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The CACCC-Binding Protein KLF3/BKLF Represses a Subset of KLF1/EKLF Target Genes and Is Required for Proper Erythroid Maturation *In Vivo*

Alister P. W. Funnell,[a] Laura J. Norton,[a] Ka Sin Mak,[a] Jon Burdach,[a] Crisbel M. Artuz,[a] Natalie A. Twine,[a] Marc R. Wilkins,[a] Carl A. Power,[b] Tzong-Tyng Hung,[b] José Perdomo,[c] Philip Koh,[d] Kim S. Bell-Anderson,[e] Stuart H. Orkin,[f] Stuart T. Fraser,[g] Andrew C. Perkins,[d,h] Richard C. M. Pearson,[a] and Merlin Crossley[a]

School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia[a]; Mark Wainwright Analytical Centre, University of New South Wales, Sydney, New South Wales, Australia[b]; Department of Medicine, St. George Clinical School, University of New South Wales, Sydney, New South Wales, Australia[c]; Division of Molecular Genetics and Development, Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland, Australia[d]; School of Molecular Bioscience, University of Sydney, Sydney, New South Wales, Australia[e]; Division of Hematology/Oncology, Children's Hospital Boston and Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Stem Cell Institute, Harvard Medical School, Boston, Massachusetts, USA[f]; Discipline of Physiology, School of Medical Science, University of Sydney, Sydney, New South Wales, Australia[g]; and Mater Medical Research Institute, South Brisbane, Queensland, Australia[h]

The CACCC-box binding protein erythroid Krüppel-like factor (EKLF/KLF1) is a master regulator that directs the expression of many important erythroid genes. We have previously shown that EKLF drives transcription of the gene for a second KLF, basic Krüppel-like factor, or KLF3. We have now tested the *in vivo* role of KLF3 in erythroid cells by examining *Klf3* knockout mice. KLF3-deficient adults exhibit a mild compensated anemia, including enlarged spleens, increased red pulp, and a higher percentage of erythroid progenitors, together with elevated reticulocytes and abnormal erythrocytes in the peripheral blood. Impaired erythroid maturation is also observed in the fetal liver. We have found that KLF3 levels rise as erythroid cells mature to become TER119$^+$. Consistent with this, microarray analysis of both TER119$^-$ and TER119$^+$ erythroid populations revealed that KLF3 is most critical at the later stages of erythroid maturation and is indeed primarily a transcriptional repressor. Notably, many of the genes repressed by KLF3 are also known to be activated by EKLF. However, the majority of these are not currently recognized as erythroid-cell-specific genes. These results reveal the molecular and physiological function of KLF3, defining it as a feedback repressor that counters the activity of EKLF at selected target genes to achieve normal erythropoiesis.

The Krüppel-like factor (KLF) transcription factors are characterized by three highly conserved C-terminal Cys_2-His_2 zinc finger motifs that bind CACCC boxes and other GC-rich elements in control regions of DNA (15). The N-terminal functional domains are less conserved, and individual KLFs are able to recruit different coregulators to function as transcriptional activators and/or repressors. For example, recruitment of the acetyltransferases P/CAF and p300/CBP by erythroid Krüppel-like factor (EKLF/KLF1) potentiates its activation of the β-*globin* gene (33, 34), while Krüppel-like factor 3 (KLF3/BKLF) utilizes the corepressor C-terminal binding protein (CtBP) to silence gene expression (5, 29).

EKLF, the founding member of the KLF family, plays an essential role in many aspects of erythropoiesis. EKLF is a potent transcriptional activator that binds to 5′-NCNCNCCCN-3′ motifs of DNA (7, 28), and as its name suggests, it is almost exclusively expressed in erythroid cells (12). Most notably, EKLF activates expression of the adult β-*globin* gene (12), and as a result, mice lacking EKLF die *in utero* at around embryonic day 14.5 from lethal β-thalassemia (13, 18). Microarray and chromatin immunoprecipitation-sequencing (ChIP-Seq) studies have further revealed that EKLF regulates the expression of many erythroid-cell-specific genes, including genes involved in heme biosynthesis, red blood cell proliferation, and membrane integrity (1, 4, 11, 19–21, 28). One gene which has consistently emerged as an EKLF target in these studies is that encoding another member of the KLF family, *Klf3*.

Klf3 was initially cloned from erythroid cells in a screen for factors with homology to the DNA-binding domain of EKLF (3, 16). EKLF and KLF3 have similar DNA-binding preferences, showing high-affinity interactions with many of the same erythroid promoter CACCC boxes *in vitro*, such as the β-*globin* gene promoter (3). Whereas EKLF can function as a potent activator of transcription, KLF3 has primarily been shown to repress transcription via the recruitment of CtBP and associated chromatin-modifying enzymes (5, 24, 29, 30).

KLF3 is expressed in a wide range of cells; however, it is particularly abundant in erythroid tissue (16). This is due in part to the fact that the *Klf3* gene possesses two promoters: an upstream promoter that is active in a range of tissues and a downstream, erythroid-cell-specific promoter that has been shown by EMSA, ChIP, and ChIP-Seq to be directly bound by EKLF (8, 28). Importantly, EKLF functionally drives *Klf3* expression, as KLF3 levels are significantly reduced in *Eklf*$^{-/-}$ fetal liver, and restoring EKLF in EKLF-deficient cell lines leads to rapid upregulation of *Klf3* transcription (8). Taken together, the high erythroid expression of KLF3, its erythroid-cell-specific promoter, and its dependence on

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Address correspondence to Merlin Crossley, m.crossley@unsw.edu.au.
A.P.W.F. and L.J.N. contributed equally to this work.
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EKLF implicate KLF3 in erythropoiesis. Furthermore, given the similar DNA-binding preferences of EKLF and KLF3, this raises the possibility that these two factors operate in a feedback circuit to fine-tune gene expression during erythroid cell maturation.

To test the physiological role of KLF3 *in vivo*, we examined erythroid development in the fetal liver and in the bone marrow, spleen, and peripheral circulation of adult *Klf3*$^{-/-}$ mice. While *Klf3*$^{-/-}$ mice have been shown to display defects in adipogenesis and B-cell lymphopoiesis (26, 31), here we report for the first time the erythroid phenotype of *Klf3*$^{-/-}$ mice. Erythroid maturation is impaired in *Klf3*$^{-/-}$ fetal liver, while reticulocytosis and an increase in erythroid nuclear inclusions (Howell-Jolly bodies) are apparent in the peripheral blood of adult *Klf3*$^{-/-}$ mice. In addition, there is a significant increase in the percentage of CD71^{+} TER119^{+} proerythroblasts in the spleen in the absence of KLF3, indicative of compensatory erythropoiesis. Taken together, these observations are consistent with loss of KLF3 leading to defective erythropoiesis and compensated anemia.

To understand the molecular mechanisms underlying KLF3's role in erythropoiesis, we employed a microarray-based approach comparing sorted *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ fetal liver erythroid populations. This analysis revealed a set of genes that are significantly derepressed in the absence of KLF3, consistent with the view that KLF3 serves predominantly as a transcriptional repressor. The comparison of sorted TER119^{-} and TER119^{+} cells also revealed that KLF3 expression is low in erythroid progenitors and is subsequently upregulated as erythroid cells mature. In agreement with this, we found that few genes are deregulated in *Klf3*$^{-/-}$ TER119^{-} progenitors, in noticeable contrast to the derepression of many target genes in more mature TER119^{+} erythroblasts.

A comparison of genes deregulated following loss of KLF3 with those known to be regulated by EKLF revealed many shared targets. The majority of these genes have no known roles in erythroid biology. This raises the possibility that KLF3 serves to focus the activity of EKLF by preventing the promiscuous activation of nonerythroid CACCC box genes during erythroid maturation. Taken together, these findings indicate that KLF3 is required for normal erythropoiesis and suggest that the balance of activating and repressing KLFs in erythroid tissue is necessary for the precise regulation of erythroid differentiation.

MATERIALS AND METHODS

***Klf3*$^{-/-}$ and *Eklf* $^{-/-}$ mice.** Ethics approval was obtained from the Animal Care and Ethics Committee, University of New South Wales, approval no. 09/128A. Generation of *Klf3*$^{-/-}$ mice has been described previously (26). Briefly, ablation of *Klf3* was achieved by replacing a genomic segment between intron 4 and exon 6 of the wild-type sequence with a neomycin resistance gene (*neo*) construct. *Klf3*$^{-/-}$ mice had previously been backcrossed for more than 10 generations to ensure a pure FVB/NJ strain (26). The *Eklf* $^{-/-}$ mouse line has also been described previously (18).

Full blood count analysis. Peripheral blood was collected from *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ mice via cardiac puncture into EDTA-coated Vacuettes (Greiner Bio-One), with heparin included at 2.5 U/ml blood. Full blood count analysis was performed by the Veterinary Pathology Diagnostic Services (VPDS) unit, University of Sydney.

Erythrocyte life span assay. *N*-Hydroxysuccinimide biotin was dissolved at 50 mg/ml in *N*,*N*,-dimethylacetamide. This was administered via a single tail vein injection at a dose of 50 mg/kg, diluted in 250 μl saline solution immediately prior to use. To assess erythrocyte life span, 30 μl peripheral blood was obtained from the tail vein of treated mice at regular intervals following *in vivo* biotin labeling. Samples were then analyzed by flow cytometry, following staining with streptavidin–R-phycoerythrin and anti-TER119-V450 antibody (BD Bioscience).

Cytological analysis. Blood smears were air dried, fixed in methanol for 15 min, stained in May-Grünwald solution (5 min) followed by Giemsa solution (15 min), and finally washed with distilled water. The slides were allowed to air dry before mounting with DePeX for storage. Spleens were dissected from litter-matched, 12-week-old *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ mice and placed into 20×-spleen-volume 10% neutral buffered formalin for 24 h. Spleens were stained with hematoxylin and eosin (H&E) and were sectioned at the Veterinary Pathology Diagnostic Services (VPDS) unit, University of Sydney.

Flow cytometry of erythrocyte populations. Flow cytometry was performed using either FACSCalibur or LSRFortessa flow cytometers (BD Bioscience), and data were analyzed using FlowJo v7.6.5 (TreeStar) software. TER119 and CD71 (clone R17217) antibodies were supplied by BD Bioscience and titrated to optimal concentrations. Reticulocytes were identified by including thiazole orange (0.1 μg/ml).

B1.6 cells. Culture of line B1.6 erythroblasts and tamoxifen-induced rescue of EKLF nuclear activity in these cells were performed as previously described (2, 8).

Purification of erythroid cell populations, cDNA synthesis, and real-time PCR. TER119^{-} and TER119^{+} cells were purified from E14.5 fetal liver using magnet-activated cell sorting with anti-TER119 Microbeads (Miltenyi Biotech) by negative depletion (LD columns) and positive selection (MS columns), respectively, as per the supplier's instructions. Total RNA was extracted using TRI reagent (Sigma, St. Louis, MO) and RNeasy kits (Qiagen, Victoria, Australia) and treated with DNase as described previously (8). cDNA was synthesized using a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). Primers were designed using PrimerExpress v3.0 (Applied Biosystems, Foster City, CA) as previously described (8) and synthesized by Sigma-Aldrich. Primers for the 18S and *Klf3* genes have been described previously (8). Primer sequences for other genes are as follows: *Klf8*, 5′-CCAAAAGCTCTCACCTGAAAGC-3′ and 5′-AGCGAGCAAATTTCCAGGAA-3′; *Lgals3*, 5′-GATCACAATCATGGGCACAG-3′ and 5′-ATTGAAGCGGGGGTTAAAGT-3′; *Fam132a*, 5′-GGTCTTCACAGTGCAGGTTCAG-3′ and 5′-GACTGCCCCAGAACTGTTGTC-3′; *Epo*, 5′-CAGCTAGGCGCGGAGATG-3′ and 5′-AATCAGTAGCAAGGAGAGTAAAAGCA-3′. Quantitative real-time PCR runs were performed in triplicate with Power SYBR green PCR Master Mix and the 7500 fast real-time PCR system (Applied Biosystems) as described previously (9). Data analysis was performed using 7500 software v2.0.4 (Applied Biosystems).

Microarray analysis. Total RNA was purified from TER119^{-} and TER119^{+} E14.5 fetal liver cells (*Klf3*$^{+/+}$ and *Klf3*$^{-/-}$) or whole E13.5 fetal liver (*Eklf* $^{+/+}$ and *Eklf* $^{-/-}$) as described above. RNA was subsequently ethanol precipitated and washed with 75% ethanol in DEPC-treated deionized water for further purification. RNA was then subjected to whole-transcript sense labeling and hybridized to Affymetrix GeneChip 1.0 ST mouse gene arrays (Affymetrix, Santa Clara, CA). Microarray preparation and scanning were performed by the Ramaciotti Centre, University of New South Wales, New South Wales, Australia. Microarray data were analyzed using Partek genomic suite v6.5 (Partek Inc., St. Louis, MO). Microarray CEL files were imported into Partek and normalized using the robust multiarray average (RMA) algorithm. After confirming array quality (Affymetrix built-in controls and principal components analysis), differential gene expression was calculated and tested for significance using a 1-way analysis of variance (ANOVA). Gene expression *P* values were corrected for multiple testing using a false discovery rate (FDR) threshold of 0.2. Genes passing the threshold and having a change greater than 2-fold were considered to be significantly differentially expressed. Hierarchical clustering was performed using this gene set with Euclidean distance and average linkage as set clustering parameters.

Assignation of putative EKLF/KLF3 shared target genes. Table S1 in the supplemental material shows the list of genes that are derepressed >2-fold (0.2 FDR) in *Klf3*$^{-/-}$ TER119^{+} E14.5 fetal liver cells. A propor-

tion of these genes have been designated as putative EKLF targets according to one or more of the following criteria: they contain or are most proximal to one or more of the set of 1,380 declared peaks in the EKLF ChIP-Seq study by Tallack et al. (28); they are occupied by EKLF and, in addition, are expressed at significant levels in either TER119$^-$ progenitor cells or TER119$^+$ erythroblasts (from E13.5 fetal liver) in the EKLF ChIP-Seq study by Pilon et al. (19); their expression is declared as significantly altered in the EKLF microarray studies by Pilon et al. (20) and Borg et al. (1); their expression is dysregulated >2-fold in the EKLF microarray studies by Hodge et al. (11) and Drissen et al. (4); they have previously been validated as an EKLF target gene *in vivo* as described by Eaton et al. (5); or they display >2-fold deregulation in our own microarray analysis of *Eklf*$^{-/-}$ E13.5 fetal liver cells or by qRT-PCR analysis of tamoxifen-induced compared to uninduced B1.6 cells.

Chromatin immunoprecipitation. ChIP was conducted on *Klf3*$^{-/-}$ murine embryonic fibroblasts, stably transduced using a murine stem cell virus retroviral delivery system containing V5-epitope tagged KLF3 or an empty vector control. Approximately 1×10^7 cells were grown to 80% confluence in Dulbecco's modified Eagle medium (DMEM), then fixed with 1% formaldehyde for 10 min, and quenched by the addition of 2 M glycine. Following Dounce treatment in 5.4 ml cytoplasmic lysis buffer (20 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA, and 1% Igepal CA-630), nuclei were resuspended in 1.8 ml sonication buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate [SDS], and 1% Igepal CA-630) and sonicated for 8 min in a Bioruptor (Diagenode, Belgium). Input samples (18 μl each) were retained for DNA extraction. Sonicate was diluted 1/10 in ChIP dilution buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 1% Igepal CA-630), and 4 μl anti-V5 antibody (Invitrogen) was added before incubation for 2 h at 4°C. A 50-μl portion of washed protein G Dynabeads (Invitrogen) was added, and incubation continued for a further 30 min. Dynabeads were washed once each with low-salt buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 1% Igepal CA-630), high-salt buffer (20 mM Tris [pH 8.0], 500 mM NaCl, 1 mM EDTA and 1% Igepal CA-630), and LiCl buffer (20 mM Tris [pH 8.0], 250 mM LiCl, 1 mM EDTA, 0.5% Igepal CA-630, and 0.5% sodium deoxycholate) and then twice with low-salt buffer. DNA was extracted from input and IP samples using an IPure kit (Diagenode, Belgium). Real-time PCR was performed on duplicate-input and IP samples using the ΔC_T method to determine fold enrichment of IP over input. Primer sequences identifying positive (+0.2 kb) and negative (−4.5 kb) control sequences in the *Klf8* promoter 1a are as previously described (5). Primer sequences for other genes are as follows: *Lgals3*, 5′-TGGAAAAACACCCGTGCCTCTGA-3′ and 5′-CAGTGCCTACGCCCAGATGACTC-3′; *Fam132a*, 5′-GATTCG CTTCCCTGGAGGTGTGG-3′ and 5′-GCCCAGTCTCTGGTCTCCTC TCT-3′.

Microarray data accession numbers. Microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo) under accession numbers GSE35592 (KLF3 E14.5 fetal liver arrays) and GSE35594 (EKLF E13.5 fetal liver arrays).

RESULTS

Loss of KLF3 results in a mild compensated anemia. We first performed full blood counts on peripheral blood collected from 20 *Klf3*$^{+/+}$ and 20 *Klf3*$^{-/-}$ age-matched adult mice (Fig. 1A to G). The examination of this cohort revealed subtle but consistent alterations in blood parameters. *Klf3*$^{-/-}$ erythrocytes contain less mean cell hemoglobin than *Klf3*$^{+/+}$ cells, and this appears to be compensated for by an increase in red blood cell count and hematocrit. The mean corpuscular volume of *Klf3*$^{-/-}$ erythrocytes is unchanged, indicating that the increased hematocrit reflects the increase in red blood cell count, rather than effects on cell size. Full blood count analysis also suggested significant reticulocytosis in *Klf3*$^{-/-}$ mice, confirmed by flow cytometric analysis of peripheral blood reticulocytes stained with thiazole orange (Fig. 1H). Additionally, peripheral blood smears from *Klf3*$^{-/-}$ mice showed an increase in the number of reticulocytes and revealed the presence of nuclear inclusions (Howell-Jolly bodies [23]) in a number of erythrocytes, again indicative of anemia (Fig. 2D). The presence of a mild compensated anemia in *Klf3*$^{-/-}$ mice is further supported by increased expression of erythropoietin (Fig. 1I). We also investigated the life span of *Klf3*$^{-/-}$ erythrocytes *in vivo* and found a significant reduction in their half-life, a probable contributory factor in the observed anemia (Fig. 1J). Finally, we also observed a significant decrease in the number of circulating platelets in *Klf3*$^{-/-}$ mice (Fig. 1G), suggesting that KLF3 may influence lineage decisions in common megakaryocyte-erythroid progenitors, as has previously been shown for EKLF (27).

Erythroid defects in the spleen and bone marrow of *Klf3*$^{-/-}$ mice. In the mouse, the adult spleen is both an erythrocyte reservoir and a site of erythropoiesis that is particularly active during periods of erythroid stress. The spleens of *Klf3*$^{-/-}$ animals appeared larger than those of *Klf3*$^{+/+}$ littermates (Fig. 2A). Measuring spleen weight, which we normalized to body weight to account for the small phenotype of *Klf3*$^{-/-}$ mice (26), confirmed the increased spleen size (Fig. 2A). We also examined H&E-stained sections of *Klf3*$^{-/-}$ spleens and found considerable disruption to the splenic architecture, with a notable increase in the proportion of erythroid red pulp, at the expense of lymphoid white pulp (Fig. 2B and C). This suggested that compensatory stress erythropoiesis occurs in the spleens of *Klf3*$^{-/-}$ mice. To confirm this, we stained splenic erythrocytes for expression of CD71 and TER119 to distinguish nonerythroid cells (CD71^{lo} TER119^{lo}) and the various maturing erythroid populations: proerythroblasts (CD71^{hi} TER119^{med}), basophilic erythroblasts (CD71^{hi} TER119^{hi}), polychromatophilic erythroblasts (CD71^{med} TER119^{hi}), and the more mature orthochromatophilic erythroblast and reticulocyte (CD71^{lo} TER119^{hi}) populations (25). We found that the absence of KLF3 leads to an increase in the percentage of all of these erythroid cells, with the proerythroblast and basophilic erythroblast populations showing the most significant changes (Fig. 3A). These changes were accompanied by a significant decrease in the nonerythroid compartment, consistent with the spleen sections. The increase in the earlier erythroid populations is suggestive of ongoing stress erythropoiesis in the spleens of *Klf3*$^{-/-}$ mice.

We also examined erythropoiesis in the bone marrow of *Klf3*$^{-/-}$ mice, again by staining for expression of CD71 and TER119 to identify maturing erythroid populations from proerythroblast to reticulocyte. Although loss of KLF3 has a less dramatic effect in bone marrow than spleen, this analysis revealed a reproducible and significant increase in the percentage of CD71^{hi} TER119^{med} proerythroblasts in *Klf3*$^{-/-}$ bone marrow (Fig. 3B), again consistent with compensatory erythropoiesis. The less severe disruption seen in the bone marrow of *Klf3*$^{-/-}$ mice may reflect the respective contributions of bone marrow and spleen to stress erythropoiesis in the mouse. While homeostatic red blood cell production occurs mainly in the bone marrow to ensure a constant steady-state supply of cells, compensatory erythropoiesis takes place predominantly in the spleen (14). Our data are consistent with loss of KLF3 leading to significant compensatory responses mediated largely by the spleen.

Reduced cellular maturation in the fetal liver of *Klf3*$^{-/-}$ mice. Fetal erythropoiesis proceeds through a number of stages that can also be defined by staining erythrocytes for surface

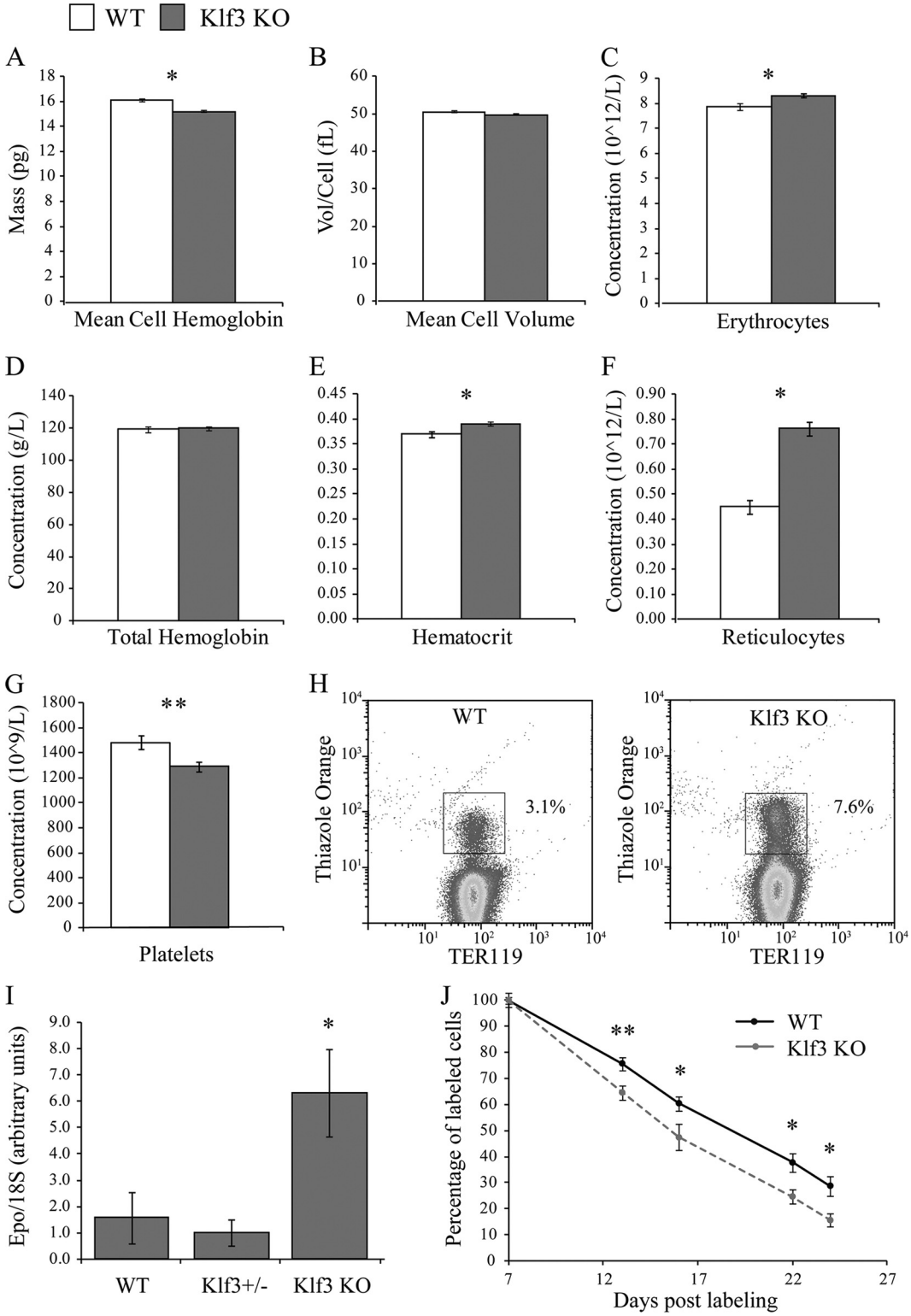

FIG 1 Peripheral red blood cell parameters of $Klf3^{-/-}$ adult mice. (A to G) Full peripheral blood count analysis was performed on 20 $Klf3^{+/+}$ and 20 $Klf3^{-/-}$ mice, 10 to 20 weeks old. (H) Representative flow cytometry plot of four $Klf3^{+/+}$ and four $Klf3^{-/-}$ mice, showing peripheral blood stained with thiazole orange and anti-TER119 antibody to determine reticulocyte percentage. (I) Expression levels of erythropoietin (Epo) in adult kidney from $Klf3^{+/+}$ ($n = 3$), $Klf3^{+/-}$ ($n = 2$), and $Klf3^{-/-}$ ($n = 2$) mice were determined by qRT-PCR. Relative expression was normalized against 18S rRNA and the expression level in $Klf3^{+/-}$ tissue was set to 1.0. (J) Plot showing the relative survival rate of WT ($n = 3$) and $Klf3^{-/-}$ ($n = 3$) erythrocytes tracked *in vivo* by biotin labeling. Error bars represent the standard errors of the means. *P* values indicate the differences between two means: *, $P < 0.05$; **, $P < 0.01$. WT, wild type ($Klf3^{+/+}$); KO, knockout ($Klf3^{-/-}$).

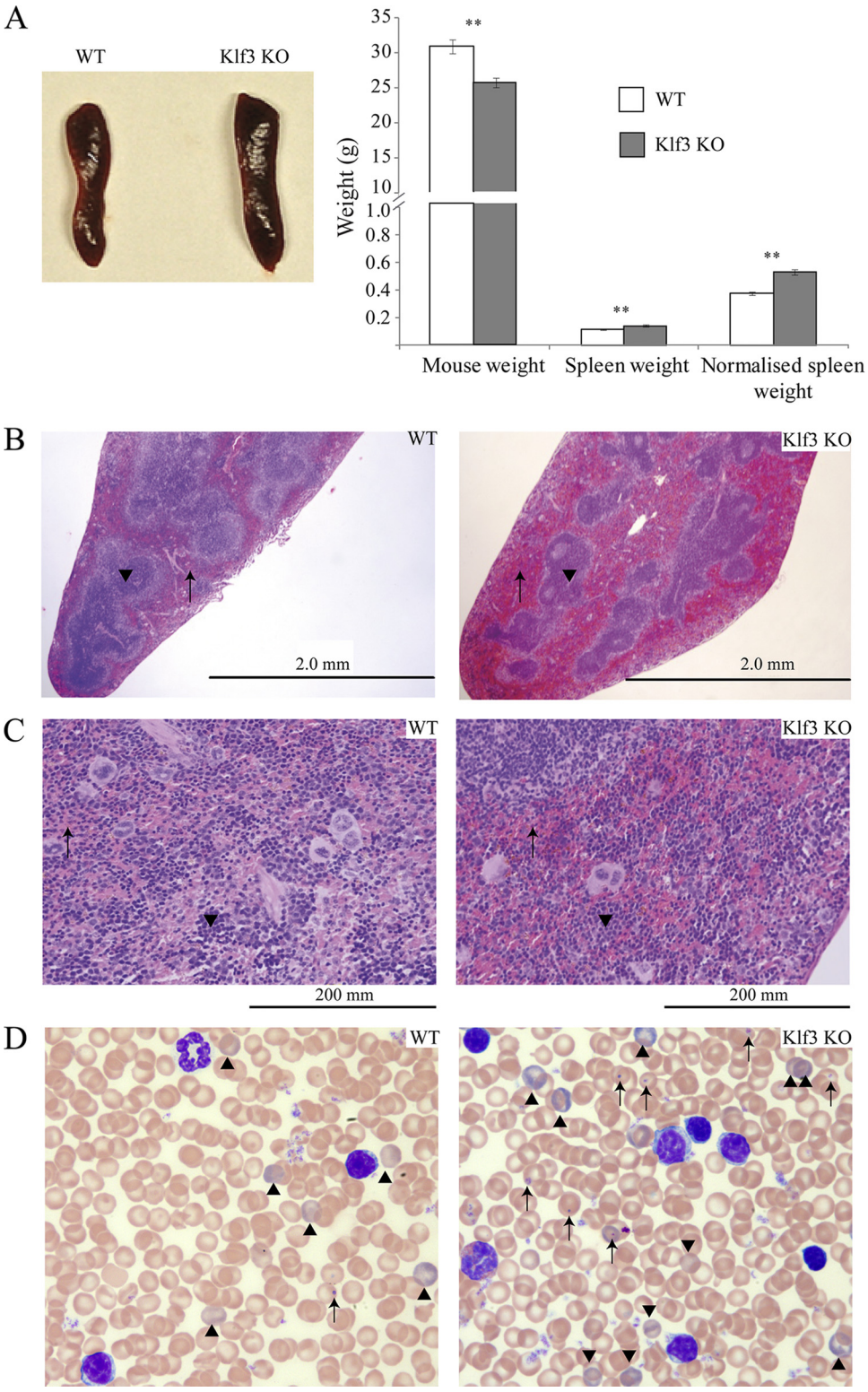

FIG 2 Abnormal splenic structure and erythropoiesis in *Klf3*$^{-/-}$ mice. (A) *Klf3*$^{-/-}$ spleens are larger than those of *Klf3*$^{+/+}$ littermates (left). *Klf3*$^{-/-}$ mice are smaller than *Klf3*$^{+/+}$ littermates (26), while normalized *Klf3*$^{-/-}$ spleen weights are significantly increased (right; $n = 42$ for each genotype). (B and C) *Klf3*$^{-/-}$ spleens have increased red pulp at the expense of white pulp. Shown are representative sections from analysis of four *Klf3*$^{+/+}$ (left) and four *Klf3*$^{-/-}$ mice (right) stained with H&E at ×40 (B) and ×100 (C) magnification. Arrows indicate red pulp, and arrowheads show white pulp. (D) Increased prevalence of Howell-Jolly bodies (arrows) and reticulocytes (arrowheads) in the peripheral blood of *Klf3*$^{-/-}$ animals. Shown are representative smears (magnification, ×1,000) comparing *Klf3*$^{+/+}$ (left) and *Klf3*$^{-/-}$ (right) peripheral blood samples from analysis of 6 mice of each genotype, 12 to 16 weeks old. Error bars show the standard errors of the means. *P* values indicate the differences between two means: **, $P < 0.01$. WT, wild type (*Klf3*$^{+/+}$); KO, knockout (*Klf3*$^{-/-}$).

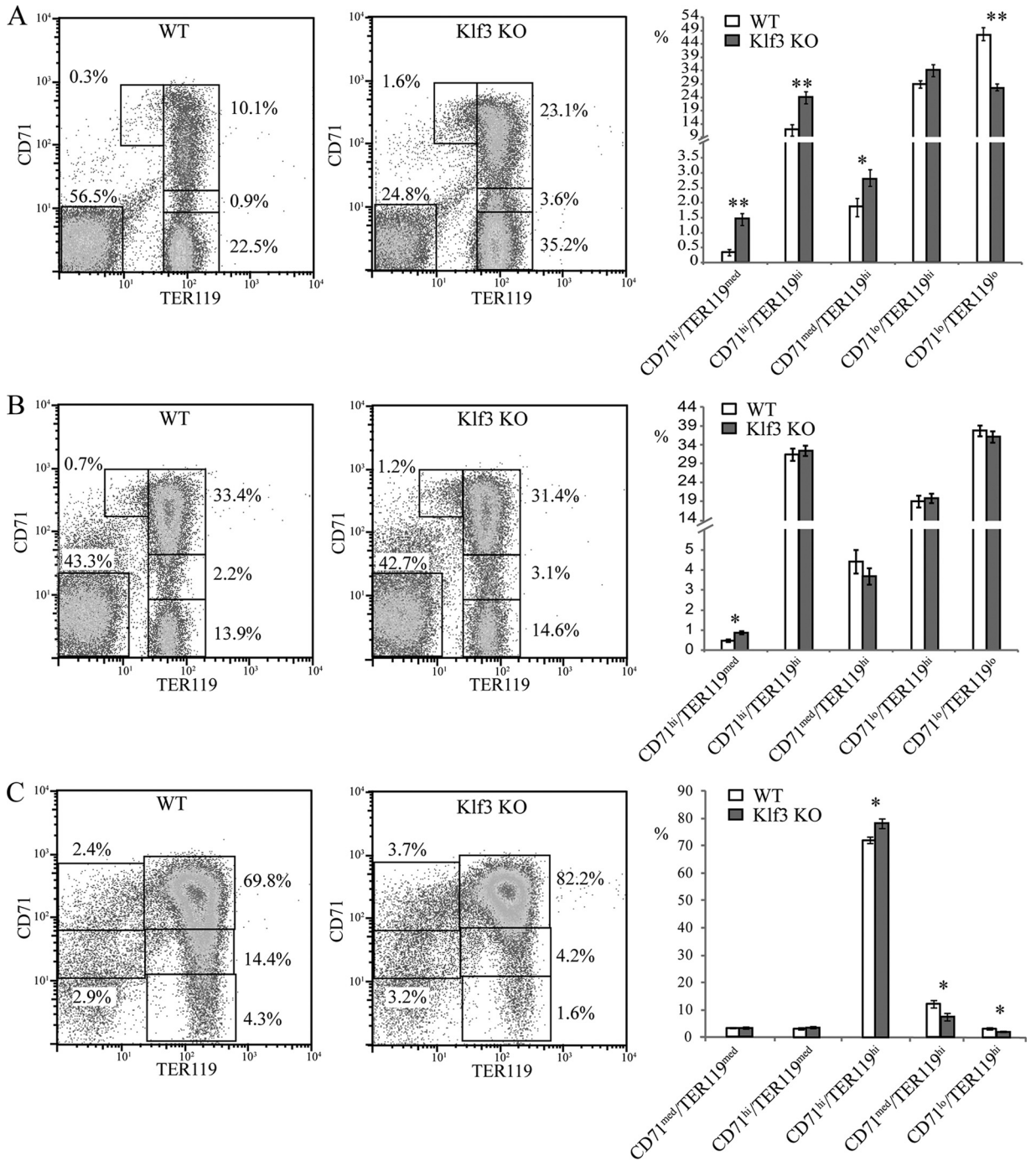

FIG 3 Disrupted erythropoiesis in the spleen, bone marrow, and fetal liver of *Klf3*$^{-/-}$ mice. Representative flow cytometry plots of *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ cells stained with antibodies against CD71 and TER119 are shown. *Klf3*$^{+/+}$ (left) and *Klf3*$^{-/-}$ (center) cells were purified from spleen (A) and bone marrow (B) of 8- to 13-week-old adults and from E16.5 fetal liver (C). (Right) Statistical analyses of flow cytometry data from several animals (spleen, $n = 10$ for each genotype; bone marrow, $n = 12$ for each genotype; fetal liver, $n = 5$ for each genotype). Error bars show standard errors of the means. *P* values indicate the differences between two means: *, $P < 0.05$; **, $P < 0.01$. WT, wild type (*Klf3*$^{+/+}$); KO, knockout (*Klf3*$^{-/-}$).

expression of CD71 and TER119 (32). At embryonic day 12.5 (E12.5), we did not detect any erythropoietic disruption in the livers of *Klf3*$^{-/-}$ mice (data not shown). However, by E14.5 some modest differences that had not reached statistical significance had emerged, with a reduced number of cells transitioning from CD71^{hi} TER119^{hi} (basophilic erythroblasts) to CD71^{med} TER119^{hi} chromatophilic and orthochromatophilic erythroblasts (see Fig. S1 in the supplemental material). By E16.5, this disruption in fetal erythropoiesis was more pronounced, with a significant increase in the percentage of *Klf3*$^{-/-}$ CD71^{hi} TER119^{hi} cells and an apparent defect in the development of the more mature CD71^{med} TER119^{hi} and CD71^{lo} TER119^{hi} erythroblast and reticu-

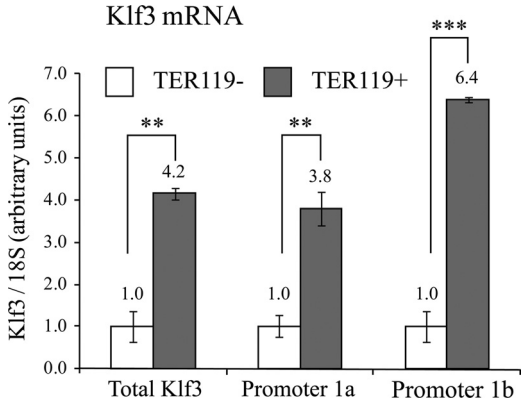

FIG 4 *Klf3* expression increases during erythroid maturation. Expression levels of *Klf3* were examined by real-time qRT-PCR in sorted TER119$^-$ and TER119$^+$ wild-type erythrocytes from E14.5 fetal liver. Shown are relative expression levels for total *Klf3* mRNA and also for transcripts emanating from the widely active promoter 1a and the erythroid-cell-specific promoter 1b (8). Relative expression was normalized against 18S rRNA, and the expression level in TER119$^-$ cells was set to 1.0. For TER119$^-$ cells, $n = 4$; for TER119$^+$ cells, $n = 2$ or 3. Error bars show standard errors of the means. *P* values indicate the differences between two means: **, $P < 0.01$; ***, $P < 0.001$ (two-tailed *t* test).

locyte populations (Fig. 3C). The accumulation of CD71^{hi} TER119^{hi} cells in the fetal livers of *Klf3*$^{-/-}$ mice is indicative of a defect in the first wave of definitive erythropoiesis, which may in part influence the later anemia seen in KLF3-deficient adult mice.

KLF3 represses genes at later stages of erythroid maturation. KLF3 thus appears to be required for normal definitive erythropoiesis in both embryonic and adult erythroid tissues. We next sought to identify the stage of erythroid maturation at which KLF3 might be most functionally relevant. We examined the levels of *Klf3* mRNA during the maturation of erythroid cells by comparing sorted TER119$^-$ progenitors to more mature TER119$^+$ erythroblasts and observed a significant 4.2-fold upregulation in *Klf3* expression (Fig. 4). Specific examination of transcripts emanating from the two *Klf3* promoters showed that the overall upregulation of *Klf3* was due to a 3.8-fold increase in expression from *Klf3* promoter 1a and a 6.4-fold increase from the more EKLF-dependent erythroid promoter 1b (Fig. 4) (8). Taken together, the results suggest that KLF3 expression rises during erythroid maturation, consistent with increasing transcriptional activity of EKLF regulating the terminal stages of erythroid differentiation (2, 28).

We next performed Affymetrix microarrays on fetal liver cells from E14.5 *Klf3*$^{-/-}$ and *Klf3*$^{+/+}$ mice and analyzed both TER119$^-$ progenitor cells and TER119$^+$ erythroblasts (see Fig. S2 in the supplemental material) in order to investigate the role of KLF3 in regulating gene expression at different stages throughout the erythroid maturation process. Here we confine our attention to genes that show a >2-fold change in expression ($P < 0.05$; FDR < 0.2). We found that in *Klf3*$^{-/-}$ TER119$^-$ erythroid progenitors, where KLF3 levels are relatively low, there was little deregulation of gene expression, with no genes being significantly altered more than 2-fold (data not shown). In contrast, we found that in the more mature TER119$^+$ erythroid cells, loss of KLF3 had a noticeable effect, with the expression of 85 genes being significantly derepressed (Fig. 5A). Interestingly, only three genes are significantly downregulated in the absence of KLF3. These results suggest that KLF3 primarily acts as a transcriptional repressor *in vivo*, consistent with previous functional studies *in vitro* (17, 22, 29).

We then compared the expression of these 85 upregulated and 3 downregulated genes across 15 independent microarrays that included TER119$^-$ and TER119$^+$ *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ samples (Fig. 5B). Analysis of this heat map revealed genes which are regulated by KLF3 and which can broadly be categorized into three groups. The first group includes genes whose expression is mildly repressed or maintained at a relatively low level during erythroid maturation (compare TER119$^-$ with TER119$^+$ wild-type samples). The second group contains genes whose expression increases during erythroid differentiation. The increased expression of these genes appears to be tempered by KLF3 during erythropoiesis, as *Klf3*$^{-/-}$ TER119$^+$ cells exhibit marked upregulation of these genes compared to wild-type TER119$^+$ cells. The final group comprises genes whose expression is strongly repressed or silenced during erythroid maturation.

These results suggest that KLF3 plays a complex role both in fine-tuning the expression of some targets by moderate repression and also in more dramatically silencing certain genes during the maturation of TER119$^-$ progenitors into TER119$^+$ erythroblasts. In each of these instances, ablation of KLF3 leads to unchecked derepression of these genes in mature erythroid cells, emphasizing KLF3's complex contribution to maintaining a proper transcriptional program in these cells.

One aspect of the role of KLF3 as a repressor that is active in the later stages of erythropoiesis can be illustrated by considering its effect on its best characterized target gene, *Klf8* (5). We have previously reported that the transcriptional repressor KLF8 is upregulated in *Klf3*$^{-/-}$ erythroid tissue (5) and as expected, we observed an 8.3-fold increase in *Klf8* expression in the *Klf3*$^{-/-}$ TER119$^+$ microarrays. We also examined *Klf8* expression during cellular maturation by qRT-PCR by comparing mRNA from sorted E14.5 TER119$^-$ progenitors and TER119$^+$ erythroblasts (Fig. 6). We found that in *Klf3*$^{+/+}$ cells, *Klf8* levels decrease 7.7-fold as progenitors mature into erythroblasts. This result fits well with the increase in expression of KLF3 during cellular maturation. In the absence of KLF3, we observed a modest 3.1-fold increase in *Klf8* in TER119$^-$ cells, contrasting with a striking 40-fold derepression in TER119$^+$ erythroblasts (Fig. 6). Thus, KLF3-mediated repression is more prominent at this later stage of cellular maturation.

KLF3 represses a subset of genes driven by EKLF. The related family member EKLF has previously been shown to be indispensable for definitive erythropoiesis in the embryo. Loss of EKLF causes erythropoiesis to arrest in the fetal liver, with erythroid progenitors failing to progress past the CD71^{hi} TER119^{lo} stage of maturation (20). *Eklf*$^{-/-}$ and *Klf3*$^{-/-}$ mice thus display impairments at similar stages during the erythropoietic differentiation program. While many EKLF target genes have been discovered primarily through microarray and ChIP-Seq analyses of *Eklf*$^{-/-}$ and *Eklf*$^{+/+}$ fetal liver cells (4, 11, 19, 20, 28), the target genes of KLF3 have hitherto largely remained unknown. Given their similar DNA-binding specificities, EKLF and KLF3 have been postulated to regulate an overlapping cohort of target genes (3, 8).

Accordingly, we asked whether any of the KLF3-dependent genes from the TER119$^+$ arrays are known to be regulated by EKLF. To do this, we first prepared a list of EKLF target genes by compiling data from several previously published studies, including microarray and ChIP-Seq experiments (1, 4, 11, 19, 20, 28). In

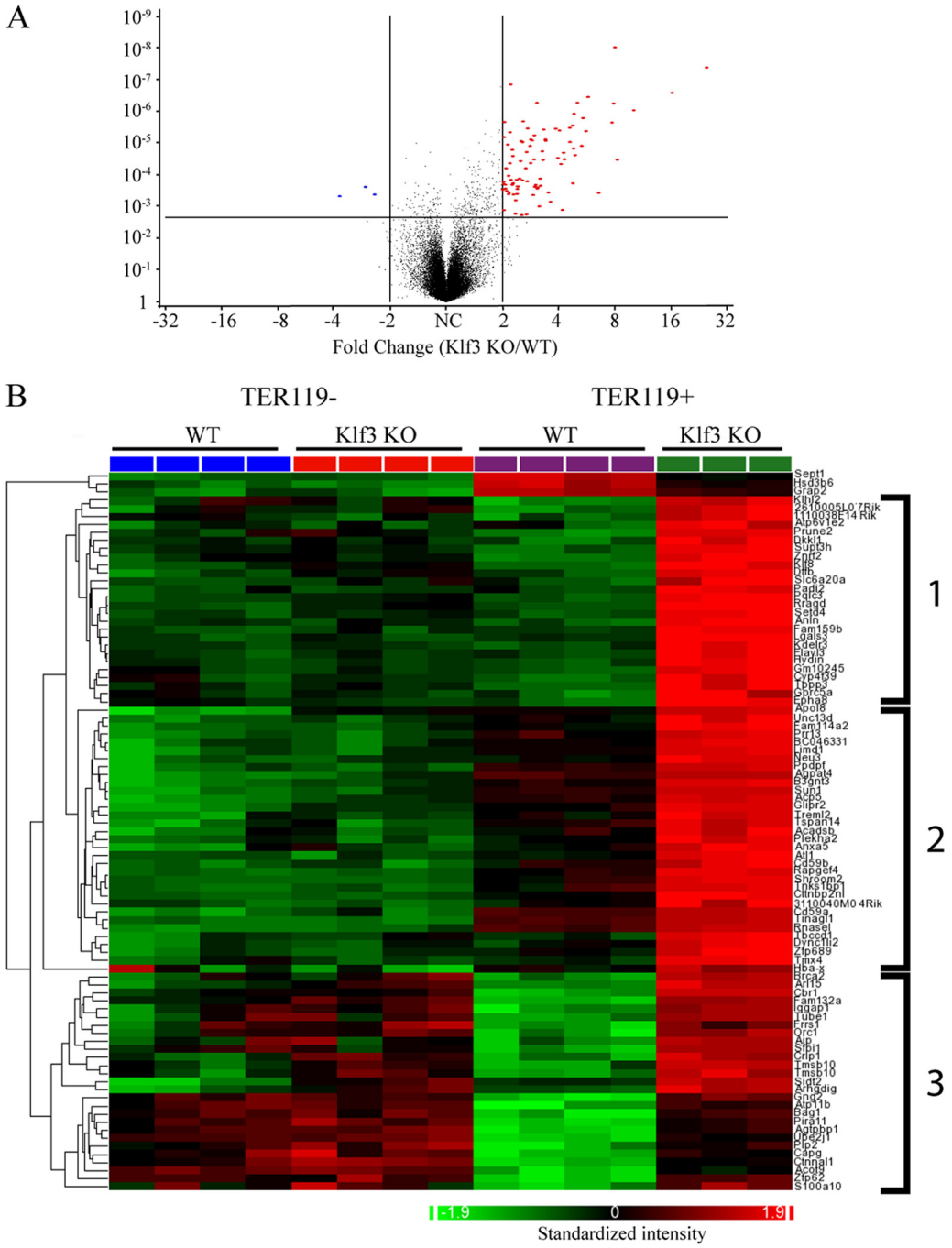

FIG 5 KLF3 represses many genes in maturing TER119$^+$ erythroblasts. RNA was extracted from TER119$^-$ (four *Klf3*$^{+/+}$ and four *Klf3*$^{-/-}$) and TER119$^+$ (four *Klf3*$^{+/+}$ and three *Klf3*$^{-/-}$) sorted cell populations from E14.5 fetal liver and was subjected to Affymetrix microarray analysis. (A) Volcano plot comparing gene expression in TER119$^+$ *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ cells. Data are for genes which are significantly differentially expressed (i.e., more than a 2-fold change) ($P < 0.05$; FDR < 0.2). Red dots represent genes that are derepressed in *Klf3*$^{-/-}$ cells, while blue dots represent genes that are downregulated. (B) Heat map comparing gene expression in TER119$^-$ and TER119$^+$ *Klf3*$^{+/+}$ and *Klf3*$^{-/-}$ sorted erythroid cells. Heat maps represent the hierarchical clustering of the set of genes that are significantly differentially expressed (as shown in panel A). Deregulated genes can broadly be grouped into three categories based on their expression during erythroid maturation: genes that are mildly repressed or stable (set 1), upregulated (set 2), or strongly repressed or silenced (set 3) in TER119$^+$ compared to TER119$^-$ wild-type cells. WT, wild type (*Klf3*$^{+/+}$); KO, knockout (*Klf3*$^{-/-}$).

addition, we also performed our own Affymetrix microarrays on three *Eklf*$^{-/-}$ and three *Eklf*$^{+/+}$ fetal livers (E13.5) to account for annotation differences and possible absences of gene probe sets on earlier microarray platforms. We then searched the list of putative KLF3 target genes and found that a striking number of these (approximately 50%) are also regulated by EKLF (see Table S1 in the supplemental material).

To further investigate the possible regulation of shared targets

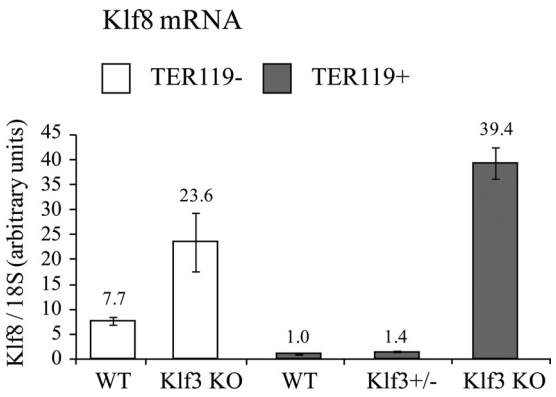

FIG 6 Increased repression of the KLF3 target gene *Klf8* during erythroid maturation. Shown are relative *Klf8* expression levels in $Klf3^{+/+}$ and $Klf3^{-/-}$ TER119$^-$ cells and in $Klf3^{+/+}$, $Klf3^{+/-}$ and $Klf3^{-/-}$ TER119$^+$ cells. Relative expression was normalized against 18S rRNA, and the expression level in $Klf3^{+/+}$ TER119$^+$ cells was set to 1.0. For TER119$^-$ cells, $n = 3$; for TER119$^+$ cells, $n = 2$ or 3. Error bars show standard errors of the means. WT, wild type ($Klf3^{+/+}$); KO, knockout ($Klf3^{-/-}$).

by EKLF and KLF3, we chose two genes, *galectin-3* (*Lgals3*) and *adipolin* (*Fam132a*), which are both highly derepressed in the TER119$^+$ $Klf3^{-/-}$ microarrays and have previously been identified as EKLF targets by ChIP-Seq (28). In hematopoietic tissue, galectin-3 is a prominent mediator of inflammation and is most strongly expressed in macrophages (10). Adipolin was recently identified as an adipokine that influences insulin sensitivity and glucose metabolism (6). We first sought to establish that these genes are indeed functionally activated by EKLF. To do this, we used the B1.6 cell line, an *Eklf*-null erythroid line which can be rescued by a tamoxifen-inducible form of EKLF (2). Consistent with functional regulation by EKLF, we found that expression of both *Lgals3* and *Fam132a* is highly induced when EKLF nuclear activity is restored by tamoxifen addition to the cells (Fig. 7A and C). Taken together with the ChIP-Seq studies, this suggests that these genes are bona fide EKLF targets.

We then compared expression of *Lgals3* and *Fam132a* in TER119$^-$ erythroid progenitors and TER119$^+$ erythroblasts (Fig. 7B and D). We found that levels of *Lgals3* and *Fam132a* mRNA are lower (2.8-fold and 10.1-fold, respectively) in TER119$^+$ than in TER119$^-$ wild-type cells, consistent with transcriptional repression occurring as KLF3 levels increase during maturation. We also observed significant derepression of *Lgals3* and *Fam132a* transcripts (15.5-fold and 11.1-fold, respectively) in $Klf3^{-/-}$ compared to $Klf3^{+/+}$ TER119$^+$ erythroblasts. Furthermore, the derepression of *Lgals3* and *Fam132a* is much less pronounced in TER119$^-$ progenitors lacking KLF3 (1.4-fold and 2.3-fold, respectively). This is consistent with our previous observation that KLF3 levels and activity are low in progenitor cells and again suggests that the major role of KLF3 is to repress genes late in maturation.

The results above suggest that EKLF and KLF3 competitively regulate *Lgals3* and *Fam132a* at a functional level. We then asked whether ChIP studies also supported direct competition. As described above, previous ChIP-Seq studies indicated that EKLF binds both the *Lgals3* and *Fam132a* loci *in vivo* (28). In order to determine whether KLF3 could also directly localize to these genes *in vivo*, we designed primers proximal to the EKLF-bound sites in these loci and then tested for KLF3 occupancy using ChIP. For these studies we used a V5-tagged KLF3 transgene that had been introduced into KLF3 null MEFs, which like erythroid cells and many other cells normally abundantly express KLF3. We observed very strong KLF3 occupancy at both the *Lgals3* and *Fam132* loci (Fig. 7E). The *Klf8* promoter served as a positive control in these experiments, and a distal region upstream of the *Klf8* gene was used as a negative control (5). This combination of functional data and occupancy as measured by ChIP analysis suggests that KLF3 directly counters the activity of EKLF at selected target genes.

Taken together, the derepression of numerous genes in $Klf3^{-/-}$ TER119$^+$ erythroid cells and the high degree of overlap between these targets and those regulated by EKLF strongly suggest that the primary erythroid function of KLF3 is to repress or temper a subset of EKLF-inducible genes during erythroid maturation. Ablation of KLF3 leads to deregulated gene expression and erythroid impairments both in the fetus and the adult, indicating that KLF3 is required for normal erythropoiesis *in vivo*.

DISCUSSION

Detailed analysis of erythropoiesis in $Klf3^{-/-}$ mice revealed consistent defects in red blood cell development. Peripheral blood smears and full blood counts demonstrated reticulocytosis and alterations in red blood cell numbers and mean cell hemoglobin content, indicative of a mild anemia. $Klf3^{-/-}$ spleens are enlarged and exhibit a considerable increase in the proportion of red pulp. Flow cytometry of splenic erythrocytes has shown a significant increase in the proportion of CD71$^+$ TER119$^+$ erythroblasts, indicating that loss of KLF3 leads to chronic compensatory stress erythropoiesis in this organ. Erythroid defects are also apparent in the fetal liver and bone marrow of $Klf3^{-/-}$ mice. In summary, loss of KLF3 leads to impairments in fetal and adult erythropoiesis, resulting in an anemia which is stabilized by compensatory stress erythropoiesis in the spleen.

Analysis of *Klf3* gene expression during erythroid maturation shows that *Klf3* levels increase as TER119$^-$ progenitor cells mature to become TER119$^+$ erythroblasts. This view of KLF3 as a late-stage repressor is further supported by recent ChIP-Seq and RNA-Seq analyses examining EKLF activity during erythroid development. KLF3 was recently confirmed as one of a set of EKLF target genes that are bound by and activated by EKLF in TER119$^+$ erythroblasts (19). Accordingly, KLF3-mediated repression of known target genes, such as *Klf8* (5), increases as erythroid cells mature, and loss of KLF3 has a more pronounced effect on gene deregulation at this later stage of development.

When one considers the set of genes regulated by KLF3, a number of things are apparent. Most notably, very few of the genes are known to play major roles in erythropoiesis, and there is a scarcity of genes with established erythroid roles, such as *Ahsp*, *Epb4.9*, and *Ermap* (11). Nevertheless, approximately half of the KLF3-dependent genes are previously reported EKLF targets, albeit without recognized erythroid-cell-specific functions. This is not entirely unexpected, in that the authors of a recent EKLF ChIP-Seq study noted that a large number of the genomic sites that are occupied by EKLF lie within or proximal to genes with no known erythroid function (28). One interpretation is that EKLF has evolved as a potent activator of CACCC-dependent genes and accumulates during erythroid maturation in order to activate targets that are essential in the later stages of erythroid differentiation. It is possible that one side effect of the potency of EKLF and its increasing activity during erythroid

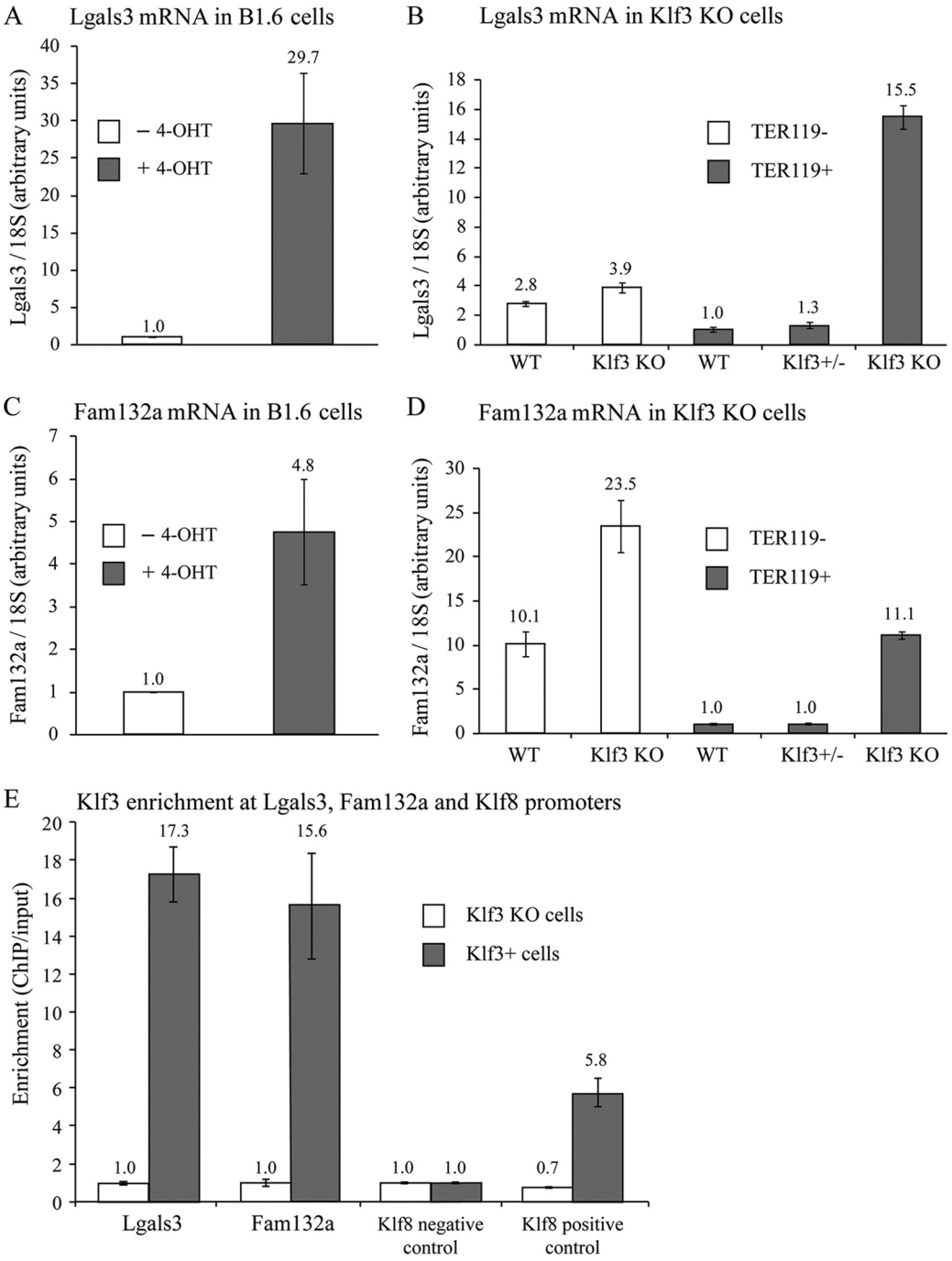

FIG 7 KLF3 and EKLF regulate shared target genes. Expression of *Lgals3* (A) and *Fam132a* (C) increases upon tamoxifen (4-OHT)-induced rescue of EKLF in the *Eklf*-null cell line B1.6 by real-time qRT-PCR. Relative expression was normalized against 18S rRNA, and the expression level in uninduced cells was set to 1.0 ($n = 3$ for *Lgals3* and 4 for *Fam132a*). Relative *Lgals3* (B) and *Fam132a* (D) expression levels in $Klf3^{+/+}$ ($n = 4$) and $Klf3^{-/-}$ ($n = 3$) TER119$^-$ cells and in $Klf3^{+/+}$ ($n = 2$ or 3), $Klf3^{+/-}$ ($n = 3$) and $Klf3^{-/-}$ ($n = 3$) TER119$^+$ cells. Relative expression was normalized against 18S rRNA, and the expression level in $Klf3^{+/+}$ TER119$^+$ cells was set to 1.0. (E) Chromatin immunoprecipitation assay showing binding of KLF3 at the *Lgals3* and *Fam132a* loci. The ChIP assays were carried out in duplicate in *Klf3*-null murine embryonic fibroblasts into which a V5-tagged *Klf3* transgene had been introduced. Fold enrichment was determined by real-time PCR and is relative to input signal. The enrichment at a site in the *Klf8* locus where KLF3 has been reported to not bind (5) was set to 1.0. *Klf8* promoter 1a was included as a positive control. Error bars show standard errors of the means. WT, wild type ($Klf3^{+/+}$); KO, knockout ($Klf3^{-/-}$).

maturation is that it has the capacity to promiscuously activate the transcription of nonerythroid CACCC-dependent genes. To counter this, EKLF may activate KLF3 to silence a subset of genes which have no specific role in erythroid cells and which, when expressed as a cohort, may even be detrimental to these cells. Indeed, many of the putative KLF3 target genes are either downregulated or maintained at low, steady levels as TER119$^-$ progenitors mature into TER119$^+$ erythroblasts (Fig. 5B). Given that KLF3 is

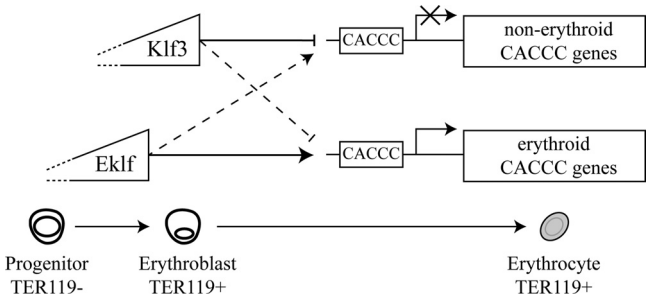

FIG 8 EKLF drives KLF3 expression to repress nonerythroid genes during erythrocyte maturation. This model proposes that EKLF drives high-level expression of KLF3 in TER119$^+$ erythroblasts both to silence nonerythroid CACCC box genes and to fine-tune the expression of a subset of EKLF erythroid targets. By this mechanism, KLF3 ensures that during red blood cell maturation, essential erythroid genes are expressed at optimal levels, while those that are not required remain silent.

a broadly expressed transcription factor present not only in erythroid cells but also in fibroblasts and other cell types, it is possible that KLF3 plays a global role in repressing these nonerythroid genes.

Another interpretation is that some of the shared EKLF/KLF3 target genes may indeed have erythroid functions, which are as yet undefined, and the role of KLF3 may simply be to fine-tune the precise expression level of particular erythroid genes in a classical negative-feedback loop. It is perhaps most likely that KLF3 does both: fine-tuning and damping the expression of some EKLF target genes and silencing other genes to refine and limit the set of EKLF targets, such that some CACCC box-dependent genes are never expressed in erythroid cells (Fig. 8). The result is satisfying, as it explains the superficially paradoxical situation where a CACCC box-dependent activator, EKLF, drives a CACCC box-dependent repressor which can counter its activity. Our results now establish EKLF and KLF3 as a pair of positively and negatively acting transcription factors that work together to orchestrate normal erythropoiesis *in vivo*.

In summary, our data indicate that KLF3 is highly expressed at the later stages of erythroid cell maturation, where it is required to repress a subset of EKLF target genes and thereby contribute to normal erythropoiesis. Thus, the molecular role of KLF3 in erythroid cells is to temper the activity of EKLF at some CACCC-dependent targets, thereby helping to refine the functional subset of EKLF-responsive genes (Fig. 8). The erythroid defects observed in the *Klf3*$^{-/-}$ mice demonstrate that KLF3 is required for normal erythropoiesis, and we conclude that the impaired erythroid maturation is due, at least in part, to the combined effects of the derepression of a cohort of KLF3-dependent genes in KLF3-deficient erythroid cells.

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A.P.W.F. designed and performed research, analyzed data, and wrote the paper; L.J.N., K.S.M., P.K., J.B., C.M.A., C.A.P., and T.H. performed research and analyzed data; N.A.T., M.R.W., and K.S.B.-A. analyzed data; J.P., S.H.O., S.T.F., and A.C.P. designed research and analyzed data; R.C.M.P. designed research, analyzed data, and wrote the paper; and M.C. devised the concept and research, analyzed data, and wrote the paper.

We declare that no competing financial interests exist.

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