Dose-dependent regulation of primitive erythroid maturation and identity by the transcription factor Eklf

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The primitive erythroid (EryP) lineage is the first to differentiate during mammalian embryogenesis. *Eklf/Klf1* is a transcriptional regulator that is essential for definitive erythropoiesis in the fetal liver. Dissection of the role(s) of *Eklf* within the EryP compartment has been confounded by the simultaneous presence of EryP and fetal liver–derived definitive erythroid (EryD) cells in the blood. To address this problem, we have distinguished EryP from their definitive counterparts by crossing *Eklf*+/− mutant and ε-*globin::histone H2B-GFP* transgenic mice. *Eklf*-deficient EryP exhibit membrane ruffling and a failure to acquire the typical discoidal erythroid shape but they can enucleate. Flow cytometric analyses of H2B-GFP+ EryP revealed that *Eklf* heterozygosity results in the loss of Ter119 surface expression on EryP but not on EryD. Null mutation of *Eklf* resulted in abnormal expression of a range of surface proteins by EryP. In particular, several megakaryocyte markers were ectopically expressed by maturing *Eklf*-null EryP. Unexpectedly, the platelet tetraspanin CD9 was detected on nucleated wild-type EryP but not on mature EryD and thus provides a useful marker for purifying circulating EryP. We conclude that *Eklf* gene dosage is crucial for regulating the surface phenotype and molecular identity of maturing primitive erythroid cells. (*Blood*. 2010;116(19):3972-3980)

Introduction

Primitive erythroid cells (EryP) are the first hematopoietic cells to appear and mature in the developing embryo.[1-3] Before the formation of the adult hematopoietic system from hematopoietic stem cells, the yolk sac–derived EryP number in the millions and are the dominant cell type of the mid-gestation mouse embryo circulation.[4,5] Larger than their definitive erythroid (EryD) counterparts, EryP express a unique set of *globin* genes and circulate, for much of their existence, as nucleated cells.[3] Shortly after midgestation in the mouse embryo, EryD are produced in enormous numbers by progenitors in the fetal liver (FL) and rapidly outnumber EryP. By birth, circulating EryP comprise only a tiny fraction of cells in the blood, and the transition to the definitive erythroid system is complete.

The genetic programs that regulate the development and maturation of the primitive erythroid compartment are being elucidated. However, this analysis is complicated by the simultaneous presence of definitive and primitive erythroid cells in the embryonic circulation after mid-gestation. To identify and monitor EryP, we have previously capitalized upon human ε-*globin* regulatory elements to drive expression of fluorescent reporters solely within this population in transgenic mice.[4,6] Here, we have asked whether null mutation of *Eklf*, a gene reported to play a critical role in erythropoiesis during embryonic development, has distinct functions in the primitive and definitive erythroid lineages.

The erythroid kruppel-like factor (Eklf, also known as Klf1)[7] is the prototype of the Krüppel-like family of zinc finger–containing transcription factors.[8] Klf family members have been shown to play crucial roles in diverse biologic processes ranging from hematopoiesis to maintenance of embryonic stem cells.[8] Originally isolated from a mouse erythroleukemia cell cDNA library[7] and proposed to regulate the fetal-to-adult *globin* gene switch,[9] *Eklf* is transcriptionally restricted to the erythroid lineage from as early as embryonic day 7.5 (E7.5) in the mouse.[10] Targeted null mutation of *Eklf* results in severe anemia and death in utero due to defective erythropoiesis.[11,12] *Eklf*-deficient EryD progenitors are generated in normal numbers but fail to mature and enucleate effectively.[12,13] Analysis of *Eklf* mutant embryos has led to the identification of a large group of target genes (in addition to *globins*) in erythroid cells (reviewed in references[13-15]). Erythroid cells lacking *Eklf* exhibit severely ruffled plasma membranes, suggesting defects in the expression and/or function of erythroid membrane and cytoskeletal proteins.[13]

It was not previously possible to focus specifically on the EryP lineage in wild-type (WT) or mutant embryos due to the lack of lineage-restricted markers that could be used to distinguish them from definitive erythroid cells. To analyze the impact of loss of one or both alleles of *Eklf* during primitive erythropoiesis, we have crossed the ε-*globin:H2B-GFP* transgenic mouse[6] onto an *Eklf* mutant background.[12] Haploinsufficiency of *Eklf* had a profound effect on the surface phenotype of EryP but not EryD, indicating that the differentiation of EryP is more sensitive to *Eklf* gene dosage than that of EryD. Null mutation resulted in a partial "identity crisis" within maturing EryP in the circulation, with up-regulation of a range of megakaryocyte-related surface antigens and transcriptional regulators. Therefore, *Eklf* regulates EryP maturation and molecular identity.

Table 1. Antibodies used for flow cytometric analyses in this study

Surface marker: fluorochrome	Clone	Source
Ter119: PE, Pacific Blue, PE-Cy7	Ter119	eBioscience
α2 integrin: APC	DX5	BioLegend
α4 integrin: PE	R1-2	eBioscience
α5 integrin: biotin	MFR5	BD Biosciences
αV integrin: PE	RMV-7	eBioscience
β1 integrin: APC	eBioHmb1-1	eBioscience
β3 integrin: biotin	2C9.G3	eBioscience
CD9: PE	eBioKMC8	eBioscience
CD24: Pacific Blue	M1/69	BioLegend
CD31: PE-Cy7	390	BioLegend
CD34: PE-Cy5	MEC14.7	BioLegend
CD41: biotin/PE-Cy7	eBioMWReg30	eBioscience
CD42: APC	1B5	Dr Barry Coller, Rockefeller University
CD44: PE	IM7	BD Biosciences
CD55: PE	RIKO-5	BD Biosciences
CD71: PE	R17217	eBioscience
CD117/c-kit: APC	2B8	BioLegend
CD147: PE	RL73	eBioscience

Conjugated antibodies were purchased from eBioscience, BioLegend, or BD Biosciences. PE indicates phycoerythrin; and APC, allophycocyanin.

Methods

Generation of human ε-*globin::H2B-EGFP* transgenic mice

The entire insert from pCX-ε-*globin*::H2B-EGFP (see supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), containing the human ε-*globin::H2B-EGFP* cassette, was excised and gel-purified to remove prokaryotic sequences. Pronuclear injections into C57BL/6 × C3H (B6C3) F1 hybrid embryos were performed by the Mount Sinai Mouse Genetics Shared Resource Facility using standard methods.[16] Two transgenic founder animals were obtained, genotyped by polymerase chain reaction (PCR), and screened for enhanced green fluorescent protein (EGFP) expression using fluorescence microscopy and flow cytometric analysis (fluorescence-activated cell sorting [FACS]). Line #38 was used for the studies reported here. The mice were maintained as homozygotes (termed *Tg/Tg*) on an ICR (outbred; Taconic) background, following Mount Sinai institutional guidelines and approval by the Mount Sinai Institutional Animal Care and Use Committee.

The *Eklf*-null mouse line has been reported previously.[12] *Eklf* heterozygotes were obtained from Dr David Bodine (National Human Genome Research Institute, Bethesda, MD) and were maintained on an ICR background. Genotyping of the targeted *Eklf* allele in embryos and adults was performed as described.[12] The ε-*globin::H2B-EGFP* transgenic mouse line was crossed with *Eklf*^{+/−} mice to obtain animals bearing the *H2B-EGFP* reporter and one null allele of *Eklf*. To generate timed *Eklf*-null embryos carrying the *H2B-EGFP* reporter allele, male and female *Eklf*^{+/−};ε::*H2B-GFP (Tg/Tg)* mice were intercrossed. The morning of detection of the vaginal plug was taken as day 0.5 of gestation.

Flow cytometry and sorting

Embryos from *Eklf*^{+/−};ε::*H2B-GFP* intercrosses were isolated at the indicated times, and blood was collected by exsanguination into FACS buffer (phosphate-buffered saline [PBS]; Gibco-BRL, Invitrogen), containing 10% fetal calf serum (Hyclone) and heparin (12.5 μg/mL; Sigma-Aldrich).[4] FLs were dissected, triturated, and filtered through a 40-μm cell strainer to produce a single-cell suspension. Cells from embryonic peripheral blood (PB) and FL were washed and stained for flow cytometric analysis.[4] The directly conjugated monoclonal antibodies used in this study are listed in Table 1. Biotinylated antibody binding was detected using streptavidin conjugated to phycoerythrin-Cy7 (eBioscience). Samples were analyzed using an LSRII cell analyzer (Becton Dickinson). Data were analyzed using the FlowJo Version 8.8.6 software package (TreeStar). Cell sorting was performed using either the Vantage Cell Sorter (Becton

Dickinson) or a Mo-Flo cell sorter (DakoCytomation). Analytical flow cytometry and cell sorting were performed at the Mount Sinai Flow Cytometry Core Facility.

Quantitative real-time RT-PCR

Total RNA was extracted from sorted cells using an RNeasy Mini Kit (QIAGEN), and cDNA was prepared using the Superscript III RT first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Gene expression was analyzed by quantitative real-time reverse transcription PCR (qRT-PCR) using SYBR-green (SYBR-green PCR master mix; Applied Biosystems), following the protocol recommended by the supplier. *Ubiquitin b (Ubb)* or *18S ribosomal RNA (Rn18s)* genes were used as internal controls for normalization. Values obtained for target gene expression were normalized to the control gene and then expressed relative to the levels in *Eklf* WT samples, which were set at a value of 1.0. The sequences of the primers used in this study are shown in Table 2.

Cytologic analysis

Collection of embryonic blood, cytocentrifugation onto glass slides, and Giemsa staining was performed as previously described.[4] For immunostaining, PB blood cells from E14.5 embryos were harvested and washed with bovine serum albumin (BSA, 0.3% wt/vol; Sigma-Aldrich)/PBS and resuspended in the same buffer. The cells were then incubated for 1 hour at room temperature with 10 μg/mL of rat anti–mouse CD9 monoclonal antibody (KMC8; BD Pharmingen; Table 1) or rat immunoglobulin G2a (IgG2a) isotype control (cat. no. 553926; BD Pharmingen) using a rotator. After washing 3× with 0.3% BSA/PBS, samples were incubated with Alexa Fluor 568–conjugated goat anti–rat IgG (Invitrogen) for 1 hour at room temperature and then washed twice with 0.3% BSA/PBS to remove unbound secondary antibody. The stained cells were centrifuged onto glass slides using a Cytospin3 cytocentrifuge (Shandon), air-dried, and mounted in Vectashield containing DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). Images were acquired using an Axioplan 2 Epifluorescence microscope (Zeiss). Lens specifications are provided in the figure legends.

Isolation of cells from BM

Single-cell suspensions were prepared from bone marrow (BM) by dissecting the femora of 6- to 8-week–old female ICR mice. Femora were dissected and flushed with 3 mL Dulbecco modified Eagle medium (Gibco BRL) using a 5-mL syringe fitted with a 20-G needle. Cells were dispersed by trituration, filtered through a 40-μm cell strainer (BD Falcon) and

Table 2. Quantitative RT-PCR primers used in this study

Target gene	Accession no.	Primer sequences	Details
Klf3	NM_008453	Forward: 5'-GAAATGTCACCCCCTTTAATGAAC-3'; reverse: 5'-CACGATGACGGAAGGATGGT-3'	Ref. 19
Gypa	NM_010369	Forward: 5'-GCCGAATGACAAAGAAAAGTTCA-3'; reverse: 5'-TCAATAGAACTCAAAGGCACACTGT-3'	ID 328119a1*
Gabpa	NM_008065	Forward: 5'-AGCGCATCTCGTTGAAGAAG-3'; reverse: 5'-TCCTGCTCTTTTCTGTAGCCT-3'	ID 4328119a1*
Fli-1	NM_008026	Forward: 5'-ATGGACGGGACTATTAAGGAGG-3'; reverse: 5'-GAAGCAGTCATATCTGCCTTGG-3'	ID 6679807a1*
Ubb	NM_011664	Forward: 5'-TGGCTATTAATTATTCGGTCTGCAT-3'; reverse: 5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'	Ref. 39
18S	NM_011664	Forward: 5'-CACGGCCGGTACAGTGAAAC-3'; reverse: 5'-AGAGGAGCGAGCGACCAA-3'	Ref. 19
Eklf	NM_010635	Quantitect primer assay; QIAGEN	Cat. no. QT01743777
CD41	NM_010575	Quantitect primer assay; QIAGEN	Cat. no. QT00117992
CD9	NM_007657	Quantitect primer assay; QIAGEN	Cat. no. QT01752513
εy	NM_008221	Forward: 5'-GCCCTCTCTCTAGCTGTCCA-3'; reverse: 5'-TGCTTTCAAGGAACAGTCCA-3'	Ref. 40

*From Primer Bank Database.[41,42]

pelleted by centrifugation. Cells were then counted using a hemocytometer. For flow cytometry, 1×10^6 cells were stained with fluorescently conjugated antibodies and then analyzed.

Results

Anomalous maturation of primitive erythroid cells lacking Eklf

Although several morphologic and molecular defects have been reported for *Eklf*-null mutant erythroid cells, it has not previously been possible to focus specifically on the primitive erythroid lineage. We were able to overcome this obstacle by analyzing an *Eklf* mutation on the background of our ε-*globin:H2B-GFP* transgenic mouse, in which expression of a histone H2B-GFP reporter is restricted to EryP.[6] Visual inspection revealed that the *Eklf*-null embryos were severely anemic at mid-gestation (supplemental Figure 1A), as reported previously.[11,12] Normal EryP maturation is characterized by a stepwise developmental progression that can be monitored by cytology[4] and is characterized by a series of discrete observable features. These include progressive loss of basophilia, loss of nucleoli, nuclear condensation, acquisition of a typical pink cytoplasmic staining, and nuclear extrusion, a hallmark of terminal erythroid differentiation.[4] Microscopic inspection of PB from *Eklf*-null mutant embryos revealed irregularly shaped EryP, with a wrinkled, nonspherical morphology, as early as E11.5 (supplemental Figure 1B-C). In contrast, EryP from WT littermates were round, with smooth contours (supplemental Figure 1C). Differentiation of *Eklf* heterozygous cells was indistinguishable from WT (not shown). The abnormal *Eklf*-null mutant EryP phenotype was particularly striking in unfixed wet preparations, with misshapen macrocytic cells that failed to attain the discoidal shape typical of stage-matched, mature WT EryP (Figure 1A).

Despite their abnormal morphology, *Eklf*-null primitive erythroid cells were able to condense their nuclei and displayed eosinophilic cytoplasmic staining with Giemsa (supplemental Figure 1C), suggesting that the molecular programs leading to chromatin condensation and hemoglobin production, respectively, were not signficantly impaired, despite reduced transcription of the ε*Y-globin* gene (see below). By E13.5-E14.5, enucleated EryP (identified by size and morphology) were detected in the PB of mutant embryos, indicating that nuclear extrusion can occur in the absence of *Eklf* (Figure 1B).

Monitoring primitive erythroid maturation in the absence of EKLF

In the studies that follow, we used the ε-*globin::H2B-EGFP* transgenic mouse line (hereafter termed ε::*H2B-GFP*; supplemen-

tal Figure 2A) in which EryP nuclei are labeled by a histone H2B-EGFP fusion protein that localizes to the nucleus.[6] Extensive characterization of the GFP-expressing cells in this transgenic mouse line confirmed the specificity of the fluorescent reporter for the primitive erythroid lineage.[4,6] The bright nuclear fluorescence in this line (Figure 1C) makes it highly amenable for imaging and flow cytometry. GFP-labeled EryP will hereafter be termed EryP/H2B-GFP.

We first examined expression of *Eklf* in WT EryP sorted from embryonic stages E8.5-E12.5. Real-time RT-PCR analysis revealed that *Eklf* is up-regulated in EryP from E8.5-E9.5 and is maintained at high levels until E11.5, when the nucleus of the EryP is condensed; transcription of *Eklf* is significantly reduced by E12.5
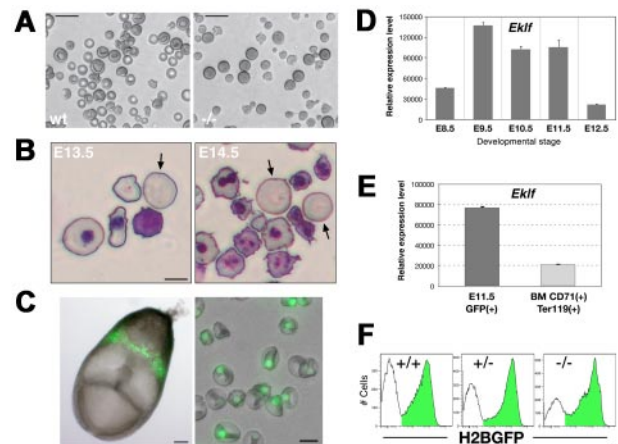

Figure 1. Eklf-deficient primitive erythroblasts show dramatic alterations in cell morphology but can complete their maturation. (A) Wet preparations of freshly harvested E14.5 PB cells reveal large *Eklf*-null mutant EryP that fail to achieve the discoidal shape observed for WT cells. (B) Detail of Giemsa-stained cytospin preparations of PB from E13.5 and E14.5 mutant embryos, showing some macrocytic cells that lack nuclei (arrows). (C) Left panel, H2B-GFP expression in an E8.0 transgenic embryo. Right panel, wet preparation of PB from an E14.5 ε-*globin::H2B-GFP* embryo. An overlay of bright field and green fluorescence is shown. Expression of the fluorescent reporter is restricted to the EryP nucleus. (D) Expression of *Eklf* mRNA in developing EryP cells. GFP⁺ cells were sorted from E8.5-12.5 embryos and real-time RT-PCR analysis of *Eklf* was performed; expression was normalized to that of *ubiquitin b*. (E) *Eklf* expression in definitive versus primitive erythroid cells. GFP⁺ EryP from E11.5 embryos or CD71⁺Ter119⁺ definitive erythroid cells from adult mouse BM were sorted and the expression of *Eklf* mRNA assessed by real time RT-PCR; expression levels are relative to *ubiquitin b*. A representative experiment (3 independent samples) is shown. (F) Histograms of ε-*globin::H2B-GFP* fluorescence intensity in E13.5 PB cells harvested from *Eklf* WT, *Eklf*⁺/⁻ or *Eklf*⁻/⁻ embryos. Images were acquired in AxioVision v4.6 using a Zeiss AxioCam digital color camera mounted on a Zeiss Axiovert 25 inverted microscope outfitted with a 20× (LD-A-Plan/0.3 NA) or 32× (LD-A-Plan/0.4 NA) objective (A-C right panel) or on a Leica MZ12 stereomicroscope outfitted with a plan apo 1.0× objective (C, left panel). Scale bars: A, 20 μm; B, 10 μm; C, left panel, 100 μm, and right panel, 10 μm.

(Figure 1D). compared with definitive erythroid cells, circulating EryP (from E11.5) were found to express *Eklf* mRNA at higher levels (approximately 3.6-fold) than do EryD at a comparable stage of development (CD71$^+$Ter119$^+$, sorted from adult BM; Figure 1E).

To evaluate the impact of the *Eklf*-null mutation on EryP development, *Eklf*$^{+/-}$ and ε*::H2B-GFP* mice were mated. *Eklf*$^{+/-}$;ε*::H2B-GFP* mice were then intercrossed to generate *Eklf*-null mutant EryP/H2B-GFP. The frequency of EryP/H2B-GFP and fluorescence intensity of the *H2B-GFP* reporter were unaffected by the loss of either one or both alleles of *Eklf* (Figure 1F; the transgene contains only a human ε-*globin* promoter but not additional upstream regulatory sequences). In contrast, transcription of the endogenous ε^Y-*globin* gene was modestly decreased in *Eklf* heterozygous EryP/H2B-GFP and, as reported by others,[17] was reduced to approximately 50% of WT levels in null mutant EryP (supplemental Figure 2B). Therefore, expression of the endogenous ε^Y-*globin* gene is regulated, in part, by *Eklf* in the EryP lineage. Whether this regulation is direct or is mediated indirectly, through additional *Eklf* target genes, remains to be evaluated. EKLF has been implicated in direct regulation of the human ε-*globin* gene.[18]

As the *Eklf*-null mutation resulted in embryonic lethality by E14.5, analyses were performed before this developmental stage. EryP/H2B-GFP were sorted from WT, heterozygous, or null mutant embryos and gene expression was examined. *Eklf* expression was reduced by nearly one-half in heterozygous EryP and was absent in *Eklf*$^{-/-}$ embryos, as expected (supplemental Figure 2C). *Klf3/Bklf* is a direct target of *Eklf*, and its expression is greatly reduced in *Eklf* mutant FL cells.[19] In EryP, the levels of *Klf3/Bklf* mRNA were reduced to 70% (heterozygotes) and 20% (null mutant) of those seen in WT embryos (supplemental Figure 2D).

Haploinsufficiency of Eklf results in the absence of surface Ter119 expression on EryP but not on EryD

The maturation of erythroblasts to enucleated reticulocytes can be monitored by examining the combined expression of the transferrin receptor CD71 and Ter119, a heavily glycosylated surface protein that is up-regulated during both primitive[4] and definitive[20] erythroid maturation. Loss of both alleles of *Eklf* results in the complete ablation of Ter119 surface expression on circulating blood or FL cells.[9,14] We, therefore, evaluated expression of CD71 and Ter119 in the EryP compartment of WT, heterozygous, and *Eklf*-null embryos. CD71 expression was not affected by the loss of one *Eklf* allele but was modestly reduced (approximately 4-fold, based on analysis of fluorescence intensity) in *Eklf*$^{-/-}$ EryP (Figure 2A), as reported by others.[14] Some heterogeneity in expression of the ε*::H2B-GFP* transgene in *Eklf*-null mutant EryP was evident (Figures 1F and 2A) but did not compromise our analyses.

Others have reported that unfractionated embryonic blood cells from *Eklf* heterozygous embryos (containing a mixture of EryP and EryD) show "intermediate" levels of Ter119 expression compared with stage-matched WT littermates.[14] To determine whether this intermediate population might in fact be heterogeneous, we took advantage of the ε-*globin::H2B-EGFP* transgene to measure Ter119 levels on each of the 2 *Eklf*$^{+/-}$ erythroid lineages. Expression of Ter119 protein on *Eklf*$^{+/-}$ EryD (GFP-negative) was normal but was almost completely absent from *Eklf*$^{+/-}$ EryP/H2B-GFP (Figure 2B-C). These results suggest a role for *Eklf* in the maturation of EryP that is distinct from its function in EryD. Both *Eklf*-null mutant EryD and EryP lacked surface expression of Ter119 (Figure 2B; *Eklf*$^{-/-}$).
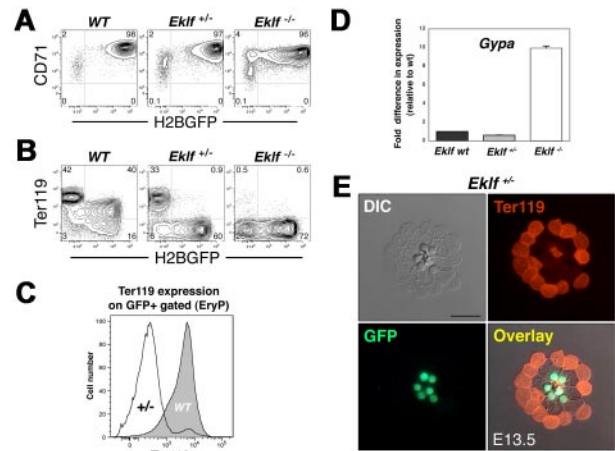

Figure 2. Haploinsufficiency of *Eklf* results in the absence of Ter119 in the primitive but not the definitive erythroid lineage. (A) FACS analysis of CD71 expression on WT, *Eklf*$^{+/-}$, and *Eklf*$^{-/-}$ embryos. CD71 is expressed on the *Eklf* mutant EryP, though at modestly reduced levels (approximately 4-fold) on null mutant cells. (B) *Eklf* dose-dependent expression of Ter119 on E14.5 EryP. WT GFP$^+$ cells (EryP) expressed Ter119 at higher levels than WT GFP$^-$ cells (EryD). In contrast, deletion of one allele of *Eklf* results in loss of expression of Ter119 on GFP$^+$ EryP but not on the GFP$^-$ EryD. Ter119 is absent from any of the null mutant cells. (C) Representative FACS histogram of Ter119 fluorescence intensity on GFP$^+$ EryP from *Eklf* WT or *Eklf*$^{+/-}$ PB, gated from samples in panel B. Expression of Ter119 on the surface of *Eklf* heterozygous cells was greatly reduced, compared with WT EryP. (D) Expression of *Glycophorin A* (*Gypa*) in E13.5 *Eklf*$^{+/+}$, *Eklf*$^{+/-}$, and *Eklf*$^{-/-}$ EryP/H2B-GFP as measured using real-time RT-PCR. mRNA expression was normalized using *18S* RNA as a control and was then normalized again, setting WT *Eklf* levels at 1.0. (E) Ter119 staining of PB cells from E13.5 ε-*globin:H2B-GFP; Eklf*$^{+/-}$ embryos. Cells were cytospun onto glass slides, fixed, and permeabilized before immunostaining. No Ter119 reactivity was detected in EryP (green fluorescent nuclei) compared with the smaller Ter119$^+$ enucleated definitive erythrocytes. Images were acquired using a Zeiss AxioCam camera mounted on a Zeiss Axioplan 2 microscope outfitted with a 63×/Plan neofluar/1.25 NA oil objective. Scale bar, 20 μm.

Ter119 is thought to be Glycophorin A (GPA, encoded by *Gypa*)[21] or a molecule that associates directly with GPA.[20] We, therefore, asked whether transcription of *Gypa* in EryP is altered by loss of one or both copies of *Eklf*. *Gypa* mRNA levels were similar in EryP from WT and *Eklf*$^{+/-}$ embryos (Figure 2D). Strikingly, however, *Gypa* mRNA levels were approximately 10-fold higher in *Eklf*$^{-/-}$ EryP.

Thus, *Eklf*$^{+/-}$ EryP express normal levels of *Gypa* mRNA but not surface Ter119, whereas transcription of the gene is greatly up-regulated in *Eklf*$^{-/-}$ EryP, on which surface Ter119 is absent. The uncoupling between transcription and Ter119 protein expression is intriguing and raises the possibility that the absence of surface Ter119 on *Eklf*$^{+/-}$ and *Eklf*$^{-/-}$ EryP might be due to aberrant protein trafficking to the plasma membrane. To address this question, we examined expression of Ter119 in permeabilized cells. In contrast with WT and *Eklf*$^{+/-}$ EryD, which express Ter119 on the cell surface (Figure 2B), no significant intracellular or extracellular signal was detected for permeabilized *Eklf*$^{+/-}$ EryP stained with Ter119 (Figure 2E; negative control shown in supplemental Figure 2E).

Eklf-null mutation results in aberrant expression of a variety of surface proteins by primitive erythroid cells

As *Eklf*-null EryP expressed CD71 but failed to express Ter119, we examined the expression of other proteins that we have found on the surface of EryP during their development.[4] Expression of the glycosylphosphatidylinositol (GPI)–anchored glycoprotein, CD24, is up-regulated on EryP as they mature.[4] In contrast to WT EryP, on
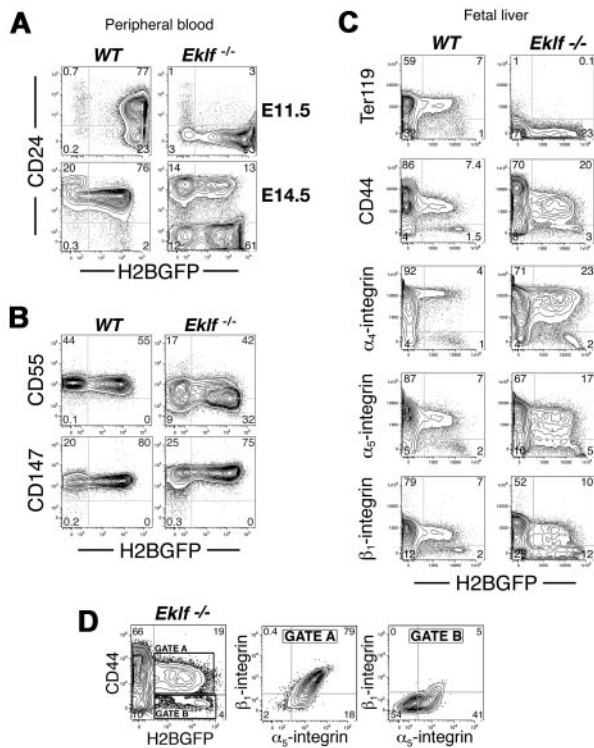
Figure 3. Aberrant surface protein expression on *Eklf*-null mutant EryP. (A) CD24 is not expressed on *Eklf*⁻/⁻ EryP at E11.5 (top panel). By E14.5, a subpopulation of EryP/H2B-GFP have activated expression of CD24 to levels seen on EryD, while the remaining EryP/H2B-GFP fail to express CD24. (B) CD55 was modestly down-regulated on *Eklf*-null mutant EryP, while CD147 was moderately up-regulated. (C) FACS analysis of FL cells isolated from WT and *Eklf* mutant embryos. In comparison with the uniform levels of expression of α4, α5, and β1 integrins and CD44 on WT EryP/H2B-GFP, heterogeneous expression was seen on *Eklf* mutant EryP. (D) EryP/H2B-GFP *Eklf*⁻/⁻ FL cells from panel C were gated according to CD44 surface expression (gate A, expressed; gate B, not expressed). The corresponding levels of integrins α5 and β1 are shown.

from the surface of paroxysmal nocturnal hemoglobinuria erythrocytes,[22] was expressed on both WT and null mutant EryP (Figure 3B). In contrast, the GPI-anchored protein CD147 was expressed at approximately 2.5-fold higher levels (as determined from mean fluorescence intensity from histograms) on *Eklf*-null mutant than on WT cells (Figure 3B). Therefore, Eklf does not appear to regulate the pathway by which GPI-proteins are modified and inserted into the cell membrane.

We have reported that EryP are found in the parenchyma of the FL, in contact with the central macrophages of erythroblastic islands, where they apparently complete their maturation and enucleate.[6] The FL phase of EryP development is accompanied by the up-regulation of several adhesion molecules, including CD44, α4-, α5-, and β1-integrins, on the surface of the cells.[6] As *Eklf*-null mutant EryP display several alterations in cell morphology and surface antigen expression,[14] we asked whether they can migrate to FL and whether they up-regulate surface antigen expression.[6] EryP/H2B-GFP were present in the FL of *Eklf*⁻/⁻;ε::H2B-GFP embryos (Figure 3C and data not shown), suggesting that, despite their morphologic abnormalities, EryP can migrate to this tissue. All GFP⁺ cells from WT FL expressed CD44 and α4-, α5-, and β1-integrins at uniform levels. However, EryP/H2B-GFP from *Eklf*-null mutant FL displayed a heterogeneous pattern of adhesion molecule expression (Figure 3C), with a distinct population that had not up-regulated any of these proteins (Figure 3D). For example, the cells that lacked CD44 also lacked or expressed only low levels of the other adhesion molecules examined. These results strongly suggest that *Eklf* regulates, directly or indirectly, the expression of adhesion molecules that may be required for the final stages of EryP maturation.[6]

Loss of *Eklf* results in aberrant expression of megakaryocyte surface proteins and transcription factors in maturing primitive erythroid cells

Eklf has been proposed to regulate a switch between the erythroid and megakaryocytic lineages.[23] *Eklf*-null mutant FL cells produce greater numbers of megakaryocyte progenitors (as detected using colony assays in vitro) than do WT embryos.[23] However, it is unclear whether this apparent lineage shift occurs solely at the level of the progenitor or whether maturing EryP/H2B-GFP also exhibit a phenotypic shift toward the megakaryocytic lineage. We addressed this issue by examining the expression of molecular hallmarks of megakaryotic differentiation in *Eklf*-null mutant EryP. The megakaryocyte/platelet marker CD41 (*Itga2b*) was aberrantly expressed on the surface of circulating E13.5 *Eklf*-null EryP (Figure 4A). Expression of *Itga2b* mRNA, as evaluated using real-time RT-PCR, was consistent with that of CD41/Itga2b
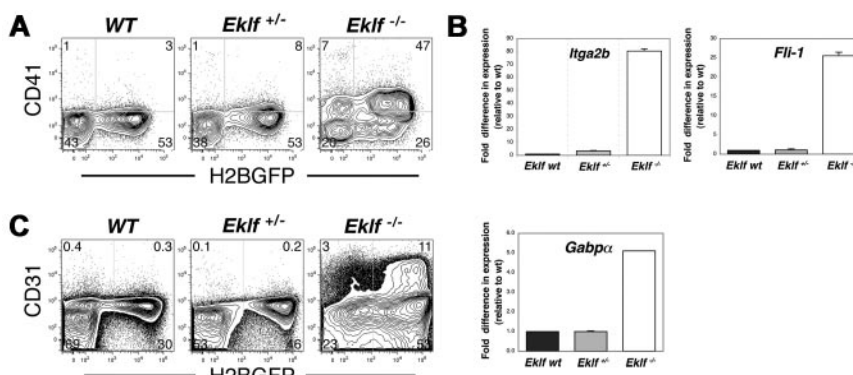
which CD24 is expressed at E11.5, *Eklf*⁻/⁻ EryP lacked expression of CD24 at this stage (Figure 3A). By E14.5, only a subpopulation (∼ 20%) of EryP/H2B-GFP had activated expression of CD24 (Figure 3A). CD24 expression on *Eklf*⁺/⁻ EryP was normal (not shown).

Several GPI-anchored molecules are lost from the surface of erythrocytes in human paroxysmal nocturnal hemoglobinuria (PNH),[22] suggesting a generalized defect in GPI-protein production. We asked whether an analogous defect might characterize *Eklf*-null mutant EryP. CD55, a GPI-anchored molecule missing

Figure 4. Aberrant expression of megakaryocyte-related adhesion proteins and transcription factors in *Eklf*-null mutant EryP. CD41 (A) and CD31 (Pecam-1, C) protein were up-regulated on EryP/H2B-GFP from *Eklf*-null E13.5 embryos. (B) Genes encoding the integrin CD41 (*Itga2b*) and the megakaryocyte transcription factors *Fli-1* and *Gabpa* were strongly up-regulated in EryP sorted from *Eklf*-null mutant E13.5 embryos, as analyzed using real-time RT-PCR.

protein, with an increase in the number of *Itga2b* transcripts of at least 80-fold over that measured for WT or *Eklf*^{+/−} cells (Figure 4B). Eklf is thought to negatively regulate expression of the transcription factor Fli-1.[23] A 25-fold increase in the level of *Fli-1* mRNA was detected in EryP lacking both alleles of *Eklf* (Figure 4B). We also observed a 5-fold increase in *GA repeat binding protein* α (*Gabpα*), which encodes a transcription factor important in the early stages of megakaryopoiesis (Figure 4B). We analyzed expression of additional megakaryocyte-related proteins on *Eklf*-null mutant EryP/H2B-GFP using FACS. CD42 (platelet glycoprotein IX), α2 integrin, αVβ3 integrin, c-kit, and CD34 were not expressed on these cells (data not shown). However, Pecam-1/CD31, which is up-regulated on FL cells in the absence of Eklf,[23] was readily detected on *Eklf*-null mutant EryP (Figure 4C). Together, these results suggest that the cellular identity of *Eklf*-deficient EryP is altered and that specification to the erythroid lineage is compromised in favor of the megakaryocyte program.

The tetraspanin CD9 is expressed by circulating nucleated WT primitive erythroid cells

In the course of examining *Eklf*-null mutant EryP for surface megakaryocyte markers, we made the unexpected discovery that the tetraspanin CD9, found on megakaryocytes, platelets, and several other cell types,[24] is also expressed on the surface of WT EryP. CD9 was detected on EryP/H2B-GFP as early as E8.5 (data not shown) and was maintained on EryP/H2B-GFP through the period of rapid enucleation (until E14.5; Figure 5A). CD9 was not found on enucleated (GFP-negative) EryP (Figure 5B and data not shown). *Cd9* mRNA was detected in EryP sorted from embryos from E8.5-12.5 (Figure 5C).

Expression of CD9 was associated with nucleated but not enucleated EryP (Figure 5B). To determine whether, like several integrins,[6] CD9 might be redistributed onto the membrane surrounding the extruded nucleus, we used a flow cytometric assay that we had developed previously in which free nuclei are identified as Side Scatter (SSC)[low]H2B-GFP[high] structures in the circulation.[6] CD9 was indeed identified on SSC[low]H2B-GFP[high] structures, although at lower levels than on nucleated EryP and was not expressed on enucleated EryP (Figure 5D). In the absence of *Eklf*, CD9 expression was heterogeneous, with approximately 50% of *Eklf*^{−/−} EryP negative for surface protein (Figure 5E). *Eklf*^{+/−} EryP exhibited WT levels of CD9 (Figure 5E).

CD9 surface expression distinguishes circulating nucleated primitive erythroid cells from circulating definitive erythroid cells

To determine whether CD9 might serve as a useful marker of nucleated primitive erythroblasts, CD9^{+} and CD9^{−} cells were sorted from PB of WT E14.5 ε::*H2B-GFP* embryos. At this stage, the blood contains a mixture of nucleated and enucleated EryP and FL-derived, enucleated EryD.[4] As shown in Figure 5F, the CD9^{+} cell fraction consisted entirely of nucleated EryP, whereas the CD9-negative fraction contained both enucleated EryP and EryD. Therefore, CD9 marks nucleated EryP in the circulation. Moreover, in combination with either the ε::*H2B-GFP* transgene (Figure 5A) or with Ter119 (Figure 5G), CD9 clearly identifies both EryP and EryD in PB through E15.5.

CD9 expression on FL and adult BM erythroid lineages

To determine whether CD9 is expressed on nucleated EryD, we examined Ter119^{+} cells from E14.5 FL (Figure 6A) and adult BM (Figure 6B). CD9 was found on approximately 40% of the Ter119^{+} cells in each population (Figure 6A). To identify the developmental stages at which CD9 is expressed during definitive erythropoiesis, we used simultaneous FACS analysis of CD71 and Ter119, a combination of markers that has been used to define maturing erythroid populations based on progressive up-regulation first of CD71 and then Ter119, followed by loss of CD71.[25] For both FL and BM, CD9 was expressed on the majority of CD71^{+}Ter119^{−} proerythroblasts and was rapidly down-regulated as these cells matured to CD71^{+}Ter119^{+} erythroblasts (Figure 6). By the reticulocyte and erythrocyte (CD71^{−}Ter119^{+}) stages, CD9 was absent from the cell surface. Therefore, CD9 is expressed on the nucleated erythroblasts of both the primitive and definitive erythroid lineages but not on mature enucleated cells.

Discussion

Delineation of transcription factor function in cells of the primitive erythroid lineage has been hampered by the simultaneous presence of primitive and definitive erythroid cells in the embryonic circulation. The generation of fluorescent transgenic reporter mouse lines that express cytoplasmic[4,26] or nuclear[6] GFP in a lineage-restricted manner has allowed us to segregate these 2 closely related erythroid lineages. It was initially believed that the primitive erythroid lineage was less severely affected than the definitive lineage by ablation of *Eklf*.[11,12] By midgestation, the blood of *Eklf*-null mutant embryos lacks mature, enucleated definitive erythrocytes and contains largely nucleated, blastlike EryD and misshapen EryP.[13,14] Previous studies had analyzed whole embryonic blood containing a mixture of both lineages, so that specific analysis of EryP was not possible. Here, we have examined in detail the consequences for development of EryP of loss of one or both alleles of *Eklf*. We find that a full complement of *Eklf* is required for normal maturation and identity restriction of primitive erythroid cells.

Eklf haploinsufficiency alters the phenotype of primitive erythroid cells

The primitive and definitive erythroid lineages are cytologically distinct. At the molecular level, the most prominent difference is in their *globin* gene and protein expression. Here we report additional differences between the lineages. By analyzing PB cells for expression of Ter119 and H2B-GFP together, so that EryP and EryD can be distinguished, we found that EryP lacking a single copy of *Eklf* fail to express Ter119, while *Eklf* heterozygous EryD expressed Ter119 at normal levels. Therefore, the Ter119 "intermediate" population reported by others[14] in fact comprises Ter119^{+} EryD and Ter119^{−} EryP. This finding highlights the utility of the ε-*globin::histone H2B-GFP* transgenic mouse line and suggests that at least some functions of *Eklf* are distinct in the 2 erythroid lineages and that EryP are more sensitive to *Eklf* gene dosage than are EryD. It is worth noting that *Runx1*-null mutant EryP, which are essentially haploinsufficient for *Eklf* (expressed at 50% of WT levels), also show reduced Ter119 protein expression.[27] In EryP, *Runx1* may activate *Eklf*, which in turn would regulate (directly or indirectly) expression of Ter119. No change in transcription of *Gypa* (the gene believed to encode Ter119) was detected in *Eklf*
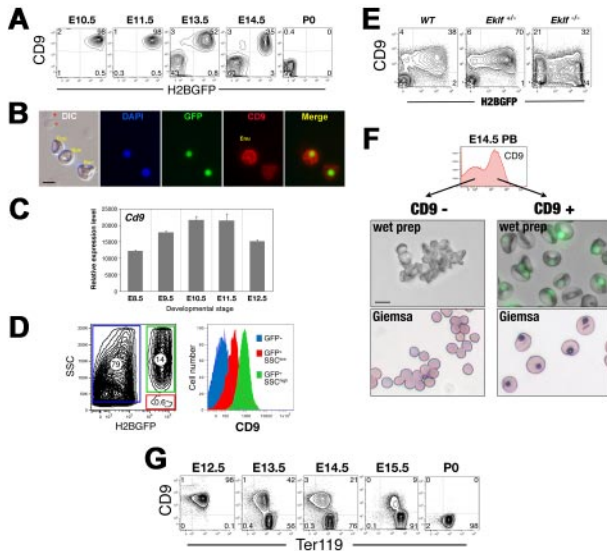
Figure 5. CD9 surface expression identifies circulating nucleated primitive erythroid cells. (A) The tetraspanin CD9 is expressed on circulating EryP/H2B-GFP. (B) Immunocytochemical staining of PB from 13.5 embryos using anti-CD9. (C) Expression of *Cd9* in EryP throughout development. GFP+ cells were sorted from E8.5-12.5 embryos and real-time RT-PCR analysis of *Cd9* mRNA expression performed. (D) CD9 is redistributed onto expelled EryP nuclei. Side scatter (SSC) and GFP can be used to identify 3 populations: SSC(high) GFP+ EryP (green), SSC(low) GFP+ free nuclei (red) and SSC(low) GFP− enucleated cells (blue) (left panel). CD9 expression on each population is shown (right panel). CD9 is present on nucleated EryP and at lower levels on free nuclei; it is not expressed on enucleated EryD. (E) CD9 expression is altered by loss of *Eklf*. While all E13.5 WT and heterozygous EryP express CD9, *Eklf*-null GFP+ cells show more heterogeneous CD9 expression, with a CD9-negative population. (F) Nucleated EryP can be isolated on the basis of CD9 expression. CD9+ and CD9− cells were sorted from E14.5 blood. Both wet preparations (middle panels) and Giemsa stains (bottom panels) clearly document that the CD9+ cells are EryP. (G) Developmental progression of CD9 expression on circulating WT erythroid cells. CD9 is expressed on EryP but not on definitive erythroid cells. Images were captured in AxioVision using a Zeiss AxioCam camera mounted on a Zeiss Axioplan 2 microscope and outfitted with a 63×/Plan neofluar/ 1.25 NA oil objective (B) or a Zeiss Axiovert 25 inverted microscope outfitted with a 32×/LD-A-Plan/0.3 NA objective (F). Scale bars, 10 μm.

heterozygous EryP. However, when both copies of *Eklf* were ablated, Ter119 protein was absent and the numbers of *Gypa* transcripts actually increased, suggesting that the failure of *Eklf* mutant EryP to express surface Ter119 might result from aberrant posttranslational processing and/or defects in protein trafficking to the cell membrane. Immunostaining of permeabilized *Eklf*+/− EryP failed to reveal intracellular Ter119 protein. While this observation suggests that the absence of expression of Ter119 on the surface of *Eklf*+/− EryP/H2B-GFP is not due to aberrant trafficking, we cannot rule out the possibility that incomplete processing of Ter119 in *Eklf*+/− EryP results in the production of protein lacking the epitope recognized by the Ter119 antibody. It is also possible that *Gypa* does not encode Ter119 (ie, Ter119 is not the mouse equivalent of human GPA); a formal demonstration that Ter119 is encoded by *Gypa* would require expression cloning of Ter119. Uncoupling of *Gypa* mRNA and surface protein expression has been reported for definitive erythroid cells.[28]

CD9 and erythropoiesis

In examining the megakaryocyte-related markers that are aberrantly up-regulated in the absence of *Eklf*, we found that the tetraspanin CD9 is expressed not only on some *Eklf*-null EryP but also on WT EryP. Surface expression of CD9 correlated strongly with the presence of a nucleus in EryP. Using CD9 surface

expression alone, we were able to identify and purify nucleated EryP from the PB of embryos at E14.5, a stage when approximately half of circulating cells are FL-derived EryD.[4] The transgenic reporters are still required for isolation of EryP from FL, a more complex milieu in which CD9 is expressed on multiple cell types, including nucleated EryD (unpublished observations). To our knowledge, CD9 is the first marker that distinguishes between nucleated EryP and definitive erythrocytes in the circulation. It will be of interest to determine whether CD9 is also expressed on developing human EryP or on maturing definitive human erythroblasts. CD9 is known to play an important role in the regulation of cell fusion and cell-cell interactions in other systems. Our work may have implications for interactions between erythroid cells and stromal or other supportive cells as well as engulfment of extruded erythroid nuclei by macrophages.

The rapid decrease in CD9 surface expression during EryP maturation suggested that, like several adhesion molecules,[6] this protein might also be lost during enucleation. Only approximately 20% of the CD9 measured on nucleated EryP is later found on the membrane surrounding the expelled nucleus. Therefore, an additional mechanism, such as exosomal release, must regulate the loss of CD9 from EryP. Indeed, CD9 is one of the most common components of exosomes.[29]

With the exception of an erythroid-specific tetraspanin, Penumbra (Pen),[30] the functions of this superfamily in erythroid cells have not been well described. Tetraspanins organize microdomains of signaling proteins, such as integrins and antigen receptor proteins. α4, α5, and β1-integrins and CD44 are expressed on the surface of maturing EryP and are redistributed onto the membrane surrounding their extruding nuclei.[4,6] Perhaps CD9 assists in the formation
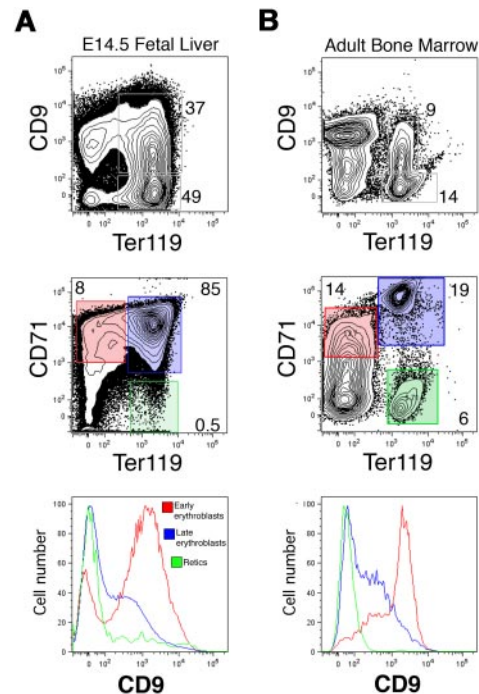

Figure 6. CD9 is expressed on definitive erythroblasts in the FL and BM. CD9 expression was measured on Ter119+ cells from E14.5 FL (A, top panel) or adult BM (B, top panel) using analytical flow cytometry. Approximately 40% of Ter119+ cells from both tissues express CD9. (Middle panels) The developmental progression of erythroid cells from FL or adult BM was identified using CD71 and Ter119 surface expression. (Bottom panels) CD9 was present on most CD71+;Ter119− (immature) erythroid cells (red), was down-regulated on CD71+;Ter119+ erythroblasts (blue), and was absent from mature CD71−;Ter119+ reticulocytes and erythrocytes (green) from both FL and adult BM.

of adhesion molecule-rich signaling complexes that mediate interactions between EryP and FL macrophages,[6] allowing them to complete their maturation and to enucleate.

A molecular "identity crisis" in primitive erythroid cells lacking Eklf

Several megakaryocyte markers (eg, CD41 and Pecam-1/CD31) were inappropriately activated in *Eklf*-deficient mutant EryP. In both primitive and definitive hematopoiesis, the erythroid and megakaryocyte lineages share a common progenitor termed the megakaryocyte-erythroid progenitor.[31] *Eklf* has been shown to regulate a switch between the erythroid and megakaryocytic lineages.[23] The megakaryocyte program is promoted in the absence of Eklf. For example, *Eklf*^{−/−} FL cells produce significantly more megakaryocyte progenitors and express the integrin CD41 and a variety of megakaryocyte genes at higher levels than do WT FL cells.[23] CD41 is also expressed on cells from the PB of *Eklf*-null mutant embryos.[32] This phenotype is thought to result from an imbalance between Eklf and Fli-1, a transcriptional regulator essential for megakaryocyte commitment and early differentiation.[23,33]

The aberrant expression of CD41, CD31/Pecam-1, and *Gabpα* observed for *Eklf*-null mutant EryP might arise, at least in part, from the increased expression of *Fli-1* that we detected in *Eklf* mutant EryP. Alternatively, or in addition, it could result from derepression, either due to the absence of Eklf/Klf1 itself or, indirectly, through the loss of an Eklf/Klf1-regulated repressor such as Klf3.[14,34] Indeed, in *Eklf*-null mutant EryP, *Klf3* transcription was significantly reduced. Using an in silico approach (see supplemental Figure 3 legend), we have identified several potential Klf binding sites within the promoter regions of *CD41/Itga2b*, *CD31/Pecam-1*, and *Gabpα* (supplemental Figure 3). Therefore, Klf3 may repress their transcription in WT cells. It is conceivable that Eklf/Klf1 also plays a role in the negative regulation of these genes, as it is known to function not only as an activator but also as a repressor.[35-37] A potential binding site for Fli-1 is present upstream of *Itga2b/CD41* and *Gabpα* but not *CD31/Pecam-1* (supplemental Figure 3) and Fli-1 may ectopically activate *Itga2b* and *Gabpα* in *Eklf*-null mutant EryP.

The expression of megakaryocyte markers on mutant EryP was not promiscuous: in addition to CD41 and CD31, other megakaryocyte-related proteins such as CD42, α2-, αV-, and β3-integrins were not detected on these cells. Moreover, they lack the erythroid lineage-specific marker Ter119. These observations suggest that *Eklf* regulates cell identity not only in megakaryocyte-erythroid progenitors but throughout EryP maturation. One of the morphologic hallmarks of *Eklf*-null mutant EryP—a ruffled cell membrane with occasional protrusions—may result from aberrant expression of megakaryocyte adhesion molecules and, therefore, alterations in the protein composition of the EryP membrane. Megakaryocytes give rise to platelets by membrane demarcation and budding.[38] The protrusions seen on *Eklf*-deficient EryP may reflect failed attempts at platelet production.

Authorship

Contribution: J.I., S.T.F., Z.H., and M.H.B. designed the experiments and prepared the figures; J.I., S.T.F., and Z.H. performed the experiments; H.Z. performed in silico analysis and prepared the supplementary figure; and J.I., S.T.F., and M.H.B. wrote the paper.

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