

Krüppel-like transcription factors KLF1 and KLF2 have unique and coordinate roles in regulating embryonic erythroid precursor maturation

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ABSTRACT

The Krüppel-like transcription factors KLF1 and KLF2 are essential for embryonic erythropoiesis. They can partially compensate for each other during mouse development, and coordinately regulate numerous erythroid genes, including the β -like globins. Simultaneous ablation of KLF1 and KLF2 results in earlier embryonic lethality and severe anemia. In this study, we determine that this anemia is caused by a paucity of blood cells, and exacerbated by diminished β -like globin gene expression. The anemia phenotype is dose-dependent, and, interestingly, can be ameliorated by a single copy of the *KLF2*, but not the *KLF1* gene. The roles of KLF1 and KLF2 in maintaining normal peripheral blood cell numbers and globin mRNA amounts are erythroid cell-specific. Mechanistic studies led to the discovery that KLF2 has an essential function in erythroid precursor maintenance. KLF1 can partially compensate for KLF2 in this role, but is uniquely crucial for erythroid precursor proliferation through its regulation of G1- to S-phase cell cycle transition. A more drastic impairment of primitive erythroid colony formation from embryonic progenitor cells occurs with simultaneous loss of KLF1 and KLF2 than with loss of a single factor. KLF1 and KLF2 coordinately regulate several proliferation-associated genes, including *Foxm1*. Differential expression of *FoxM1*, in particular, correlates with the observed *KLF1* and *KLF2* gene dosage effects on anemia. Furthermore, KLF1 binds to the *FoxM1* gene promoter in blood cells. Thus KLF1 and KLF2 coordinately regulate embryonic erythroid precursor maturation through the regulation of multiple homeostasis-associated genes, and KLF2 has a novel and essential role in this process.

Introduction

Erythropoiesis in the mouse and other mammals proceeds in two distinct waves during development.¹ First, primitive erythroid cells appear in the yolk sac, an extra-embryonic membrane, in regions known as blood islands.^{2,3} Large, nucleated primitive erythroid cells begin to circulate at about mouse embryonic Day 8.25 (E8.25).^{4,5} Mouse primitive erythroid progenitor cells form colonies (EryP-CFC) in semi-solid culture media⁴ which can first be detected at E7. Primitive erythroid cells mature in circulation in a synchronous manner and eventually undergo enucleation.⁶ The second wave, or definitive erythropoiesis, begins in the fetal liver and results in mature, enucleated definitive erythroid cells by around E12.¹

The erythroid developmental program is regulated by a network of key transcription factors,⁷ including Krüppel-like factor 1 (KLF1 or EKLF, Erythroid Krüppel-like factor). Members of the KLF family have three Cys2-His2 zinc fingers near their C-terminus that recognize a core 5'-CACCC-3' DNA sequence.⁸ KLF1 is erythroid-specific and required for the normal expression of the embryonic and adult β -like globin genes.⁹⁻¹¹ *KLF1*^{-/-} mice develop β -thalassemia and die by E16.^{9,10} KLF1 regulates the expression of numerous other erythroid genes, including those encoding cytoskeletal and membrane proteins, and heme synthesis enzymes.^{2,13} In addition,

KLF1 regulates cell cycle control, proliferation and apoptosis.¹⁴⁻¹⁶ Pathogenic mutations in *KLF1* can cause congenital dyserythropoietic anemia in humans.^{17,18}

To date, 17 mammalian KLFs have been identified, and they have diverse roles in development.⁸ The zinc finger domain of KLF2 is 89% similar to KLF1. KLF2 is necessary for primitive erythropoiesis, and positively regulates embryonic globin gene expression, as shown in *KLF2* knockout and erythroid-conditional knockout mouse embryos.^{11,19,20} Global gene expression assays indicate that many genes that are important in the processes of development, differentiation and cell migration are dysregulated in *KLF2*^{-/-} compared to wild-type primitive erythroid cells.²¹ Ablation of KLF2 causes lethality between E11.5 and E14,^{5,22,23} which can be due to intra-embryonic hemorrhaging²⁴ or heart failure.^{23,25}

KLF1 and KLF2 share functional roles in globin gene regulation. KLF1 and KLF2 can partially compensate for each other, as is evident from the reduced embryonic β -like globin gene expression of *KLF1/KLF2* double (*KLF1*^{-/-}*KLF2*^{-/-}) compared to single knockout embryos.¹¹ There is also more aberrant morphology, consistent with increased apoptosis, in double compared to single knockout primitive red blood cells.

In addition, E10.5 *KLF1*^{-/-}*KLF2*^{-/-} embryos are strikingly pale and appear anemic, whereas E10.5 *KLF1*^{-/-} and E10.5 *KLF2*^{-/-} mice are grossly normal. *KLF1*^{-/-}*KLF2*^{-/-} mice die by E11.5, which is earlier than *KLF1*^{-/-} or *KLF2*^{-/-} embryos.

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Gene expression profiling of E9.5 erythroid cells was used to identify genes that are synergistically regulated by KLF1 and KLF2.¹² These genes are principally involved in cellular pathways that control homeostasis, hemopoiesis, apoptosis and proliferation. Erythroid conditional knockout and other functional studies identified Myc as an important downstream target of KLF1 and KLF2. Additional direct or indirect targets include other genes governing proliferation and/or apoptosis, such as *Cd24a* antigen,²⁶ forkhead box M1 (*FoxM1*),²⁷ sphingosine kinase 1 (*Sphk1*)²⁸ and parathyroid hormone 1 receptor (*Pthr*).²⁹

In this work, we further assess how KLF1 and KLF2 coordinately control primitive erythropoiesis. E10.5 *KLF1/KLF2* double erythroid-conditional knockout embryos are anemic like *KLF1-/-KLF2-/-* mice. A surprising new discovery was made in that restoring one copy of the KLF2 (*KLF1-/-KLF2+/-*) but not the *KLF1* gene (*KLF1+/-KLF2-/-*) ameliorates this phenotype. Anemia in E10.5 *KLF1+/-KLF2-/-* and *KLF1-/-KLF2-/-* mice correlates with a drastic reduction in the number of peripheral blood cells. EryP-CFC assays identified a novel role for KLF2 in the maintenance of primitive erythroid progenitor potential. KLF1 can partially compensate for KLF2 in this function, but its pivotal role is in the regulation of erythroid precursor cell proliferation. *KLF1-/-KLF2-/-* embryos have more severely impaired colony forming ability than single knockouts. The data indicate that KLF1 and KLF2 coordinately control the maturation of primitive erythroid precursors through the regulation of multiple cell cycle- and proliferation-associated genes, including *FoxM1*.

Methods

Generation of mice

The *KLF1* and *KLF2* knockout (KO) mouse models have been described previously.^{9,22} The *KLF1* and *KLF2* loci are linked on mouse chromosome 8. A model with the *KLF1* and *KLF2* null alleles on the same chromosome, *KLF1+/-KLF2+/-*(R), was developed.¹¹ Mice with a floxed *KLF2* allele were obtained from Dr. Jerry Lingrel.³⁰ Mice carrying the *KLF1* null allele and the *KLF2* floxed allele on the same chromosome were created by screening for recombinants as previously described.¹¹ The *Tie2-Cre* mouse model has been described previously.³¹ All experiments involving mice were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (VCU IACUC) under protocol number AM10347. The mice used for this study are of mixed genetic background.

Tissue collection and processing

Peripheral blood cells were collected by severing the vitelline and umbilical vessels of E9.5 or E10.5 mouse embryos.²⁰ Whole-mount embryos were photographed with an Olympus SZ2-ILST microscope (Olympus America, Center Valley, PA, USA), using an Olympus Q-Color 3 camera and QCapture 2.81.0 software (Quantitative Imaging, Surrey, BC, USA). Embryonic yolk sacs were embedded and sectioned as previously described.¹¹ Images of yolk sac sections were captured with an Olympus BX41 compound microscope and Olympus DP71 digital camera. cDNA synthesis and quantitative reverse-transcriptase PCR (qRT-PCR) were performed as previously described.^{11,12,19,20} Primer sequences for qRT-PCR are listed in *Online Supplementary Table S1*.

Erythroid progenitor assays

To study primitive erythroid progenitors, E8.25-E8.5 (6-12

somite pairs) implants including embryo and yolk sac were dissected in PB2,³² and dissociated into single cells using 0.01% trypsin (Worthington Biochemical Corporation, NJ, USA) and mechanical trituration. Half of the cells from each implant were plated in 1% methylcellulose supplemented with 10% plasma derived serum (Animal Technologies, TX, USA), 5% protein-free hybridoma medium (Gibco/BRL), IL-3 (20 ng/mL), IL-6 (20 ng/mL), stem cell factor (60 ng/mL) (IL-3, IL-6 and SCF-Peprotech, NJ, USA), erythropoietin (2 U/mL), MTG and L-glutamine. The remaining cells were used for genotyping. Erythroid colonies were stained using benzidine and counted either on Day 2 or Day 6 of culture. Colony images were captured using an Olympus IX70 microscope and Q-Color 3 camera (Olympus America). Two-dimensional colony areas were calculated using Image J software.

Cell cycle analysis

Cell cycle profiles were assessed using the APC BrdU Flow kit (BD Biosciences). The protocol was adapted from Malik *et al.*³³ Briefly, E9.5 erythroblasts were cultured in erythroid maturation medium containing BrdU for 90 min, fixed, and stained with APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD). The BD FACSCanto™ II Analyzer was used to measure total DNA content and BrdU incorporation using 7-AAD and APC fluorescence intensity, respectively. Gating was applied to include only single cells and exclude clumped cells.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described.²⁰ Primer sequences for qPCR are listed in *Online Supplementary Table S2*.

Statistical analysis

The Student's t-test was used for statistical analyses. $P < 0.05$ was considered significant.

Results

KLF1/KLF2 double conditional KO embryos recapitulate the *KLF1-/-KLF2-/-* anemia phenotype

KLF1 is erythroid-specific, but KLF2 has a more widespread tissue distribution. In order to determine whether the anemia observed in *KLF1-/-KLF2-/-* embryos results from erythroid cell-specific ablation of KLF2, the ideal mouse model would be an erythroid conditional knockout of *KLF2* on a *KLF1* null background. Unfortunately, Cre expression under the control of the erythroid-specific erythropoietin receptor (EpoR) promoter in ErGFP-Cre mice^{20,34} is dependent on KLF1 (*Online Supplementary Figure S1*). Consequently, this model was untenable. β -Cre,³⁵ with the β -globin promoter, was also unworkable because it is linked to the *KLF1* gene. *Tie2-Cre* mice express Cre in erythroid cells by E7.5, using the *Tie2* promoter.³⁶ Therefore, this model was used, with the caveat that KLF2 is ablated in both erythroid and endothelial cells.

KLF1+/-KLF2F/+Tie2-Cre and *KLF1+/-KLF2F/F* mice were mated to obtain E10.5 embryos of the following genotypes: *KLF1-/-KLF2F/F;Tie2-Cre* (*KLF1/KLF2* double conditional KO or dcKO), *KLF1-/-KLF2F/F* (*KLF1* KO) and *KLF1+/-KLF2F/+* (wild-type control). E10.5 *KLF1/KLF2* dcKO embryonic blood cells have substantially reduced KLF2 mRNA compared to wild-type (*Online Supplementary Figure S2*). At E10.5, wild-type (Figure 1A), *KLF1* KO (Figure 1B) and *KLF2* KO¹¹ embryos are surrounded by yolk sacs with distinctly red blood vessels. In comparison, E10.5 *KLF1/KLF2* dcKO mice (Figure 1C) are pale and

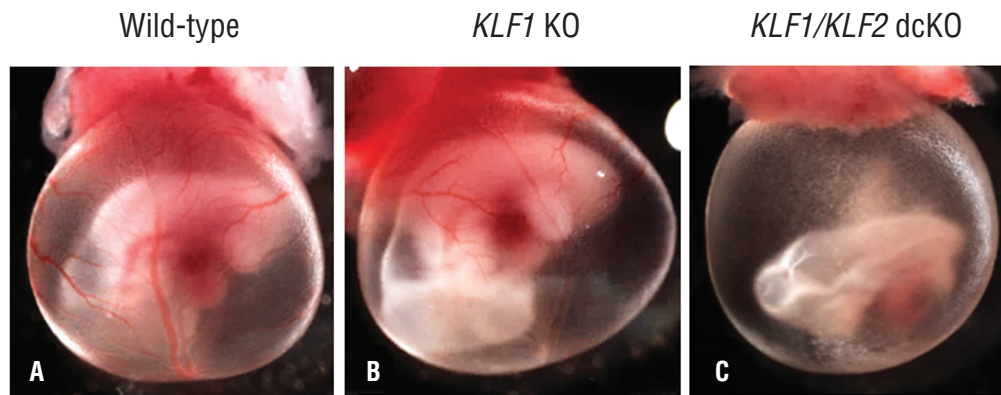


Figure 1. *KLF1/KLF2* double conditional KO embryos recapitulate the *KLF1*^{-/-}*KLF2*^{-/-} anemia phenotype. E10.5 whole-mount embryos surrounded by yolk sacs. (A) Wild-type embryos (*KLF1*^{+/+}*KLF2F*^{+/+}, n=3) have distinct red blood vessels. (B) *KLF1* KO (*KLF1*^{-/-}*KLF2F*^F, n=4) embryos are similar in appearance to wild-type embryos. (C) *KLF1/KLF2* double conditional KO embryos (*KLF1*^{-/-}*KLF2F*^F, Tie2-Cre, n=4) appear anemic in contrast to A and B. Photographs were taken at 15X magnification.

appear anemic, similar to *KLF1*^{-/-}*KLF2*^{-/-} embryos.¹¹ As a control to rule out Cre toxicity, it was determined that E10.5 whole-mount Tie2-Cre embryos do not show an anemia phenotype (*data not shown*).

To determine whether the pale appearance of *KLF1/KLF2* dcKO embryos is due to a defect in the development of blood vessels, yolk sac tissue sections were studied. Blood islands consisting of blood cells (Ery) surrounded by endothelial cells (En) are situated between the epithelium (Ep) and mesothelium (Me), as shown for wild-type in Figure 2A and D. The yolk sac vasculature of *KLF2* KO,¹⁹ *KLF1* KO (Figure 2B and E) and *KLF1/KLF2* dcKO embryos (Figure 2C and F) appears normal, with well-defined blood vessels lined by endothelial cells. This suggests that aberrant erythroid rather than vascular development causes the anemia phenotype. Although it is difficult to accurately assess the relative number of blood cells in yolk sac sections, *KLF1/KLF2* dcKO vessels appear to contain fewer cells than the other genotypes.

The globin mRNA amounts in E10.5 wild-type, *KLF1*^{-/-} and *KLF1/KLF2* dcKO peripheral blood cells were determined. E10.5 *KLF2* erythroid conditional KO (*KLF2F*^F*ErGFP*^{-Cre}) blood cells have approximately 20% less embryonic globin mRNA than wild-type.^{20,34} *KLF1*^{-/-} embryos have an approximately 35% reduction in β h1- and Ey-globin mRNA (*Online Supplementary Figure S3A* and *B*), while β h1- and Ey-globin mRNA in *KLF1/KLF2* dcKO embryos is reduced to approximately 50% of wild-type (*Online Supplementary Figure S3A* and *B*). Surprisingly, *KLF1/KLF2* dcKO embryos do not display drastic decreases in globin mRNA correlating with the observed anemia phenotype.

E10.5 *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos have fewer peripheral blood cells than *KLF1*^{-/-} *KLF2*^{+/-}

Rare recombinant embryos of the genotypes *KLF1*^{-/-}*KLF2F*^{+/+}, Tie2-Cre and *KLF1*^{+/-}*KLF2F*^F, Tie2-Cre were obtained. E10.5 *KLF1*^{-/-}*KLF2F*^{+/+}, Tie2-Cre embryos, with one *KLF2* allele, have yolk sacs with visibly red blood vessels like wild-type (*Online Supplementary Figure S4A*), but a *KLF1*^{+/-}*KLF2F*^F, Tie2-Cre embryo, with one *KLF1* allele, appears anemic like *KLF1/KLF2* dcKO (*Online Supplementary Figure S4B*). To extend this surprising finding to a system that is more easily manipulated, several

E10.5 *KLF1*^{-/-}*KLF2*^{+/-} and *KLF1*^{+/-}*KLF2*^{-/-} embryos were obtained. *KLF1*^{-/-}*KLF2*^{+/-} whole mount embryos appear similar to wild-type, with distinct red blood vessels (Figure 3A), but *KLF1*^{+/-}*KLF2*^{-/-} embryos appear anemic (Figure 3B). These findings confirm that one functional *KLF2*, but not *KLF1*, allele can ameliorate the anemia phenotype.

E10.5 *KLF1*^{-/-} embryos have approximately 2-fold fewer peripheral blood cells than wild-type.¹² In order to understand the contributions of *KLF1* and *KLF2* to the starkly different erythropoietic phenotypes of E10.5 *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{+/-} embryos, circulating blood cell numbers were determined. *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos have fewer peripheral blood cells than wild-type (Figure 3C). Of particular interest was the observation that *KLF1*^{+/-}*KLF2*^{-/-} embryos have significantly fewer peripheral blood cells than *KLF1*^{-/-}*KLF2*^{+/-} embryos (Figure 3C). The number of blood cells in E10.5 *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos, for which whole-mounts appear anemic, is similar and is several-fold less than wild-type.

The amount of embryonic β h1- and Ey-globin mRNA in blood cells from these embryos was determined using qRT-PCR. *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} blood cells have approximately 50% of the β h1-globin (Figure 3D) and Ey-globin mRNA (Figure 3E) compared to wild-type. The *KLF1*^{-/-}*KLF2*^{-/-} amount correlates with the findings in *KLF1/KLF2* dcKO embryos (*Online Supplementary Figure S3*). There are no significant differences in the amounts of β h1- and Ey-globin mRNA between *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos. Therefore, disparities in globin gene expression do not explain the major difference in the anemia phenotypes of *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} compared to *KLF1*^{-/-}*KLF2*^{+/-} whole-mount embryos. The measurements of the amount of globin mRNA per cell and the number of blood cells per embryo were multiplied to calculate the total embryonic β -like globin mRNA per embryo (*Online Supplementary Table S3*). *KLF1*^{-/-}*KLF2*^{+/-} embryos have approximately 4-fold less, whereas *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos have 10- to 15-fold less total globin mRNA per embryo than wild-type. Based on these findings, a combined reduction in globin per cell and in number of blood cells contributes to the anemia phenotype of E10.5 *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos.

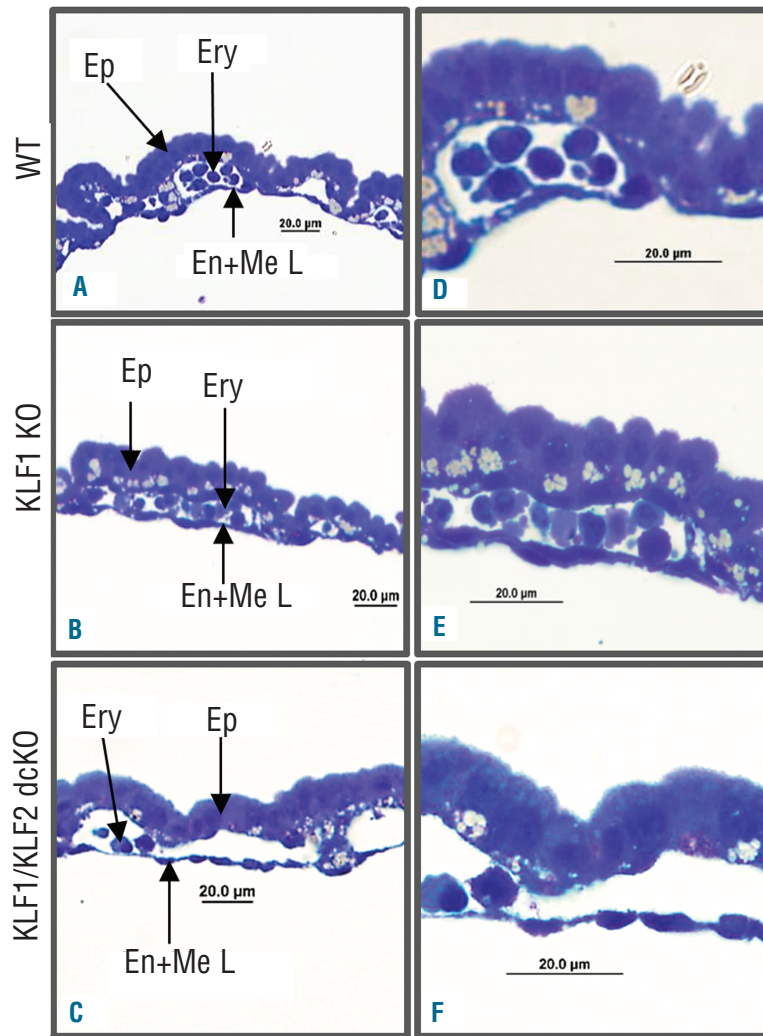


Figure 2. *KLF1/KLF2* double conditional KO embryos have normal yolk sac blood vessels. Blood islands from wild-type (A and D), *KLF1* KO (B and E) and *KLF1/KLF2* double conditional KO (C and F) yolk sacs. $n=3$ for each genotype. A, B and C are 400X; and D, E and F are 1000X magnification. The abbreviations used are columnar epithelium (Ep), blood cells (Ery) and endothelial/mesothelial layer (En + Me L).

E10.5 *KLF1*^{-/-}*KLF2*^{+/-} embryos may escape anemia because there is a compensatory increase in *KLF2* mRNA in the absence of *KLF1*, ameliorating the phenotype. To test this hypothesis, the amount of *KLF2* mRNA in blood cells of E10.5 wild-type and *KLF1*^{-/-} embryos was quantified by qRT-PCR. An approximate 2.5-fold increase in *KLF2* mRNA amount was observed in *KLF1*^{-/-} blood cells compared to wild-type (Online Supplementary Figure S5), supporting this postulate.

***KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} erythroblasts display impaired G1- to S-phase cell cycle progression**

Primitive and definitive *KLF1*^{-/-} blood cells show cell cycle defects.^{14,15} Furthermore, *KLF1* directly regulates *E2f2* and *p18*, genes that control cell cycle progression.^{14,15,37} It was, therefore, of interest to determine if *KLF1* and *KLF2* have overlapping functions in cell cycle regulation, and whether decreased proliferation is responsible for the reduced blood cell number in *KLF1*^{-/-}*KLF2*^{-/-} embryos. Therefore, the cell cycle profiles of E9.5 erythroblasts from *KLF1*^{-/-}*KLF2*^{-/-}, *KLF1*^{-/-}, *KLF2*^{-/-} and wild-type embryos were compared. After 90 min in cell culture medium containing BrdU, an average of 18.8% of wild-type erythroblasts are in G0/G1, 79.7% are in S-phase and 1.5% are in G2/M (Figure 4A). Similar results have been reported by Malik *et al.* for E10.5 erythroblasts.³³ The cell cycle distri-

bution of *KLF2*^{-/-} erythroblasts is similar to wild-type, suggesting that *KLF2* ablation does not affect cell cycle progression. *KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos have an approximately 20% reduction in cells in S-phase compared to wild-type, and a concomitant 2-fold increase in cells in G0/G1 phase, suggesting a block in S-phase entry. There were no significant differences between the cell cycle profiles of *KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} E9.5 erythroblasts, suggesting that *KLF1* has a unique role in regulating cell cycle progression that is not shared by *KLF2*. Simultaneous ablation of *KLF2* does not exacerbate the cell cycle disruption in *KLF1*^{-/-} red blood cells. This work with E9.5 *KLF1*^{-/-} erythroblasts extends and confirms other reports of cell cycle defects in *KLF1*^{-/-} blood cells.^{14,15}

The relative expression of certain genes important for G1- to S-phase progression, including *E2f2*, *E2f4*, *p18* and *p27*, was assessed in wild-type, *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} blood cells, to assess whether the observed gross phenotypic differences are associated with differential gene regulation. Recent studies have indicated that *KLF1* haploinsufficiency results in dysregulation of a subset of its target genes and hence *KLF1*^{+/-} blood cells were included as an additional control.^{38,39} The amount of *E2f2* (Figure 4B), *E2f4* (Figure 4C) and *p18* (Figure 4D) mRNA is significantly reduced in *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} blood cells

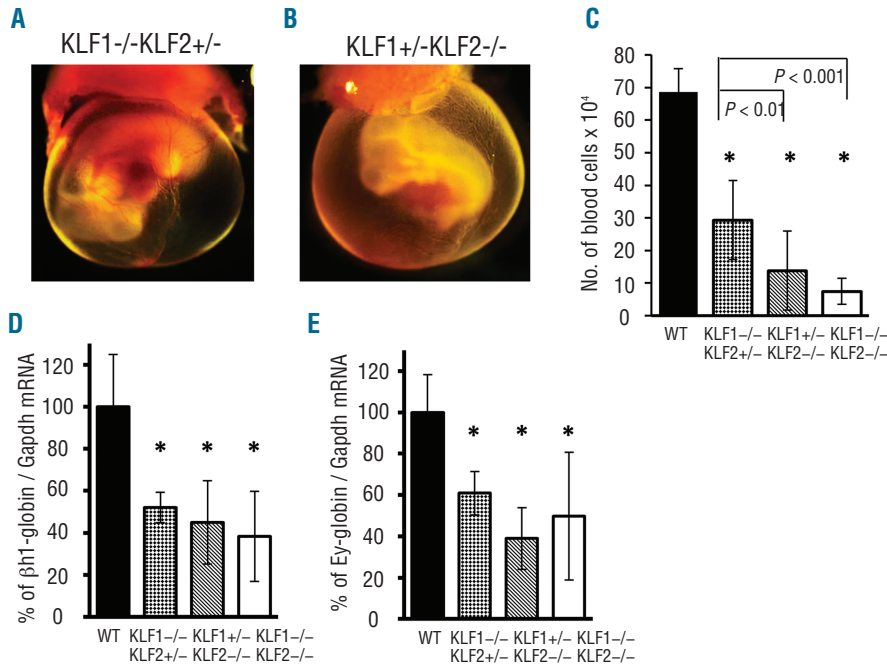


Figure 3. E10.5 *KLF1*^{+/-}/*KLF2*^{-/-} and *KLF1*^{-/-}/*KLF2*^{+/-} embryos have fewer peripheral blood cells than *KLF1*^{-/-}/*KLF2*^{+/-} embryos. (A and B) E10.5 whole-mount embryos surrounded by yolk sacs. Embryos with one normal *KLF2* gene, (A) *KLF1*^{-/-}/*KLF2*^{+/-} (n=3) appear grossly normal; whereas (B) *KLF1*^{+/-}/*KLF2*^{-/-} (n=3) embryos, with one functional *KLF1* gene, appear anemic. Photographs were taken at 15X magnification. (C) *KLF1*^{+/-}/*KLF2*^{-/-} and *KLF1*^{-/-}/*KLF2*^{-/-} embryos have fewer peripheral blood cells than *KLF1*^{-/-}/*KLF2*^{+/-}, correlating with the anemia phenotype. **P*<0.005 compared to wild-type. Other significant *P*-values are as shown by brackets. The amount of mouse embryonic (D) βh1-globin and (E) Eγ-globin mRNA is reduced in *KLF1*^{-/-}/*KLF2*^{+/-}, *KLF1*^{+/-}/*KLF2*^{-/-} and *KLF1*^{-/-}/*KLF2*^{-/-} blood cells compared to wild-type (WT) (* = *P*<0.05). *Gapdh* mRNA was used as an internal standard for qRT-PCR. The globin-to-*Gapdh* mRNA ratio for WT was taken as 100%. n=4-10 for each genotype. Error bars indicate standard deviation.

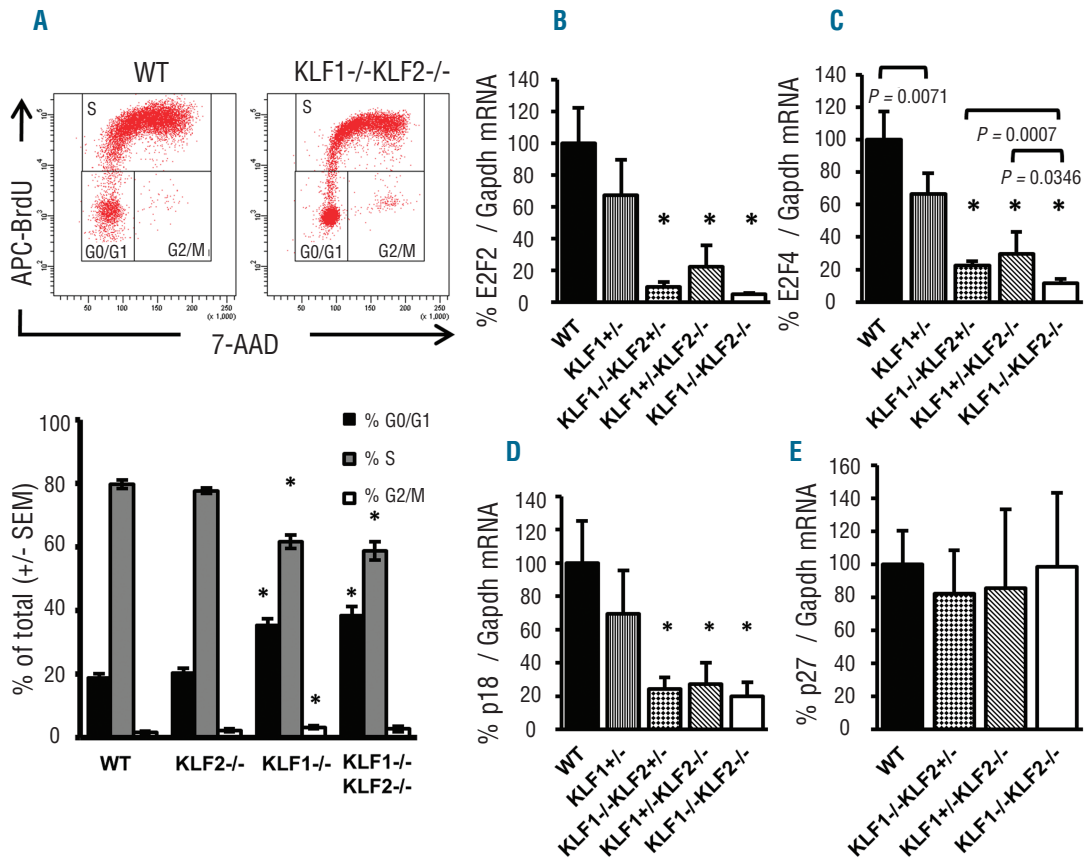


Figure 4. *KLF1*^{-/-} and *KLF1*^{-/-}/*KLF2*^{-/-} blood cells show aberrations in G1- to S-phase progression during cell division. (A) Representative flow cytometry plots measuring DNA content (7-AAD) and BrdU incorporation (APC-BrdU) are shown for WT and *KLF1*^{-/-}/*KLF2*^{-/-} embryos. The graph below depicts analyses of the percentage of cells in G0/G1, S and G2/M phases of the cell cycle in wild-type (WT), *KLF2*^{-/-}, *KLF1*^{-/-} and *KLF1*^{-/-}/*KLF2*^{-/-} E9.5 erythroblasts. n = 3-9 for each genotype. Error bars indicate standard error. **P*-value <0.01 compared to wild-type. Expression of genes (B) *E2f2* (C) *E2f4* (D) *p18* and (E) *p27* was compared in wild-type (WT), *KLF1*^{+/-}, *KLF1*^{-/-}/*KLF2*^{+/-}, *KLF1*^{+/-}/*KLF2*^{-/-} and *KLF1*^{-/-}/*KLF2*^{-/-} blood cells by qRT-PCR. *Gapdh* was used as an internal standard for qRT-PCR. The gene-to-*Gapdh* mRNA ratio for WT was taken as 100%. n = 4-10 for each genotype. **P*-value <0.01 compared to wild-type and *KLF1*^{+/-}. Other significant *P*-values are shown using brackets. Error bars indicate standard deviation.

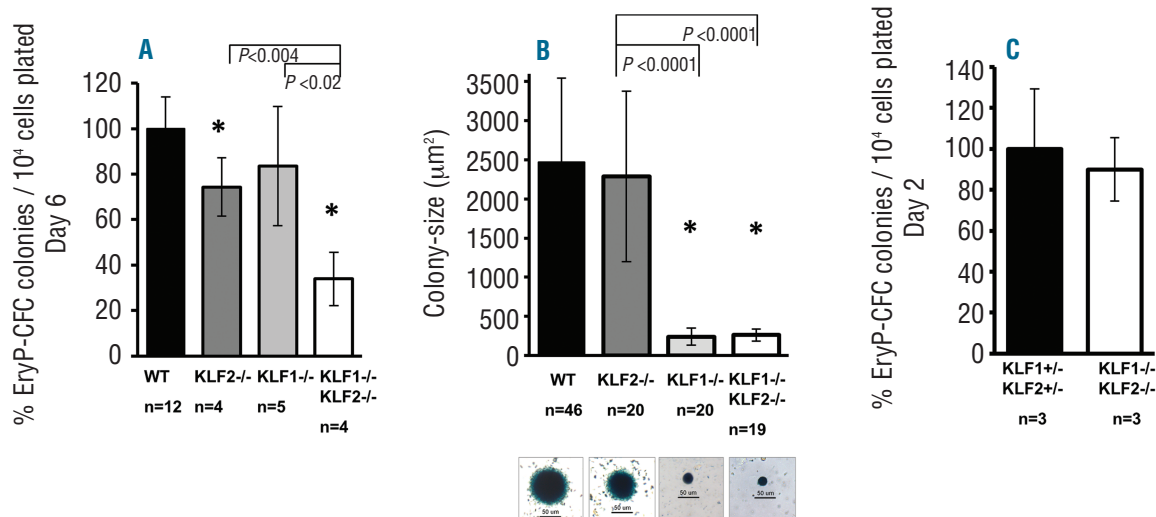


Figure 5. *KLF1*^{-/-}*KLF2*^{-/-} embryos have defects in primitive erythroid precursor maturation. E8.25 - E8.5 implants were dissociated into single cells using trypsin and plated in methylcellulose supplemented with growth factors. Erythroid colonies were stained with benzidine and counted on Day 2 or Day 6 of culture. (A) Comparison of number of EryP-CFC colonies formed per 10⁴ cells plated from wild-type (WT), *KLF2*^{-/-}, *KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} implants on day 6 of culture. The average number of colonies formed per 10⁴ cells plated from wild type implants was set to 100% for each litter. 'n' indicates number of embryos. (B) The average colony size was determined by measuring the two-dimensional area of colonies from captured images. Average colony size of *KLF2*^{-/-}, *KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} colonies was compared to wild-type colonies. 'n' indicates number of colonies analyzed from 4 to 6 embryos of each genotype. Representative colonies from each genotype are shown below the respective genotype (100X magnification, scale bar is 50 μm). (C) Comparison of number of EryP-CFC colonies formed per 10⁴ cells plated from *KLF1*^{+/-}*KLF2*^{+/-} and *KLF1*^{-/-}*KLF2*^{-/-} implants on day 2 of culture. The average number of colonies formed per 10⁴ cells plated from *KLF1*^{+/-}*KLF2*^{+/-} implants was set to 100. n: indicates number of embryos. *P-value <0.01 compared to wild-type. Other significant p-values are shown using brackets. Error bars indicate standard deviation.

compared to both wild-type and *KLF1*^{+/-} blood cells. *KLF1* and *KLF2* both contribute to the expression of these three cell cycle-related genes. The expression of p27 mRNA in the mutant genotypes is not different than wild-type (Figure 4E). None of these genes show significantly different expression in *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{+/-} blood cells, so there is no correlation with the gross phenotypic differences between these embryos.

***KLF1*^{-/-}*KLF2*^{-/-} embryos have defects in primitive erythroid precursor maturation**

The paucity of peripheral blood cells in *KLF1*^{-/-}*KLF2*^{-/-} embryos may result from a decrease in the number or a defect in the differentiation of erythroid progenitors. To determine whether *KLF1* and *KLF2* control the primitive erythroid progenitor (EryP-CFC) compartment, we performed colony-forming assays on E8.25 - E8.5 wild-type, *KLF1*^{-/-}, *KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos. At this developmental stage, EryP-CFC colony numbers reflect the number of primitive erythroid progenitors present in the yolk sac. On Day 4 of culture, visibly red primitive erythroid colonies are observed in wild-type (n=10) but not *KLF1*^{-/-}*KLF2*^{-/-} (n=4) cultures, indicating a defect in the erythroid compartment in the absence of *KLF1* and *KLF2* (data not shown).

To further assess this defect, colonies in methylcellulose were stained with benzidine for hemoglobin, at Day 6 of culture. *KLF1*^{-/-} embryos gave rise to normal numbers of EryP-CFCs (Figure 5A), but the colonies are approximately 10-fold smaller than wild-type (Figure 5B). This finding correlates with the data indicating that *KLF1*^{-/-} erythroid cells exhibit cell cycle defects (Figure 4A). Interestingly, *KLF2*^{-/-} embryos produce significantly fewer EryP-CFCs than wild-

type (Figure 5A), but colony size is indistinguishable from wild-type (Figure 5B). The number of EryP-CFCs derived from *KLF1*^{-/-}*KLF2*^{-/-} embryos is significantly less than wild-type, *KLF2*^{-/-} and *KLF1*^{-/-} (Figure 5A). The *KLF1*^{-/-}*KLF2*^{-/-} colonies are also smaller than wild-type, although similar in size to *KLF1*^{-/-}. It was of interest to distinguish whether there are fewer than normal progenitors in *KLF1*^{-/-}*KLF2*^{-/-} embryos, or if they are unable to produce colonies that survive to Day 6 of culture. Therefore the method of Malik *et al.* was used to count nascent colonies on Day 2 of culture.³³ There are similar numbers of EryP-CFC in *KLF1*^{-/-}*KLF2*^{-/-} and *KLF1*^{+/-}*KLF2*^{+/-} on Day 2 (Figure 5C). This suggests that normal numbers of primitive erythroid progenitors are specified in *KLF1*^{-/-}*KLF2*^{-/-} embryos, but the simultaneous ablation of *KLF1* and *KLF2* leads to a stochastic defect in maturation of erythroid precursors, leading to fewer colonies on Day 6. The *KLF1* and *KLF2* genes interact to synergistically control colony number. Thus, aberrant precursor potential and proliferation capacity likely contribute to the reduced number of blood cells in E10.5 *KLF1*^{-/-}*KLF2*^{-/-} embryos.

***KLF1* and *KLF2* modulate the expression of genes controlling proliferation**

Microarray analyses were used previously to demonstrate that the expression of five proliferation-associated genes, *Foxm1*, *Cd24a*, *Myc*, *Sphk1* and *Pthr*, is progressively reduced in wild-type, *KLF1*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} E10.5 blood cells.¹² Therefore, qRT-PCR was used to assess expression of these genes in wild-type, *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} E10.5 blood cells. The amounts of all five mRNAs are significantly reduced in *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-}

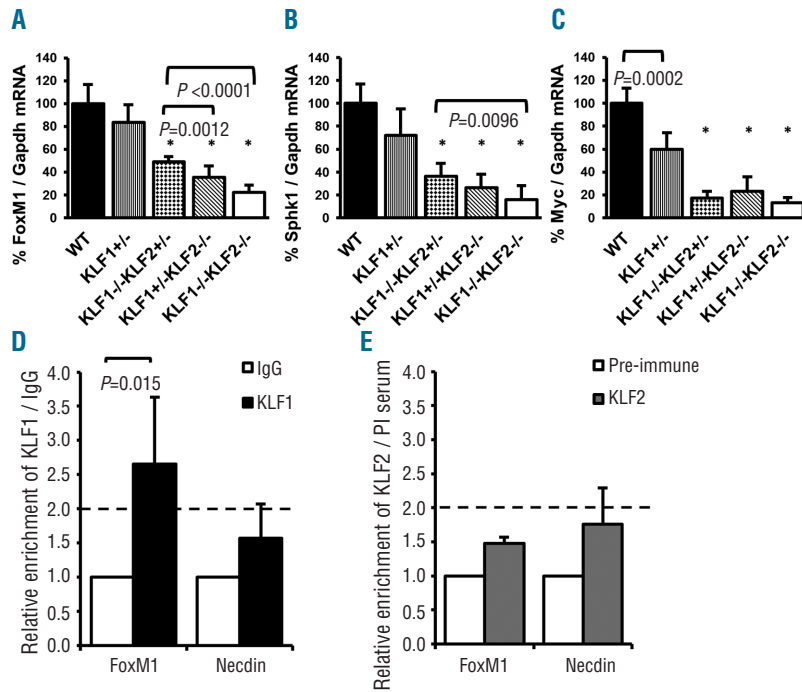


Figure 6. KLF1 and KLF2 modulate the expression of genes involved in proliferation. qRT-PCR was used to determine the amount of (A) *Foxm1*, (B) *Sphk1* and (C) *cMyc* mRNA in wild-type (WT), *KLF1*^{+/-}, *KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} E10.5 blood cells. Gapdh mRNA was used as an internal standard. Wild-type was taken as 100%. n = 4-10 per genotype. All of the test genotypes (*KLF1*^{-/-}*KLF2*^{+/-}, *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-}) were significantly different from wild-type and *KLF1*^{+/-} (**P* < 0.0001). Other significant p-values are as shown by brackets. Error bars indicate standard deviation. ChIP assays were performed on E10.5 wild-type erythroid cells. Polyclonal antibodies specific for (D) KLF1 and (E) KLF2 were used to determine binding/enrichment of the respective transcription factor at the *FoxM1* gene promoter. Non-specific IgG or pre-immune serum (PI serum) were used as controls for antibody specificity as indicated. The y-axis represents the relative fold enrichment. The mean IgG or PI serum enrichment was set to 1.0, and the enrichment of KLF1 and KLF2 were scaled appropriately. Based on the negative controls, a greater than 2-fold enrichment was considered a minimal requirement for binding, indicated by the dotted line. The x-axis indicates the gene promoter primers used for qPCR (described in *Online Supplementary Table 2*). Primers specific for the *Necdin* gene were used as a negative control. n = 3, error bars indicate standard deviation.

blood cells compared to wild-type and *KLF1*^{+/-} blood cells, verifying the results of the microarray analyses (Figure 6A-C and *Online Supplementary Figure S6A* and *B*). Only *Foxm1* mRNA is significantly reduced in *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} compared to *KLF1*^{-/-}*KLF2*^{+/-} (Figure 6A), specifically correlating with the genotypes that display the anemia phenotype and reduced peripheral blood cell number. However, it seems unlikely that dysregulation of a single downstream target gene is responsible for the anemia phenotype in E10.5 *KLF1*^{+/-}*KLF2*^{-/-} and *KLF1*^{-/-}*KLF2*^{-/-} embryos. Rather it is probably due to the cumulative effects of reduced expression of multiple proliferation-associated genes, including *FoxM1*.

The *Foxm1* gene has two consensus CACCC KLF binding sites within 500 bp upstream of the transcriptional start site. To assess whether KLF1 and KLF2 directly bind to the *Foxm1* promoter, ChIP assays were performed using wild-type E10.5 blood cells, followed by qPCR to quantify enrichment of KLF1 and KLF2. Based on the negative controls, a greater than 2-fold enrichment was considered a minimal requirement for binding. KLF1 is approximately 2.5-fold enriched compared to the negative control (Figure 6D), suggesting that it directly binds and regulates the *Foxm1* gene. Binding of KLF2 was not detected within this *Foxm1* promoter region (Figure 6E); however, it could bind to the gene at another site.

Discussion

The *KLF1* and *KLF2* genes interact to control globin gene regulation and primitive erythroid precursor maturation. Interactions between related transcription factor

genes in erythroid cell development have previously been reported. *Gata1* null embryos have normal primitive erythroid cell numbers.⁴⁰ *Gata2* knockout embryos have fewer primitive erythroid cells than wild-type.⁴¹ *Gata1/Gata2* double knockout embryos show a more severe defect in primitive erythropoiesis than either single knockout, with yolk sac blood islands almost completely devoid of erythroid cells.⁴² Functional overlap has also been described for KLF3 and KLF8, which act as transcriptional repressors. *KLF3* knockout mice have a mild erythroid phenotype.^{43,44} *KLF8* knockout mice have normal erythroid parameters.⁴⁵ The *KLF3/KLF8* double knockout causes embryonic lethality, and greater dysregulation of fetal liver gene expression than either single knockout.⁴⁵

The reduced number of E10.5 *KLF1*^{-/-}*KLF2*^{-/-} peripheral blood cells stems from a defect in erythroid precursor maintenance and maturation. Primitive erythroid progenitor colonies (EryP-CFC) derived from E8.5 *KLF1*^{-/-} embryos are similar in frequency but significantly smaller in size than wild-type. Studies of definitive fetal liver (adult) erythroid progenitors have produced inconsistent results with respect to the frequency of BFU-E and CFU-E colony forming units. Perkins *et al.* found a similar number of BFU-E and CFU-E in E15 *KLF1*^{-/-} and wild-type mice.⁹ Pilon *et al.* used sorted E13.5 *KLF1*^{-/-} fetal liver erythroid progenitors (CD71^{LO}, Ter119^{NEC}), and also found normal numbers of BFU-E.¹⁴

However, Pilon *et al.* observed an increased frequency of CFU-E in *KLF1*^{-/-} compared to wild-type, which was attributed to a block in erythropoiesis. Interestingly, they observed that *KLF1*^{-/-} fetal livers have fewer cells than wild-type.¹⁴ In addition, *KLF1*^{-/-} BFU-E and CFU-E colonies contain less hemoglobin and require 24-48 h

longer to reach the same size as wild-type, suggesting a defect in proliferation, analogous to our findings for embryonic progenitors.¹⁴ Thus KLF1, though present in a lower amount in primitive than in definitive erythroid cells,^{20,46} plays a similar role in proliferation at both stages.

A novel role for KLF2 in the maintenance of mouse primitive erythroid precursors was identified in this work. This correlates with ENCODE data from the Ross Hardison laboratory, which indicates that *KLF2* is robustly expressed in mouse adult megakaryocyte-erythroid progenitors (MEPs) (UCSC Accession: wgEncodeEM003184). Investigations into the role of KLF2 in definitive erythroid progenitors have produced discordant results. Wani *et al.* reported a severe reduction in the number of CFU-E derived from E11.5 *KLF2*^{-/-} fetal liver progenitors compared to wild-type.²² However, Kuo *et al.* found normal numbers of erythroid colonies in *KLF2*^{-/-} fetal liver cultures.²⁴ These studies could be complicated because the demise of the *KLF2*^{-/-} embryos is imminent. Our studies reveal that E8.25-E8.5 *KLF2*^{-/-} embryos have fewer primitive erythroid progenitor colonies than wild-type embryos on Day 6 of culture. KLF1 can partially compensate for the lack of KLF2, because fewer EryP-CFC are obtained from *KLF1*^{-/-}*KLF2*^{-/-} than from *KLF2*^{-/-} embryos. Interestingly, there are normal numbers of erythroid colonies derived from *KLF1*^{-/-}*KLF2*^{-/-} embryos on Day 2 of culture. It is possible that a stochastic selection event occurs beyond Day 2, causing only some *KLF1*^{-/-}*KLF2*^{-/-} colonies to be maintained. *KLF2* ablation leads to increased apoptosis in E10.5 yolk sac erythroid cells,¹⁹ and there is some evidence of a modest increase in apoptosis in *KLF1*^{-/-} definitive erythroid cells.¹⁶ Although it is not possible to study apoptosis directly in E8.5 erythroid progenitor cells, the stochastic event in *KLF1*^{-/-}*KLF2*^{-/-} cultures might logically involve apoptosis.

KLF1 and *KLF2* gene dosage is important for the normal regulation of several proliferation genes. The transcription factor Foxm1 stimulates proliferation by promoting S-phase entry in the cell cycle.⁴⁷ *Foxm1*^{-/-} embryos have approximately 3-fold fewer cardiomyocytes, with defects in DNA replication and mitosis,⁴⁸ but no increase in apoptosis was noted.^{27,48} Similarly, embryos with *KLF1* mutations have fewer blood cells that display aberrant S-phase entry, coincident with reduced *Foxm1*. *Foxm1* mRNA is present in lower amounts in *KLF1*^{+/-}*KLF2*^{-/-} than in *KLF1*^{-/-}*KLF2*^{+/-} embryonic erythroid cells, suggesting that it is more responsive to KLF2 than KLF1, and correlating with the anemia phenotype in E10.5 *KLF1*^{+/-}*KLF2*^{-/-} but not *KLF1*^{-/-}*KLF2*^{+/-} whole-mount embryos. Although the difference in *Foxm1* expression between *KLF1*^{-/-}*KLF2*^{+/-}

and *KLF1*^{+/-}*KLF2*^{-/-} embryos is modest, we speculate that small changes could have large effects in a milieu where the expression of multiple proliferation genes is dysregulated. In addition, there is a modest increase in *KLF2* mRNA in *KLF1*^{-/-} blood cells, possibly ameliorating the *KLF1*^{-/-}*KLF2*^{+/-} phenotype.

In summary, the Krüppel-like transcription factors KLF1 and KLF2 are critical for embryonic erythropoiesis. They are expressed at similar amounts and display functional compensation in primitive erythroid cells. This is evident in the dramatic anemia phenotype observed in *KLF1*/*KLF2* double knockout but not in *KLF1* or *KLF2* single knockout embryos at E10.5. In this study, we show that E10.5 anemia is primarily due to a reduction in the number of peripheral blood cells. Surprisingly, the anemia and the number of erythroid cells observed is dose-dependent, with more peripheral blood cells in *KLF1*^{-/-}*KLF2*^{+/-} than in *KLF1*^{+/-}*KLF2*^{-/-} embryos. KLF1 and KLF2 have a greater than additive positive effect on maintaining the colony forming ability of E8.5 erythroid progenitor cells, but KLF2 depletion alone leads to a reduced number of colonies compared to wild-type. These data suggest that certain genes involved in the maturation or proliferation of red blood cells are preferentially responsive to regulation by KLF2, as opposed to KLF1. Further studies of the roles of KLF1 and KLF2 in erythroid precursor maturation may lead to novel therapeutic strategies for the anemias.

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