietic Deficits in CH Embryos

(A) Model depicting stage-specific requirement for *Gata2* -77 and +9.5 enhancers during developmental hematopoiesis. As the +9.5 enhancer deletion abrogates HSC genesis in the AGM of +9.5^{-/-} embryos, whether it exerts essential activities in fetal liver hematopoietic cells was unclear.

(B) Genotypes of WT, +9.5^{+/-}, -77^{+/-} and CH embryos at timed developmental stages and at time of weaning. The numbers of dead embryos at each developmental stage are shown in parentheses. Significance was analyzed using the Chi-squared test.

(C) Representative E13.5 CH embryos exhibiting reduced liver size and hemorrhage (arrows). (D) Representative whole-mount staining of E10.5 WT and CH embryos showing CD31⁺ cells (magenta) and c-Kit⁺ cells (green) within the dorsal aorta (DA). Scale bar, 100 μ m. Quantitation of c-Kit⁺ cells within the DA (WT [n = 5], +9.5^{+/-} [n = 4] and CH [n = 4]). Graphs depict mean \pm SEM; **p < 0.01, ***p < 0.001. Significance was analyzed using the 2-tailed unpaired Student's t test.

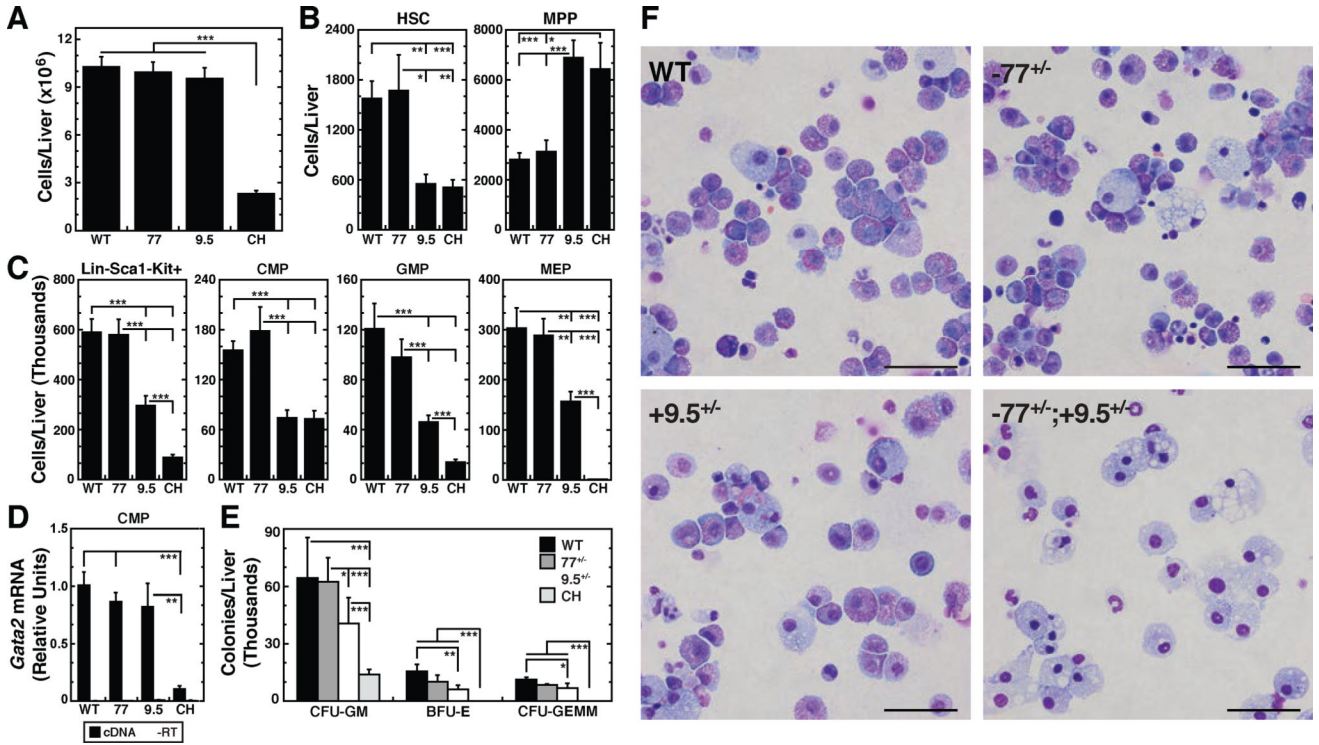


Figure 2. Myelo-erythroid Progenitor Functional Defect in CH Fetal Liver

(A) Total fetal liver cells in E13.5 fetal livers (WT [n = 23]; -77^{+/-} [n = 19]; +9.5^{+/-} [n = 32]; CH [n = 27]).

(B) Quantitation of HSCs (Lin⁻Mac1⁺CD41⁻CD48⁻CD150⁺Sca1⁺Kit⁺) and MPPs (Lin⁻Mac1⁺CD41⁻CD48⁻CD150⁻Sca1⁺Kit⁺) from flow cytometric analysis of E13.5 fetal livers (WT [n = 12]; -77^{+/-} [n = 6]; +9.5^{+/-} [n = 6]; CH [n = 9]).

(C) Quantitation of Lin⁻Sca1⁻Kit⁺ cells, CMPs (Lin⁻CD34⁺FcR^{low}Kit⁺Sca1⁻), GMPs (Lin⁻CD34⁺FcR^{high}Kit⁺Sca1⁻), and MEPs (Lin⁻CD34⁻FcR^{low}Kit⁺Sca1⁻) from E13.5 fetal livers (WT [n = 20]; -77^{+/-} [n = 8]; +9.5^{+/-} [n = 20]; CH [n = 20]).

(D) *Gata2* mRNA quantitation in E13.5 fetal liver CMPs (WT [n = 19]; -77^{+/-} [n = 8]; +9.5^{+/-} [n = 8]; CH [n = 6]).

(E) CFU activity of E13.5 fetal liver hematopoietic progenitors. Graphs are adjusted for fetal liver cellularity. Graphs show mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

Significance was analyzed using the 2-tailed unpaired Student's *t* test.

(F) Representative images of Wright-Giemsa stained cells isolated from all colonies at time of counting. Scale bars, 50 μm.

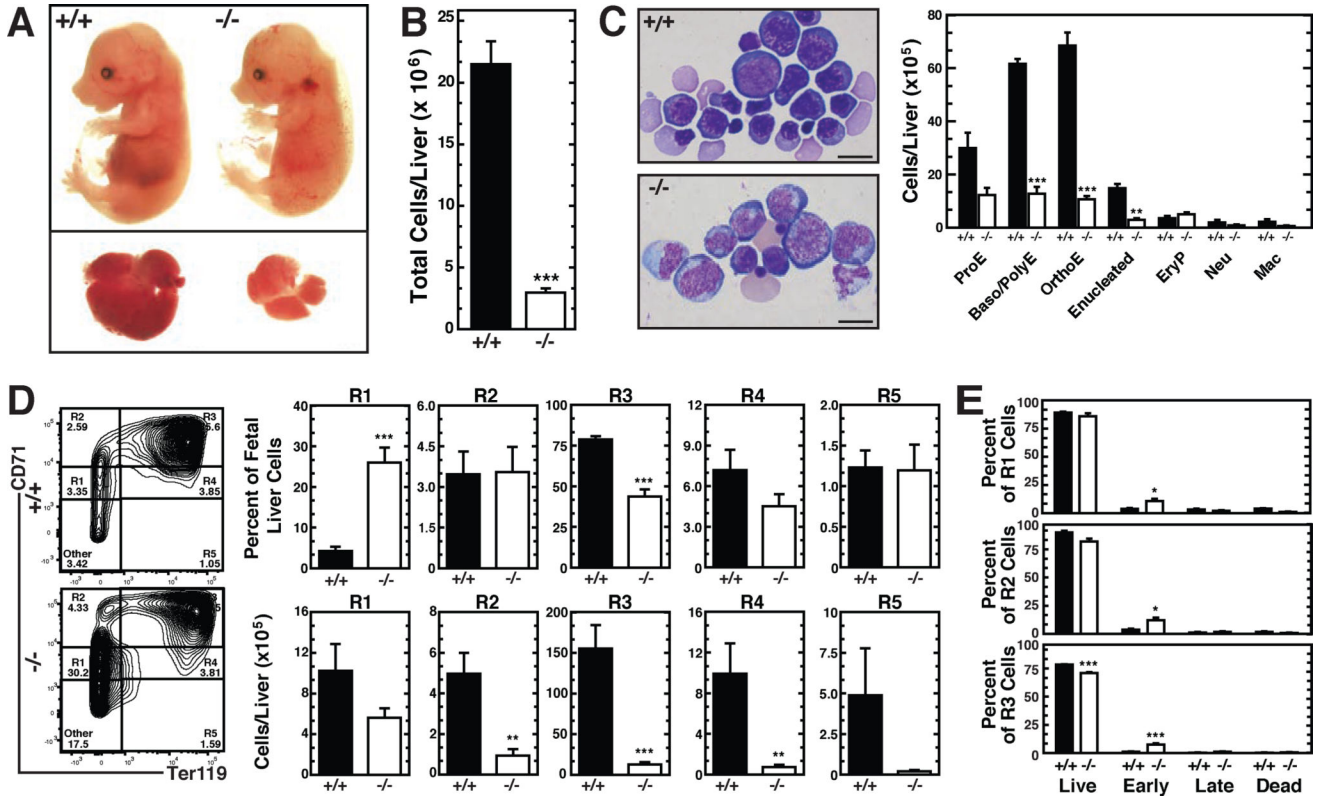


Figure 3. -77 *Gata2* Enhancer Promotes Erythroid Maturation *In Vivo*
 (A) Representative E14.5 $-77^{+/+}$ and $-77^{-/-}$ embryos and fetal livers.
 (B) Quantitation of cell numbers in E14.5 fetal livers ($-77^{+/+}$ [n = 7], $-77^{-/-}$ [n = 7]).
 (C) Representative Wright-Giemsa images and quantitation of $-77^{+/+}$ and $-77^{-/-}$ E14.5 total fetal liver populations. Scale bar, 20 μ m. ProE, proerythroblast; Baso/Poly, basophilic/polychromatophilic erythroblast; OrthoE, orthochromatic erythroblast; EryP, primitive erythroid; Neu, neutrophil; Mac, macrophage. ($-77^{+/+}$ [n = 3], $-77^{-/-}$ [n = 3]).
 (D) Representative flow cytometric analysis and quantitation of R1-R5 populations from E14.5 fetal livers ($-77^{+/+}$ [n = 7], $-77^{-/-}$ [n = 7]).
 (E) Quantitation of early (AnnexinV⁺DRAQ7⁻) and late (AnnexinV⁺DRAQ7⁺) apoptotic cells and dead cells (Annexin V⁻DRAQ7⁺) from R1-R3 populations by flow cytometry ($-77^{+/+}$ [n = 4]; $-77^{-/-}$ [n = 5]). Graphs show mean \pm SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Significance was analyzed using the 2-tailed unpaired Student's *t* test.

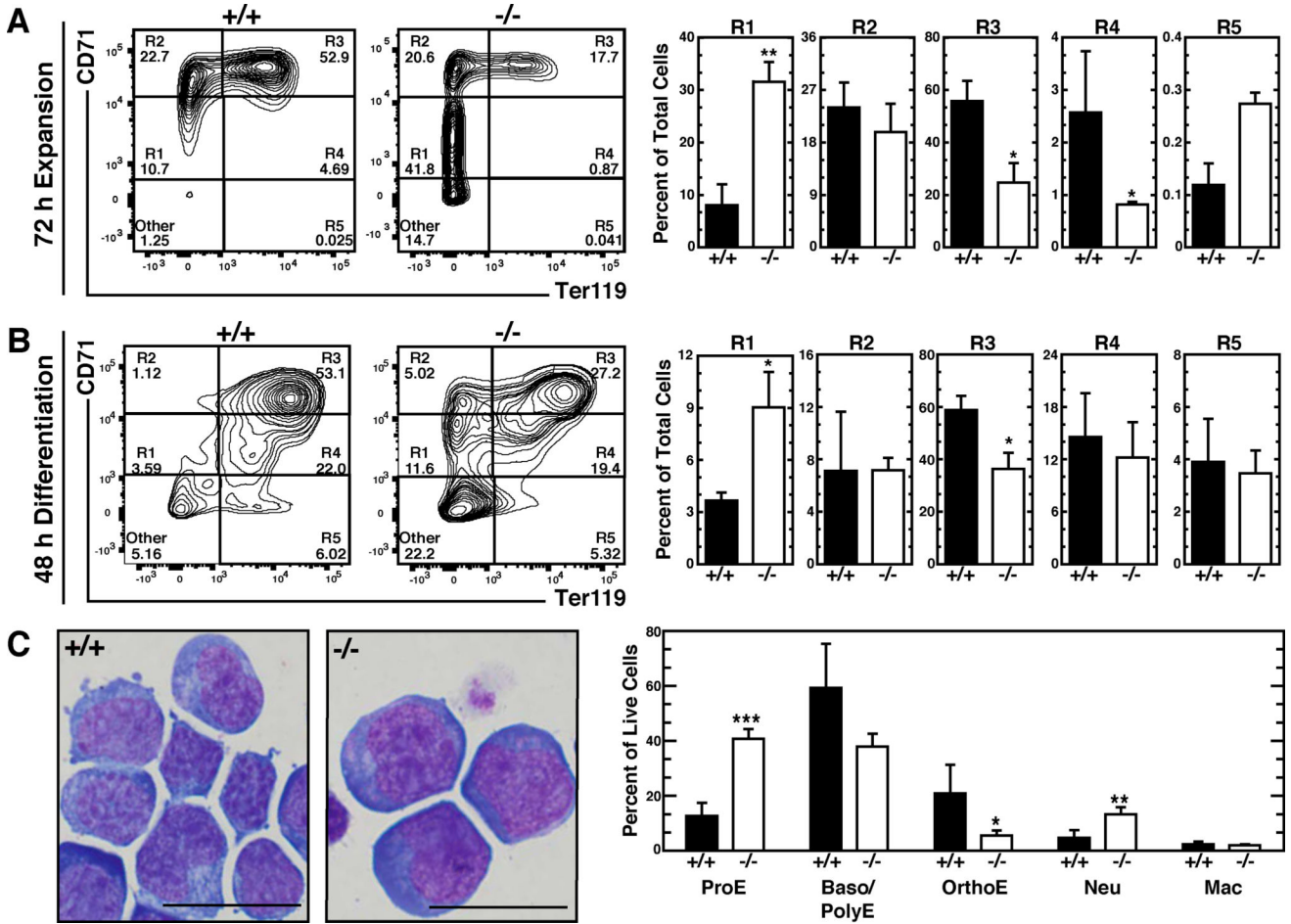


Figure 4. -77 *Gata2* Enhancer Promotes Erythroid Maturation *Ex Vivo*

Representative flow cytometric analysis and quantitation of R1–R5 populations from E14.5 fetal livers cultured in (A) expansion medium for 72 h ($-77^{+/+}$ [n = 5]; $-77^{-/-}$ [n = 4]) or (B) expansion media for 72 h then differentiation medium for 48 h ($-77^{+/+}$ [n=4]; $-77^{-/-}$ [n = 4]).

(C) Representative Wright-Giemsa images and quantitation of $-77^{+/+}$ and $-77^{-/-}$ E14.5 lineage negative cells cultured in expansion system for 72 h. Scale bar, 20 μ m. ProE, proerythroblast; Baso/Poly, basophilic/polychromatophilic erythroblast; OrthoE, orthochromatic erythroblast; Neu, neutrophil; Mac, macrophage ($-77^{+/+}$ [n = 6]; $-77^{-/-}$ [n = 6]). Graphs show means \pm SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Significance was analyzed using the 2-tailed unpaired Student's *t* test.

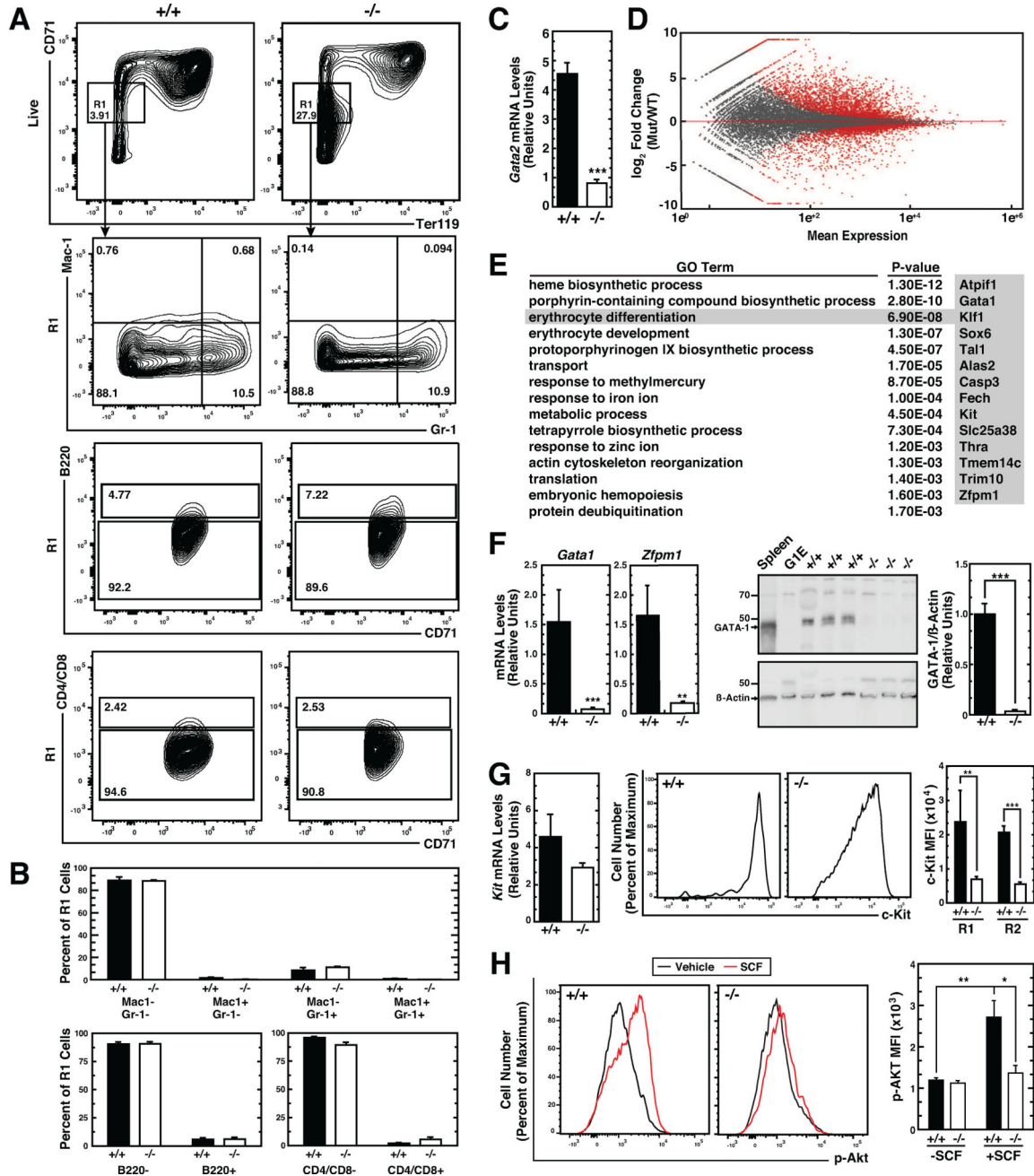


Figure 5. -77 *Gata2* Enhancer-dependent Transcriptome Establishes Developmental Signaling

(A) Representative flow cytometric analysis of erythroid cells from E14.5 fetal liver using cell surface markers CD71 and Ter119. The R1 (CD71^{low}Ter119⁻) population was also evaluated for presence of monocytes (Mac1⁺Gr1⁻), granulocytes (Mac1⁺Gr1⁺), B cells (B220⁺) and T cells (CD4/CD8⁺).

(B) Quantitation of Mac1-Gr1, B220, and CD4/CD8 cells in R1 populations (-77^{+/+} [n = 4]; -77^{-/-} [n = 4]).

(C) Quantitation of *Gata2* mRNA levels in E14.5 fetal liver R1 cells (-77^{+/+} [n = 4]; -77^{-/-} [n = 6]).

(D) MA plot of RNA-seq-based comparison of R1 transcriptomes from $-77^{+/+}$ and $-77^{-/-}$ E14.5 fetal livers ($-77^{+/+}$ [n = 3]; $-77^{-/-}$ [n = 3]). Red points indicate down- or up-regulated genes (false discovery rate (FDR) <0.05).

(E) Gene Ontology analysis of genes downregulated in $-77^{-/-}$ R1 cells. The most enriched biological processes are shown with corresponding p values. Genes comprising the erythrocyte differentiation category are shown.

(F) Quantitation of *Gata1* and *Zfpml1* mRNA levels ($-77^{+/+}$ [n = 4]; $-77^{-/-}$ [n = 6]) and GATA-1 protein levels ($-77^{+/+}$ [n = 6]; $-77^{-/-}$ [n = 5]) in E14.5 fetal liver R1 cells.

(G) Quantitation of *Kit* mRNA levels ($-77^{+/+}$ [n = 4]; $-77^{-/-}$ [n = 6]) and cell surface c-Kit mean fluorescence intensity (MFI) ($-77^{+/+}$ [n = 8]; $-77^{-/-}$ [n = 5]) in E14.5 fetal liver R1 cells.

(H) Quantitation of p-Akt MFI after stimulation with 10 ng/ml SCF in $-77^{+/+}$ and $-77^{-/-}$ Ter119⁻ cells (WT [n = 7]; $-77^{-/-}$ [n = 6]). Graphs show mean \pm SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Significance was analyzed using the 2-tailed unpaired Student's *t* test.

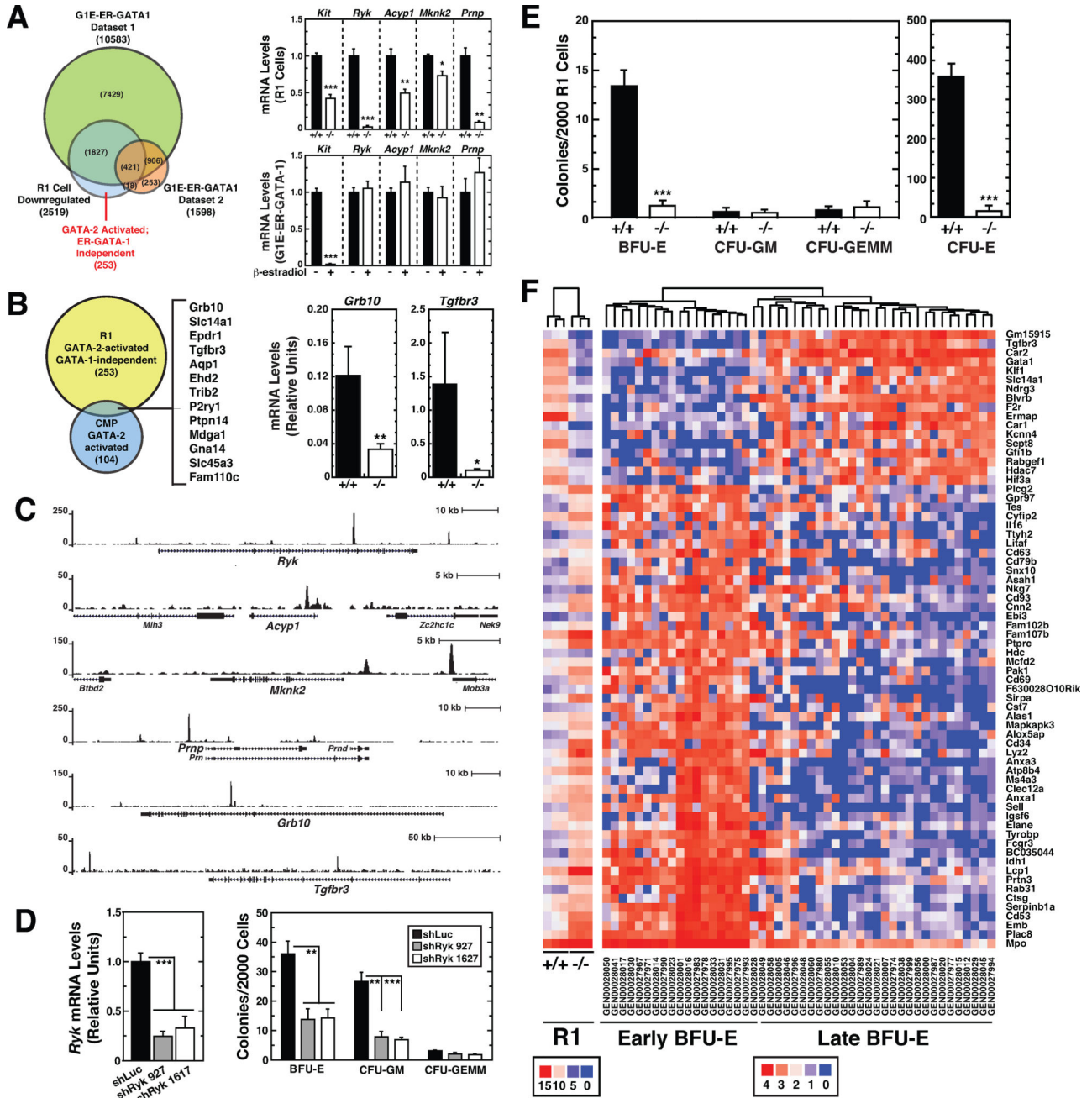


Figure 6. -77 *Gata2* Enhancer Requirement for Burst Forming Unit-Erythroid
 (A) Venn diagram depicting overlap between genes regulated by GATA-1 in G1E-ER-GATA-1 cells and by the -77 enhancer in R1 cells. Changes in R1 cell RNA-seq read counts are shown for representative GATA-2-activated genes. Also shown are the relative mRNA levels of these genes in G1E-ER-GATA-1 cells with and without β -estradiol treatment. G1E-ER-GATA-1 Dataset 1; Gene expression changes following 48 h induction of GATA-1 activity by β -estradiol quantified by RNA-seq. G1E-ER-GATA-1 Dataset 2; Gene expression changes following 24 h induction of GATA-1 activity by β -estradiol. Transcripts were

quantified by Agilent-028005 SurePrint G3 Mouse GE 8×60K Microarray (Pope and Bresnick, 2013).

(B) Venn diagram depicting overlap between GATA-2-activated genes in R1 cells and CMPs (GSE69786). Genes common to both categories are listed. Reductions in *Grb10* and *Tgfb3* mRNA expression in $-77^{-/-}$ R1 cells were confirmed by qRT-PCR ($-77^{+/+}$ [n = 4]; $-77^{-/-}$ [n = 6]).

(C) GATA-2 ChIP-seq profiles in GATA-1-null G1E erythroid precursor cells mined from dataset GSM722387.

(D) CFU activity of lineage-depleted E14.5 fetal liver cells following shRNA-mediated knockdown of *Ryk*. *Ryk* mRNA was quantified by qRT-PCR 3 days post-infection with control (shLuc) or *Ryk* shRNA-expressing retrovirus. 2000 cells were plated in methylcellulose within 16 h post-infection. Colonies were enumerated on day 8 (n = 6). (E) CFU activity of E14.5 fetal liver R1 cells quantified after 2 (CFU-E) or 8 (BFU-E, CFU-GM and CFU-GEMM) days in methylcellulose. Graphs show mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. Significance was analyzed using the 2-tailed unpaired Student's *t* test. (F) Cluster analysis of $-77^{+/+}$ and $-77^{-/-}$ R1 gene expression and comparison with “early” and “late” BFU-E gene expression signature (Gao et al., 2016).

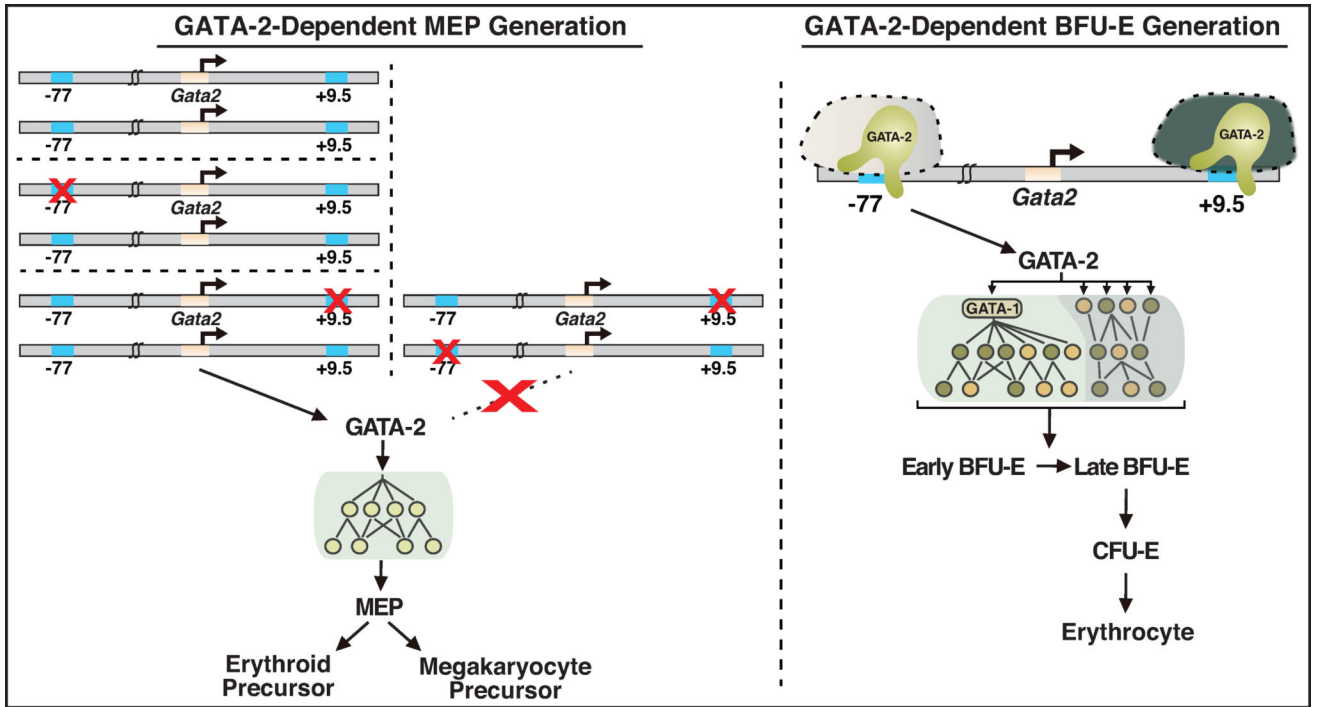


Figure 7. Model for *Gata2* Enhancer-dependent Megakaryocytic Erythrocyte Progenitor and Burst Forming Unit-Erythroid Generation

Left, GATA-2-dependent MEP generation. Both -77 and $+9.5$ enhancers must reside on a single allele to activate *Gata2* transcription to establish a genetic network that supports the genesis and/or survival of MEPs, the precursor to erythrocytes and megakaryocytes. Right, GATA-2-dependent BFU-E generation. The -77 enhancer activates *Gata2* transcription in erythroid precursors, resulting in a GATA-2-dependent genetic network that supports the genesis and/or survival of BFU-E, the vital erythroid precursor required for erythrocyte development and regeneration. This network parses into two components: (i) GATA-2 activates *Gata1* transcription, and GATA-1 regulates a large ensemble of genes that establish the erythroid cell transcriptome. (ii) GATA-2 activates a smaller target gene cohort without a requirement to upregulate GATA-1. In aggregate, the genes establish signaling pathways that support proliferation, differentiation and survival and the vast constituents that comprise the unique phenotype of erythroblasts and their reticulocyte and erythrocyte progeny.