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The Erythroid Phenotype of EKLF-Null Mice: Defects in Hemoglobin Metabolism and Membrane Stability

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Development of red blood cells requires the correct regulation of cellular processes including changes in cell morphology, globin expression and heme synthesis. Transcription factors such as erythroid Krüppel-like factor EKLF (Klf1) play a critical role in erythropoiesis. Mice lacking EKLF die around embryonic day 14 because of defective definitive erythropoiesis, partly caused by a deficit in β-globin expression. To identify additional target genes, we analyzed the phenotype and gene expression profiles of wild-type and EKLF null primary erythroid progenitors that were differentiated synchronously in vitro. We show that EKLF is dispensable for expansion of erythroid progenitors, but required for the last steps of erythroid differentiation. We identify EKLF-dependent genes involved in hemoglobin metabolism and membrane stability. Strikingly, expression of these genes is also EKLF-dependent in primitive, yolk sac-derived, blood cells. Consistent with lack of upregulation of these genes we find previously undetected morphological abnormalities in EKLF-null primitive cells. Our data provide an explanation for the hitherto unexplained severity of the EKLF null phenotype in erythropoiesis.

The anemias, caused by failure of erythropoiesis, constitute the most common human genetic disorders. Most of these disorders are caused by mutations or deletions in the coding sequences or regulatory elements of the α- and β-globin genes (37). A number of these mutations have marked the importance of specific promoter elements, such as the β-globin CACC box. Mutations within this box result in severe down regulation of β-globin gene expression (7, 8, 16). The CACC box motif is found in many erythroid gene promoters (29); it is a binding site for the erythroid Krüppel-like factor EKLF (or KLF1), a member of the SP/XKLF transcription factor family (8, 20). An 81 amino acid DNA binding domain, found close to the C termini of all members, defines this family. The binding domain consists of three conserved Cys_2His_2-type zinc fingers (27). The expression of EKLF is largely restricted to the erythroid lineage (20, 32), although expression in macrophages has been reported recently (19). Mice lacking EKLF die in utero around embryonic day 14 (E14) from severe anemia associated with a marked deficit in β-globin expression (23, 26). The time of death coincides with the stage of development in which the fetuses become dependent on definitive, fetal liver-derived, erythroid cells that take over the oxygen transport from the primitive, yolk sac-derived cells. So far, no abnormalities in $EKLF^{-/-}$ primitive cells have been described. However, EKLF is expressed in these cells (32). Although the expression of the embryonic εy and βh1 globin genes is unaffected by the absence of EKLF, the low expression rate of the adult βmaj-globin gene in these cells is EKLF dependent (35). Furthermore, LacZ reporter transgenes have been used to demonstrate that EKLF can act as a transcriptional activator in embryonic erythropoiesis (34).

The fatal anemia caused by the absence of β-type globin protein in definitive erythrocytes can not be rescued by expression of exogenous γ-globin (the human fetal β-type globin) despite an efficient production of hybrid α2γ2 hemoglobin (25). Moreover, it was demonstrated that $EKLF^{-/-}$ ES cells injected into blastocysts do not contribute to the mature erythrocyte compartment, although $EKLF^{-/-}$ cells were found as erythroid progenitors. This phenotype was ameliorated but not completely rescued by γ-globin expression in these $EKLF^{-/-}$ cells (18). The synthesis of heme is tightly coupled to the expression of globin genes (21). Heme is synthesized from succinyl coenzyme A (succinyl-CoA) and glycine in seven enzymatic steps. It has been suggested that EKLF plays a role in the control of expression of some of these genes (33). The genes encoding two critical enzymes of the heme synthesis pathway, Alas2 and Pbgd, contain potential EKLF binding sites (28, 30). Alas2, the erythroid cell-specific isoform of Alas, is upregulated during erythroid differentiation (9). This is critical for erythropoiesis and cannot be compensated for by expression of Alas1 (38).

In addition, EKLF is likely to regulate other genes that are of vital importance in definitive erythropoiesis. To study the role of EKLF in erythropoiesis and identify novel EKLF-dependent genes, we employed in vitro cultures of primary erythroid progenitors that undergo a synchronized differentiation program (5, 36). Progenitors from mouse fetal livers proliferate in serum-free medium under the control of erythropoietin (Epo), stem cell factor (SCF) and the glucocorticoid hormone dexamethasone (Dex). When exposed to Epo in absence of

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SCF and Dex, the cells undergo terminal differentiation. Differentiation of wild type progenitors mimics in vivo erythropoiesis as the cells undergo three to four rapid cell divisions accompanied by a decrease in cell size and the accumulation of hemoglobin. Finally the cells expel their nucleus (36). In this process, erythroid genes are expressed in the appropriate temporal order (5). This differentiation model of primary cells provides a unique opportunity to study gene expression in the absence of EKLF.

In this paper, we describe the phenotype of differentiating primary definitive erythrocytes in the absence of EKLF. Our data show that EKLF is dispensable for erythropoiesis up to the pro-erythroblast stage, but is essential for completing the terminal differentiation program. We demonstrate that EKLF is required for the activation of erythroid cell-specific genes that are important for hemoglobin metabolism and stabilization of the cells. Furthermore, we show that expression of these novel EKLF-dependent genes is severely reduced in EKLF$^{-/-}$ primitive erythroid cells, strongly indicating that EKLF is a positive regulator of endogenous genes in these cells. We find morphological abnormalities in EKLF$^{-/-}$ primitive cells that are consistent with the reduced expression of these genes. Collectively, our data provide an explanation for the hitherto unexplained severity of the EKLF-null phenotype in definitive cells.

MATERIALS AND METHODS

Cultivation of mouse erythroid progenitors. Fetal livers of E12.5 mouse embryos were disrupted and seeded into Stem-Pro-34 medium supplemented with human recombinant erythropoietin (Erypo; Cilag AG, Switzerland; 1 U/ml), murine recombinant stem cell factor (100 ng/ml; R&D Systems, Minneapolis, MN) and dexamethasone (Sigma; 10^{-6} M) (36). The cultures of erythroid progenitors were subjected to daily partial medium changes and addition of fresh factors. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY-1; Schärfe-Systems, Reutlingen, Germany). Progenitors were enriched through Ficoll (lymphocyte separation medium, 1078 g/cm^3; Eurobio, France) by centrifugation when the cultures contained $> 40\%$ dead and/or differentiated cells as estimated by size distribution. Cell density was kept at $\sim 2 \times 10^6$ cells/ml.

Terminal differentiation of mouse erythroid progenitors. Progenitors were washed twice in phosphate-buffered saline (PBS) and seeded at 1.5×10^6 cells/ml in differentiation medium (Stem-Pro-34 medium) supplemented with Epo (10 U/ml) and iron-saturated human transferrin (Sigma; 1 mg/ml). Cells were maintained at densities of 2×10^6 to 4×10^6 cells/ml, requiring dilution with fresh medium twice daily between 24 and 50 h. Cell number and size distribution were determined using an electronic cell counter. Hemoglobin content was quantitated in a photometric assay as described previously (1).

Real-time PCR. The real-time PCR assay was performed on a Bio-Rad Icycler. For the PCRs, the Eurogentec qPCR Corekit for SYBR Green was used. Reactions were performed in 25 μl mix as described by the manufacturer. Amplification program: 10 min at 95°C, 40 cycles of denaturation at 95°C for 20 s, annealing and elongation at 60°C for 45 s. After each elongation step, the fluorescence signal was measured at 75°C. To confirm amplification specificity, the PCR products were subjected to melting curve analysis. Primer sequences are as follows: Alas2, 5′-CACCTATGCTTAAGGAGCCA-3′ and 5′-CAGAAGCA CACAGGAAAGCA-3′; Alad, 5′-CTTTGATCTCAGGACTGCTG-3′ and 5′-A ACAGCTGCGGTGCAAAGTA-3′; Pbgd, 5′-TACTTCTGGCTTCCAAGTG C-3′ and 5′-CAAGGTGAGGCATATCTTCC-3′; Urod, 5′-ATCCCTGTGCCT TGTATGCA-3′ and 5′-AGGTTGGCAATTGAGCGTTG-3′; Cpox, 5′-CAA TTTGAAGCCAGTCCGTG-3′ and 5′-CTGGACTAGAACTCCCTTTG-3′; Ppox, 5′-ATTCCAGCTTCAGAGCTCAG-3′ and 5′-TACTGCAGATTCACC ACAGC-3′; Fech, 5′-ACCAGTGACCACATTGAGAC-3′ and 5′-GGCCTTG GAGAACAATGGAT-3′; Hprt, 5′-AGCCTAAGATGAGCGCAAGT-3′ and 5′-ATGGCCACAGGACTAGAACA-3′; β-globin, 5′-ATGCCAAAGTGAAG GCCCAT-3′ and 5′-CCCAGCACAATCACGATCAT-3′; Ahsp, 5′-GGATCAG CAGGTCTTTGATG-3′ and 5′-AGAGTACTCAGCTCTTGCTG-3′; Kcnn4, 5′-AAGCACACTCGAAGGAAGGA-3′ and 5′-TTCCGGTGTTTCAGCCGT A-3′; Epb4.9: 5′-TGCTCAAGACCCAAGGCTTA-3′ and 5′-TCCTATCTG GTTTTGCCTGG-3′; Gapdh, 5′-CCTGCCAAGTATGATGACAT-3′ and 5′-G TCCTCAGTGTAGCCCAAG-3′; CA1: 5′-AGAGTCTGCAGTTCCAGTTC-3′ and 5′-GCCAGGTCATAATTGAGGAC-3′.

cDNA array hybridizations and analysis. Total RNA from cultured cells and fetal livers was extracted using TRI Reagent (Sigma) following the manufacturer's instructions. This RNA was used to hybridize microarrays containing ~17,000 expressed sequence tag (EST) sequences and a custom-made hematopoietic microarray containing ~9,000 cDNAs that were enriched for erythroid cell- and T-cell-specific cDNAs by subtracting cDNA of expanding I/11 cells and quiescent CD4$^+$ T cells from cDNAs prepared from 3T3 fibroblasts and EpH4 epithelial cells (15). For a single hybridization, 30 μg total RNA was reverse transcribed into cDNA using Cy5-UTP or Cy3-UTP (CyDye; Amersham Biosciences). The microarrays were hybridized and analyzed as described previously (15). The scanning was performed using a Genepix 400A scanner (Axon Instruments, Inc.), and the analysis was performed using the GenePix program, Microsoft Excel, and Access.

Inducible EKLF cultures. Livers were isolated from E12.5 EKLF$^{-/-}$::EKLF-lbd fetuses. Single cell suspensions were cultured for 16 h in StemPro-34 containing 1% bovine serum albumin (BSA), 1% glutamine, and 10 U/ml EPO, but without serum supplement. The EKLF-lbd was activated by supplementing the medium with 250 nM 4-hydroxy-tamoxifen with or without 20 μg/ml cycloheximide. After 16 h of culture, cells were harvested and RNA was isolated for preparing cDNA.

Western blotting. From wild-type and EKLF$^{-/-}$ E12.5 fetuses, livers were disrupted and embryonic blood cells washed in PBS–1 mM EDTA. The cells were spun down and lysed in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8.0) supplemented with a protease inhibitor cocktail (Sigma). Proteins were separated on SDS–10% polyacrylamide gel electrophoresis (PAGE) gels under reducing conditions and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were probed with anti-dematin (BD Biosciences; catalog no. 611062) or anti-eIF2α (Santa Cruz Biotechnology; catalog no. FL-315) antibodies. Second-step reagents were horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG from Dako (Glostrup, Denmark). Peroxidase activity was visualized by enhanced chemiluminescence (ECL) using Western blotting detection reagents from Amersham Biosciences.

Cytospins, sections and staining. E12.5 embryonic blood cells were collected in PBS–1 mM EDTA and cytocentrifuged. The preparations were stained with neutral benzidine and histological dyes as described (2). For the sections of embryonic blood cells, wild-type and EKLF$^{-/-}$ E12.5 yolk sacs were immersion fixed in 3% paraformaldehyde–1% glutaraldehyde in Millonig's buffer (0.1 M Na-PO_4 buffer, pH 7.2) at 4°C overnight or longer. Then they were rinsed with Millonig's buffer and postfixed in 1% OsO_4 at 4°C. The yolk sacs were dehydrated through an ascending acetone series and embedded in epon. Sections were cut at 1 μm and stained with toluidine–methylene blue. Images of the cytospins and sections were acquired with an Olympus BX40 microscope. The lens used was Olympus Plan 100×/1.25.

RESULTS

EKLF is required for the in vitro differentiation of erythroid progenitors. To study the role of EKLF in definitive erythropoiesis, we expanded erythroid progenitors from fetal livers and studied their expansion and differentiation kinetics (5, 36). E12.5 fetal livers of wild-type and EKLF$^{-/-}$ embryos were resuspended in serum-free medium supplemented with Epo, SCF, and Dex, and erythroid progenitors were expanded for 10 days. The absence of EKLF did not influence the proliferation capacity of the cultured progenitors. Both wild-type and EKLF$^{-/-}$ progenitors doubled every 24 h, and the cells had similar morphologies (T = 0, Fig. 1C). Differentiation of the cultures was induced by replacing the renewal factors SCF and Dex with transferrin and high concentrations of Epo. For the wild-type erythroid progenitors this resulted in the execution of the terminal differentiation program, which is characterized by three to four accelerated "differentiation" divisions, cell size reduction, accumulation of hemoglobin, and finally enucle-

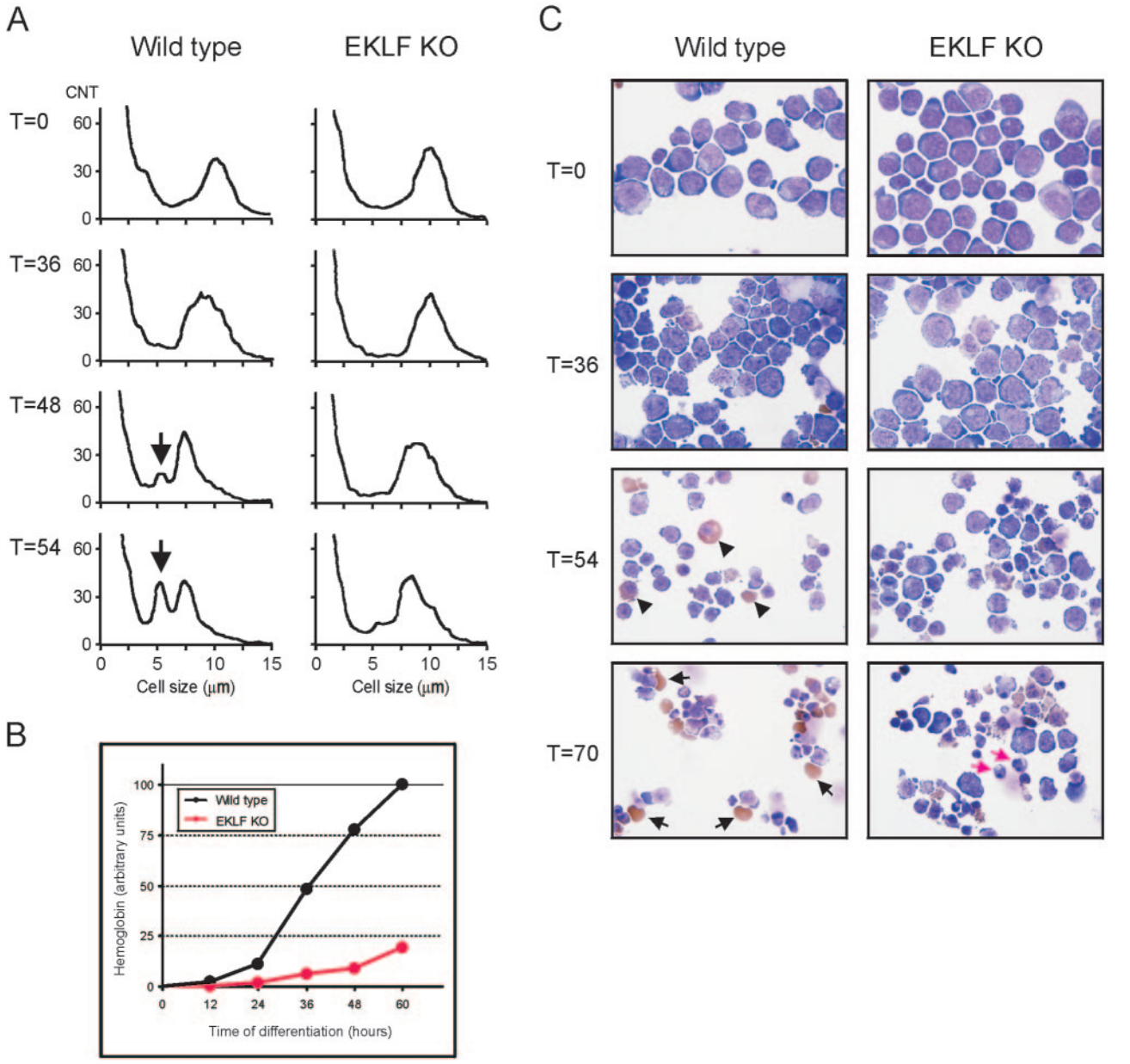

FIG. 1. Differentiation of primary erythroid progenitors. (A) Cell size distribution of wild-type and EKLF$^{-/-}$ erythroblast cultures during differentiation. The size of wild-type cells decreases during differentiation and after 48 h enucleated cells appear (arrow). (B) Hemoglobin per cell volume was measured with intervals of 12 h in wild-type and EKLF$^{-/-}$ cultures after induction of differentiation. (C) Morphological analysis of wild-type and EKLF$^{-/-}$ erythroblasts during differentiation. Aliquots of the cultures were cytocentrifuged onto glass slides and stained with both cytological dyes and with neutral benzidine for hemoglobin (brownish stain). The absence of EKLF does not affect progenitors (T = 0). At T = 36 a decrease of cell size compared to cells at T = 0 is observed in the wild-type culture, but not in the EKLF$^{-/-}$ culture. Only in the wild-type cultures, hemoglobin is detected, first at T = 54 (arrowheads), and these cells can complete the terminal differentiation to enucleated erythrocytes (black arrows). EKLF$^{-/-}$ cells do not accumulate hemoglobin, and their nuclei become pycnotic (red arrows). Original magnification, ×100. T, time in hours; KO, knockout.

ation (Fig. 1). Although EKLF$^{-/-}$ cells displayed some aspects of the differentiation program, such as α-globin expression (6) and the appearance of pycnotic nuclei (Fig. 1C), they failed to undergo normal terminal differentiation. First, the cell size distribution barely changed upon induction of differentiation and the cells failed to undergo enucleation (Fig. 1A). Second, the amount of hemoglobin formed was 10-fold lower than in wild-type cells (Fig. 1B). Third, although cytospins of the differentiating cultures show no morphological differences between wild-type and EKLF$^{-/-}$ cells under renewal conditions (T = 0) (Fig. 1C), they show that upon induction of differentiation EKLF$^{-/-}$ cells initially retained a large cell size and did not mature to erythrocytes (Fig. 1C). FACs analysis showed that the percentage of cells that are positive for annexin V, an early marker for apoptotic cells, is comparable in EKLF null and wild-type or heterozygous cultures (data not shown). This

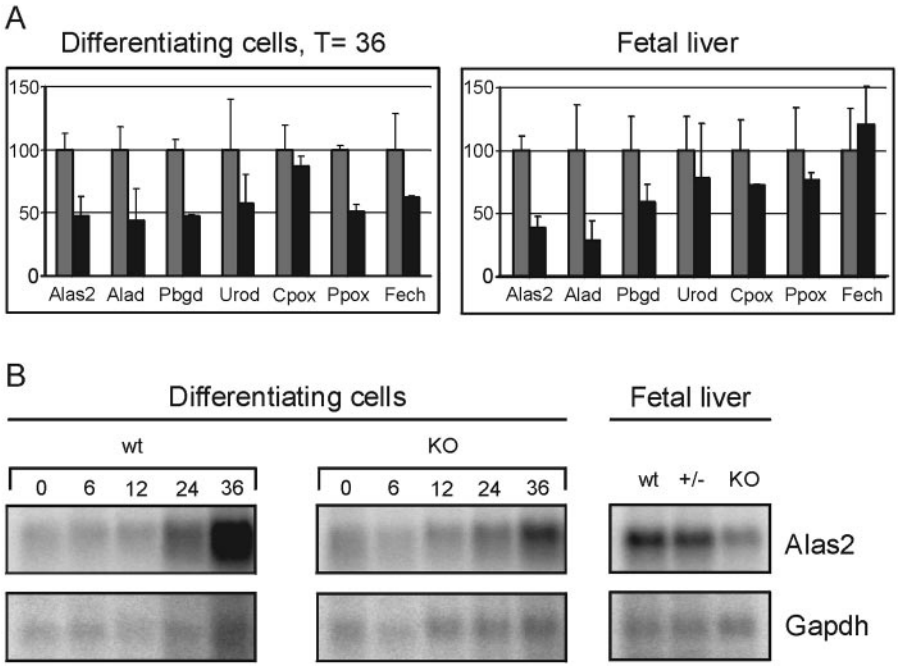

FIG. 2. Expression of genes involved in heme synthesis in EKLF-deficient erythroid cells. (A) Expression of genes involved in heme synthesis was measured by real time PCR on cDNA of wild-type and EKLF KO cultures 36 h after induction of erythroid differentiation, and of fresh E12.5 fetal livers. Values were normalized to Hprt. The wild-type values (grey) were set to 100%, EKLF KO values are displayed in black. Error bars indicate the standard deviation. (B) Northern blot showing the expression of Alas2 in differentiating wild-type and EKLF KO cultures and in E12.5 fresh fetal livers of wild-type, heterozygous, and EKLF KO embryos. Gapdh was used as a loading control. T, time in hours; wt, wild-type; +/−, heterozygous for the EKLF knockout; KO, EKLF knockout. Abbreviations for the heme synthesis enzymes are given in the text.

indicates that EKLF is not required for survival of erythroid cells. In conclusion, the data show that the anemia of EKLF-deficient mice (23, 26) is not due to a failure of erythroid progenitors to expand but that EKLF is specifically required to execute the terminal differentiation program. Furthermore, our results validate the use of this culture system for the detection of EKLF-dependent genes.

Reduced expression of genes involved in heme synthesis in EKLF$^{-/-}$ erythroid cells. The in vitro expansion and induction of terminal differentiation of erythroid progenitors provides a unique opportunity to study EKLF-dependent gene expression. In addition to insufficient β-globin synthesis, the failure of EKLF-deficient cells to accumulate hemoglobin could be due to reduced heme synthesis, as both processes are tightly linked. Heme is synthesized from succinyl-CoA and glycine in seven enzymatic steps, catalyzed by aminolevulinic acid synthase (Alas), aminolevulinate dehydratase (Alad), porphobilinogen deaminase (Pbgd), uroporphyrinogen decarboxylase (Urod), coproporphyrinogen oxidase (Cpox), protoporphyrinogen oxidase (Ppox), and ferrochelatase (Fech), respectively. We performed real-time PCR experiments to detect the expression of these genes in in vitro expanded erythroid progenitors 36 h after induction of differentiation and in fresh E12.5 fetal livers (Fig. 2A). Expression of Alas2 and Alad is significantly decreased in the absence of EKLF both in in vitro expanded progenitors and in fetal livers. Because the first step in the heme synthesis pathway is rate limiting, we were particularly interested in the expression of Alas2. Figure 2B shows the expression pattern of Alas2 during the first 36 h of differentiation in wild-type and EKLF null cells. As expected, the gene is upregulated during erythroid differentiation of wild-type cells. Although the gene is also upregulated in the absence of EKLF, the expression is reduced compared to wild-type cells. Furthermore, expression of Alas2 in EKLF$^{-/-}$ fetal livers is strongly reduced compared to wild-type and heterozygous fetal livers. We conclude that the expression of enzymes involved in heme synthesis is partially dependent on EKLF. Secondary effects could be involved, e.g., reduced expression of the rate-limiting enzyme Alas2 might be part of an adaptation mechanism to compensate for the strongly diminished expression of β-globin.

Screening for new EKLF-dependent genes. To find other genes that may be regulated by EKLF, we performed gene expression profiling of primary fresh E12.5 fetal liver cells and in vitro expanded and differentiated cells. We used two previously described custom made cDNA microarrays (15). One contains approximately 17,000 EST sequences (17K microarray), and the second contains approximately 9,000 hematopoietic cell-enriched transcripts (9K microarray). RNA was isolated from wild-type and EKLF$^{-/-}$ cells from fresh E12.5 fetal livers and from primary progenitors that were expanded for 10 days (T = 0) and 12 h after induction of differentiation (T = 12). cDNAs were generated from these RNAs, Cy5 or Cy3 labeled, and hybridized to both 9K and 17K microarrays. The Cy3/Cy5 ratios were calculated for each spot as ^{2}log value. Table 1 lists these values for genes that appeared to be regulated by EKLF. The largest differences in gene expression between wild-type and EKLF$^{-/-}$ RNA samples were found in

TABLE 1. Genes that are differentially expressed in the absence of EKLF

Gene[a]	Symbol	Gene expression[b] in:		
		Cultured cells		Fetal liver
		T = 0	T = 12	
Downregulated in EKLF KO				
Heme synthesis				
● Aminolevulinic acid synthase 1	Alas1	0.1	0.2	0.2
● Hydroxymethylbilane synthase/porphobilinogen deaminase	Hmbs/PBGD	0.2	0.5	1.1
● Uroporphyrinogen decarboxylase	Urod	−0.1	0.3	0.6
● Coproporphyrinogen oxidase	Cpox	0.0	−0.3	0.0
● Protoporphyrinogen oxidase	Ppox	0.7	0.3	0.8
● Ferrochelatase	Fech	−0.2	0.0	0.6
Erythroid cell specific				
● Hemoglobin, beta adult major chain	Hbb-b1	0.9	0.8	3.5
▲ Hemoglobin, beta adult major chain	Hbb-b1	0.7	0.5	3.0
● Hemoglobin Z, beta-like embryonic chain	Hbb-bh1	0.7	0.3	1.6
● Hemogen	Hemgn	−0.2	0.6	1.5
▲ Alpha hemoglobin stabilizing protein	Eraf	1.0	2.5	3.6
Cell membrane				
● Erythrocyte protein band 4.9	Epb4.9	0.4	0.5	3.7
● K^+ intermediate/small conductance Ca^{2+}-activated channel, subfamily N, member 4	Kcnn4	0.8	1.0	2.5
▲ Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Slc3a2	0.2	1.2	2.9
● Solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	−0.2	−0.6	1.5
● Solute carrier family 22 (organic cation transporter), member 4	Slc22a4	0.2	0.3	1.7
● Solute carrier family 43, member 1	Slc43a1	0.5	0.7	1.8
● Kell blood group	Kel	−0.1	0.4	1.9
● CD24a antigen	Cd24a	0.3	0.3	1.5
● Transferrin receptor	Tfrc	0.2	0.8	2.0
● Intercellular adhesion molecule 4, Landsteiner-Wiener blood group	Icam4	−0.1	0.0	1.7
Other				
● Protease, serine, 25	Prss25	0.9	1.0	4.7
● Sin3 associated polypeptide	Sap30	0.2	0.8	2.1
● Interferon-induced protein with tetratricopeptide repeats 3	Ifit3	0.4	0.4	2.1
● Ubiquitin associated domain containing 1	Ubadc1	0.4	0.4	1.6
● unc-84 homolog A (*C. elegans*)	Unc84a	0.8	0.6	2.2
● RIKEN cDNA 1110063G11 gene	1110063G11Rik	0.1	0.8	1.9
● RIKEN cDNA 1200006I17 gene	1200006I17Rik	−0.4	0.1	1.8
● Monoglyceride lipase	MgII	0.0	0.3	1.4
● Cat eye syndrome chromosome region, candidate 2 homolog (human)	Cecr2	1.0	0.7	1.8
● Lectin, galactose binding, soluble 1	Lgals1	0.7	1.0	1.5
● Myeloid differentiation primary response gene 116	Myd116	0.3	0.2	1.4
● Guanine nucleotide binding protein, alpha inhibiting 2	Gnai2	1.2	0.9	4.0
▲ Guanine nucleotide binding protein, alpha inhibiting 2	Gnai2	1.0	0.9	3.2
▲ Mitogen-activated protein kinase kinase 5	Map2k5	0.7	0.7	3.1
▲ Hypothetical guanylate-kinase-associated protein	Dlg7	1.2	1.0	3.6
▲ CCR4-NOT transcription complex, subunit 7	Cnot7	0.4	0.4	4.0
● *Mus musculus* RIKEN cDNA 2700084L06 gene	2700084L06Rik	0.7	1.0	1.3
● Peroxiredoxin 2	Prdx2	0.4	0.6	1.2
● Microsomal glutathione *S*-transferase 3	Mgst3	0.0	0.3	1.9
● Nuclear factor of kappa light chain gene enhancer in B-cell inhibitor, alpha	Nfkbia	0.6	1.0	1.6
● Glutathione peroxidase 4	Gpx4			1.4
Upregulated in EKLF KO				
● Coagulation factor II (thrombin) receptor	F2r	−1.0	−0.4	−1.3
● Enolase 1, alpha non-neuron	Eno1	0.0	−0.4	−1.2
● Heme oxygenase (decycling) 2	Hmox2	0.0	−0.2	−2.9
● Phosphoglycerate mutase 1	Pgam1	−0.1	−0.1	−1.2
● RIKEN cDNA 1110014J01 gene	1110014J01Rik	−0.3	0.0	−2.3
● Kelch domain containing 3	Klhdc3	0.1	−0.1	−1.8
● 24-Dehydrocholesterol reductase	Dhcr24	−0.3	0.3	−1.9
● RIKEN cDNA 6720456B07 gene	6720456B07Rik	0.0	0.1	−2.1
● Thrombospondin 1	Thbs1	0.1	−0.4	−2.5
● Guanosine monophosphate reductase	Gmpr	−0.7	−0.2	−1.6
● Ethanolamine kinase 1	Etnk1	−0.7	−0.5	−1.6
● Metastasis suppressor 1	Mtss1	0.2	−0.1	−1.6
▲ Carbonic anhydrase 1	Car1	−1.2	−1.3	−2.5
▲ Pyruvate kinase, muscle	Pkm2	−1.6	−1.1	−1.7

[a] KO, knockout; ● and ▲, from the 17K and erythroid cell-enriched 9K microarrays, respectively.

[b] Gene expression in wild-type and EKLF KO cultured cells at 0 and 12 h after differentiation and in wild-type and EKLF KO fetal livers was determined with cDNA microarrays. Values are the ^{2}log of the wild-type/EKLF KO ratio. The average of values was taken when genes were represented more than once on the chips. Genes with ^{2}log ratios higher than 1.2 or lower than −1.2 in fetal livers and genes involved in heme synthesis are displayed.

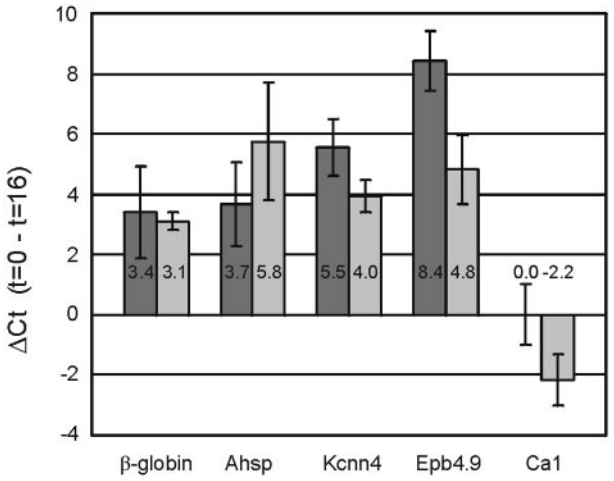

FIG. 3. Gene regulation by activating EKLF. EKLF null fetal liver cells carrying an EKLF-lbd transgene were cultured for 16 h. Gene expression was measured by real time quantitative PCR. The graph shows the difference in PCR cycles at which the PCR products reach the threshold level between T = 0 (fresh liver cells) and T = 16 (cultured cells). The medium of the cultured cells contained 4-OHT with (dark bars) or without (light bars) CHX. Expression of Hprt or Gapdh was used to normalize the data. Error bars indicate the standard deviation.

fresh fetal livers. Spots representing genes involved in heme synthesis gave relatively low ratios between wild-type and EKLF$^{-/-}$ samples, in agreement with our real-time PCR data. The erythroid cell-specific Alas2 gene was not present on the microarrays used. Many of the genes that appeared to be upregulated by EKLF can be classified as erythroid cell-specific and/or cell membrane specific (Table 1). Among the erythroid cell-specific genes is the known EKLF target gene βmaj globin (23, 26). As expected, high ratios were found for this gene. Other particularly high ratios were found for the alpha hemoglobin stabilizing protein (Ahsp), erythrocyte protein band 4.9 (Epb4.9), and the K^+ intermediate/small conductance Ca^{2+}-activated channel (Kcnn4 or Gardos channel).

Activation of target genes by a tamoxifen-inducible version of EKLF. To study the direct involvement of EKLF in the expression of these putative target genes, we made use of a tamoxifen (4-OHT)-inducible fusion protein consisting of EKLF and a modified estrogen receptor ligand-binding domain (EKLF-lbd) (6). In transgenic mice this construct is expressed under the control of the β-globin locus control region driving an α-globin promoter. To demonstrate EKLF-dependent expression of the target genes, we isolated EKLF$^{-/-}$ fetal liver cells carrying the EKLF-lbd transgene and cultured the cells for 16 h in differentiation medium containing 4-OHT. Furthermore, to exclude secondary pathways leading to gene expression we used the protein synthesis inhibitor cycloheximide (CHX). Gene expression of the cultured cells was studied by real time quantitative PCR. The differences in PCR cycles at which the PCR products reach the threshold level are shown in Fig. 3. The expression of βmaj, Ahsp, Kcnn4 and Epb4.9 are upregulated after 16 h of differentiation in the presence of 4-OHT and CHX (Fig. 3, dark bars). These genes are also upregulated in the presence of 4-OHT only (Fig. 3, light bars). Ca1 is one of the genes that might be downregulated by EKLF (Table 1, lower part). However, in these experiments Ca1 is downregulated only in the absence of CHX, indicating that EKLF is indirectly involved in its downregulation. Next, we focused our attention on two newly identified EKLF-dependent genes in the erythroid cell-specific- and membrane-specific categories, Ahsp and Epb4.9 respectively.

Alpha hemoglobin stabilizing protein. Ahsp (Eraf, Edrf) is an abundant erythroid cell-specific protein playing a role in the hemoglobin metabolism by forming a stable complex with free α-globin (14). An imbalanced production of α- and β-globin, as observed in thalassemia patients, can lead to precipitation of the free globins forming cytotoxic Heinz bodies (22). By binding free α-globin, Ahsp prevents α-globin denaturation and precipitation. Ahsp was represented 10 times on the erythroid cell-specific microarray chip, and the average ratio between wild-type and EKLF$^{-/-}$ expression at T = 0 was 2-fold, at T = 12 was 6-fold, and in fresh fetal livers was 12-fold. Thus, Ahsp expression is consistently downregulated in EKLF$^{-/-}$ erythroid cells.

The low expression of Ahsp in absence of EKLF was confirmed by Northern blot analysis (Fig. 4A). Ahsp mRNA is not detected in undifferentiated wild-type progenitors (T = 0) but increases to high levels during differentiation. However, in the absence of EKLF Ahsp is not detected at any time during differentiation. Similarly, Ahsp is not expressed in fresh EKLF$^{-/-}$ E12.5 fetal livers. No obvious difference in expression in EKLF$^{+/-}$ compared to wild-type fetal livers is found.

Since the expression of Ahsp appears to be strongly dependent on EKLF, we were interested in the expression of Ahsp in primitive erythroid cells in embryonic blood. Primitive cells express α-globin, and therefore expression of Ahsp can be expected. Notably, EKLF is also expressed in these cells, although to date no endogenous EKLF-dependent genes have been described in primitive cells. We performed Northern blot analysis for Ahsp on RNA isolated from wild-type, EKLF$^{+/-}$, and EKLF$^{-/-}$ primitive blood cells. Figure 4A (right panel) shows that Ahsp expression is readily detected in wild-type and EKLF$^{+/-}$ primitive blood cells. However, no expression of Ahsp is observed in the absence of EKLF.

In adult blood of Ahsp$^{-/-}$ mice, precipitates of α-globin are detected as Heinz bodies (14). Because of the absence of β-globin in EKLF$^{-/-}$ fetuses, the presence of Heinz bodies in erythrocytes would not provide useful information on the consequences of the absence of Ahsp. The excess of α-globin chains would result in the formation of globin chain precipitates regardless of the functional status of Ahsp. However, since EKLF has no effect on the expression of the embryonic globin genes, we examined whether precipitates could be found in EKLF$^{-/-}$ primitive blood cells. E12.5 yolk sacs (7 wild-type or EKLF$^{+/-}$ and 8 EKLF$^{-/-}$) were fixed and embedded in epon, and 1-μm sections were stained with toluidine/methylene blue. We observed blue spots, generally located in apparent close proximity to the cell membrane, only in the EKLF$^{-/-}$ yolk sacs (Fig. 4B). These blue spots likely represent inclusion bodies resulting from the precipitation of α-globin chains in the absence of Ahsp. Morphologically they appear similar to the Heinz bodies found in definitive erythrocytes of Ahsp-deficient mice (14). Collectively, our results demonstrate that EKLF positively regulates the expression of Ahsp in both definitive and primitive erythroid cells. Absence of Ahsp in

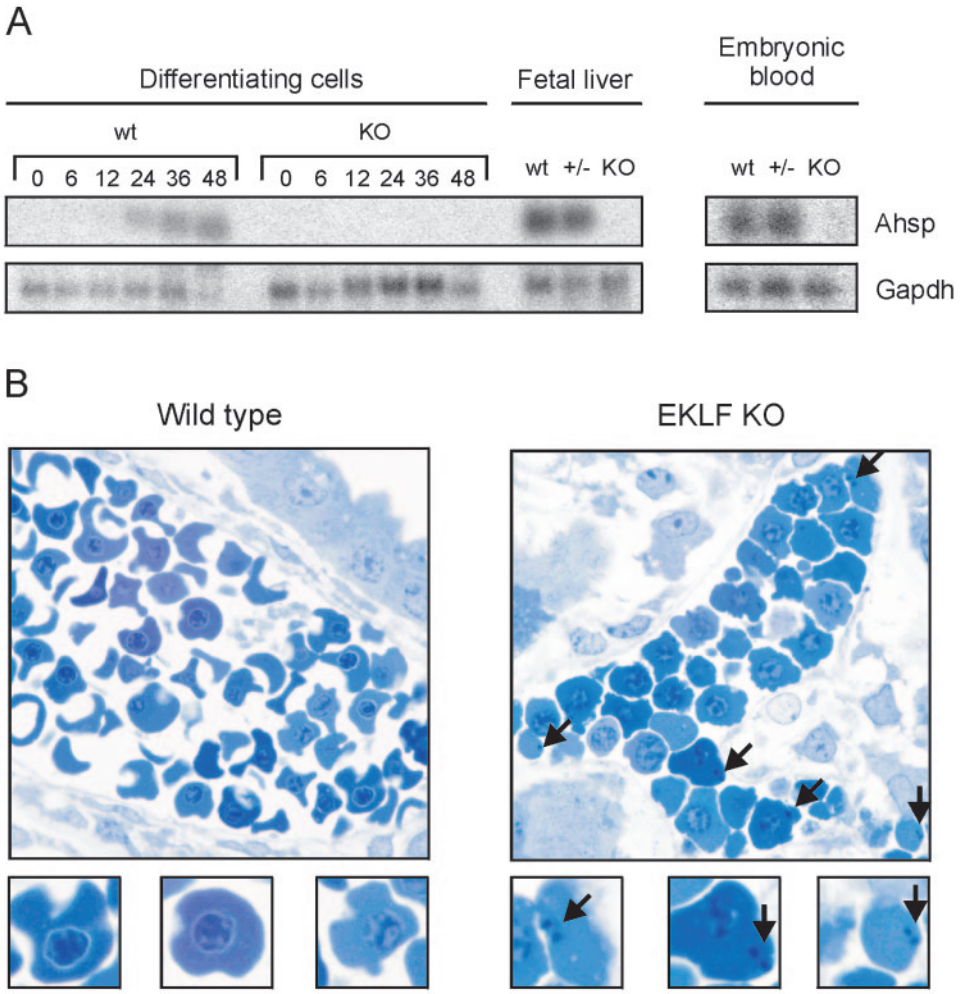

FIG. 4. Ahsp expression in EKLF null erythroid cells. (A) Northern blot showing accumulation of Ahsp in wild-type cells during differentiation but not in EKLF$^{-/-}$ cultures. Furthermore, Ahsp expression is EKLF-dependent in E12.5 fetal livers and in embryonic blood. Gapdh was used as a loading control. (B) Apparent inclusion bodies detected as blue spots (indicated by arrows) in EKLF$^{-/-}$ embryonic blood cells in toluidine/methylene blue-stained semithin sections of fixed E12.5 yolk sacs. These blue spots were not detected in wild-type embryonic blood cells. Original magnification, ×100. Other details are as for Fig. 2.

EKLF$^{-/-}$ primitive blood cells appears to result in the formation of α-globin precipitates. The enhanced formation of such precipitates in definitive blood cells will contribute significantly to the severity of the phenotype of the EKLF knockout.

Erythrocyte protein band 4.9. Epb4.9 is the most differentially expressed membrane-specific gene found in the gene expression profiling experiments, comparing wild-type and EKLF$^{-/-}$ fetal livers (Table 1). These data were confirmed by Western blot with whole cell protein extract from E12.5 fetal livers. Epb4.9 expression is not detectable in EKLF null fetal livers (Fig. 5A). Interestingly, Epb4.9 expression in primitive erythrocytes is also dependent on the presence of EKLF (Fig. 5A). Epb4.9, or dematin, is an actin-binding and bundling protein of the erythrocyte membrane skeleton. It is required for the maintenance of the shape and mechanical properties of the erythrocyte membrane (13). Mice expressing a truncated form of the protein (dematin headpiece null mice) develop compensated anemia with spherocytosis (13), indicating that erythroid cells require this protein. We analyzed the morphology of EKLF$^{-/-}$ primitive cells on cytospins of blood isolated from E12.5 embryos (Fig. 5B). The wild-type cells appear smooth, and in general, the cells are round shaped (arrowheads). In contrast, the EKLF$^{-/-}$ cells have a wrinkled appearance and the cells are deformed (arrows). This suggests that the cell membrane is less stable and malleable, very similar to the morphological abnormalities observed in definitive erythrocytes of Epb4.9 mutant mice (13). We conclude that the expression of the Epb4.9 gene in erythroid cells is strictly dependent on the presence of EKLF. Since lack of Epb4.9 expression adversely affects the functioning of the erythroid cell membrane, this would contribute to the severity of the EKLF knockout phenotype.

DISCUSSION

EKLF is required late in the erythroid differentiation program. We have demonstrated that EKLF is despensible for expansion of erythroid progenitors but required for terminal

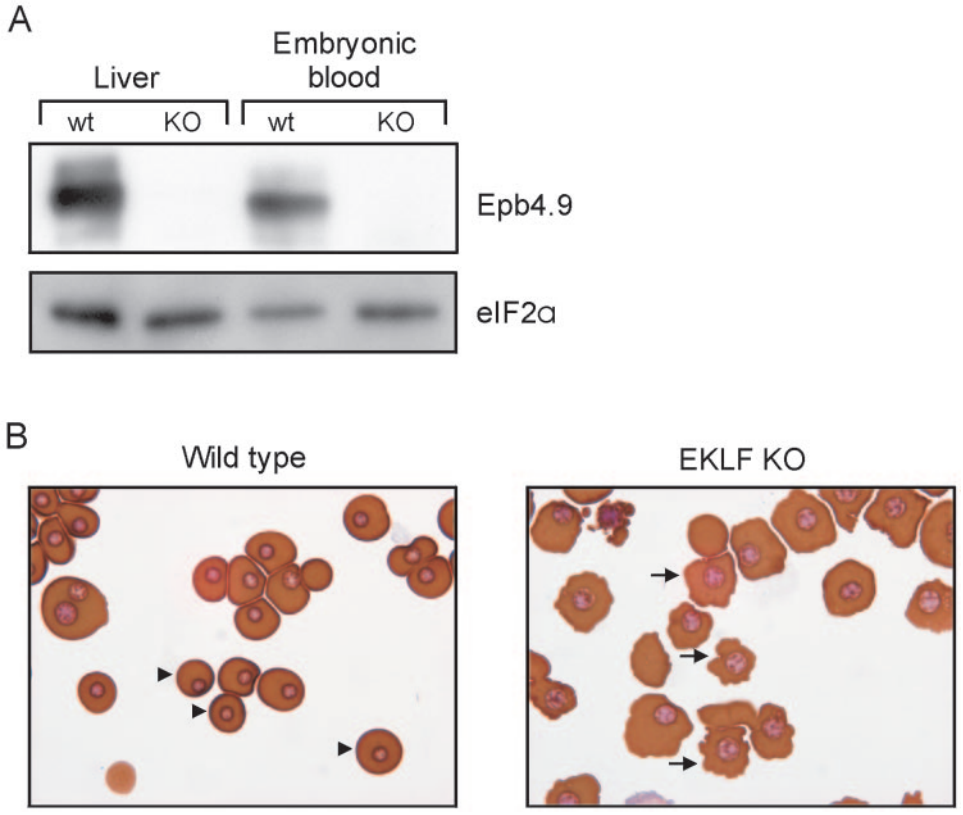

FIG. 5. Epb4.9 expression in EKLF null erythroid cells. (A) Western blot of whole cell protein extracts. Expression of Epb4.9 is detected in E12.5 fetal livers and in embryonic blood of wild-type embryos but not in EKLF$^{-/-}$ embryos. Antibody against translation elongation factor eIF2α was used as a loading control. (B) Cytospins of primitive blood cells, stained with neutral benzidine and histological dyes, showing morphological abnormalities of EKLF KO cells. Membranes of wild-type blood cells appear smooth, and the cells are round shaped (arrowheads). The membranes of EKLF KO blood cells are wrinkled, and the cells are deformed (arrows). Other details are as for Fig. 2.

differentiation. This is in agreement with the observation that EKLF$^{-/-}$ ES cells contribute to erythroid progenitors but not to the mature erythrocyte compartment in chimeric mice (18). It was postulated that this phenotype was due to the lack of β-globin expression. However, expression of γ-globin in these cells only partially rescued the phenotype, suggesting that EKLF regulates more genes that are important for erythroid differentiation. Here, we describe a number of novel EKLF-dependent genes that have important functions in the physiology of erythroid cells. In definitive erythroid cells these genes display an expression pattern similar to that of β-globin, since they become highly expressed late in erythroid differentiation. Thus, EKLF is an activator of essential erythroid genes that are upregulated during terminal erythroid differentiation.

Heme synthesis enzymes. In the absence of EKLF, expression of heme synthesis genes is reduced but not abrogated. Apparently, expression of these genes is not completely dependent on the presence of EKLF. This is not surprising since the enzymes of the heme synthesis pathway are ubiquitously expressed. Heme is an important molecule in many metabolic processes. Cell growth and in particular mRNA translation are controlled by the availability of nutrients and mitogenic factors, and also by the availability of heme. Heme-regulated eIF2α kinase (HRI) phosphorylates and inactivates eukaryotic initiation factor 2α (eIF2α) in the absence of heme (3), thereby coordinating heme and globin synthesis (11). In E12.5 EKLF$^{-/-}$ fetal livers we did not observe an elevated phosphorylation of eIF2α compared to wild-type fetal liver (data not shown), indicating that heme synthesis is still sufficient for the basic metabolic requirements of erythroblasts. Since a network of control mechanisms ensures the coordinate expression of the components required for the synthesis of hemoglobin, reduced expression of the rate-limiting enzyme Alas2 might be part of the adaptive response to the strongly diminished expression of β-globin.

EKLF is functional in primitive blood cells. In addition to the heme synthesis genes, we have focused on two genes, Ahsp and Epb4.9, that are strictly dependent on EKLF for their expression in definitive erythroid cells. Interestingly, their expression is also EKLF dependent in primitive cells. This strongly suggests that EKLF functions as a positive regulator in both definitive and primitive cells. Previously, no abnormalities have been described for EKLF null embryonic cells. However, the absence of Ahsp and Epb4.9 is likely to contribute significantly to the observed phenotypic changes in primitive EKLF-deficient cells, i.e., the presence of protein precipitates reminiscent of Heinz bodies and apparent membrane instability. Mice lacking Ahsp are viable, but Heinz bodies are present in definitive erythrocytes caused by precipitated α-globin chains (14). In the absence of Ahsp, α-globin precipitates inflict membrane damage on the erythrocytes leading to an increased turnover of the cells (14).

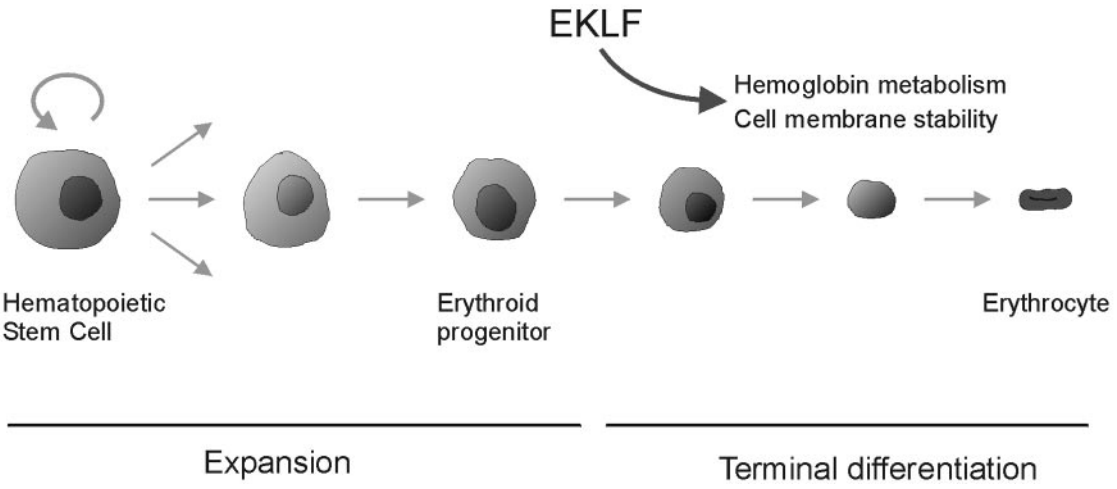

FIG. 6. EKLF regulates genes necessary late in the erythroid differentiation program. The hematopoietic stem cell gives rise to all blood cell types, including the erythroid lineage. In the erythroid differentiation program, EKLF is dispensable for the expansion of erythroid progenitors. To complete the differentiation program, EKLF is required for the proper expression of genes involved in cell membrane stability (e.g., Epb4.9) and hemoglobin metabolism (e.g., β-globin, heme synthesis enzymes, and Ahsp), in order to enable appropriate terminal differentiation to erythrocytes.

Epb4.9 plays an important role in the stability of the erythroid membrane skeleton. Erythrocytes of Epb4.9 mutant mice have a more fragile membrane skeleton and reduced deformability, caused by reduced association of spectrin and actin to the plasma membrane (13). Embryonic blood cells lacking EKLF and therefore lacking Epb4.9 expression, appear to have similarly weakened cell membranes. Furthermore, this phenotype can be aggravated by the lack of Ahsp. Ahsp and Epb4.9 mutant mice are viable, but the of lack of expression of both genes, as is the case in the absence of EKLF, is likely to further reduce the half-life of erythrocytes, consistent with observations on EKLF$^{-/-}$ adult erythrocytes (18). Notably, EKLF$^{-/-}$ primitive blood cells appear to function sufficiently, since EKLF$^{-/-}$ embryos die only at the time when they become dependent on definitive blood cells. This suggests that the large, nucleated primitive cells are relatively flexible in their ability to cope with disturbed functionality, compared with the highly specialized enucleated definitive cells. This, in addition to the fact that they last for only 4 or 5 days, may explain why the phenotype of EKLF$^{-/-}$ primitive cells is relatively subtle, allowing them to carry out their essential tasks during embryogenesis.

Additional EKLF-dependent genes. Our studies focused on EKLF-dependent genes that are particularly informative on the role of EKLF in primitive and definitive erythropoiesis. However, the gene expression profiling analysis revealed a number of additional differentially expressed genes (Table 1). EKLF$^{-/-}$ cells show reduced expression of the transferrin receptor and peroxiredoxin 2 (Prdx2). Reduced expression of the transferrin receptor, a protein that transports iron into the cells, is in line with impaired heme and globin synthesis. Prdx2 is an important enzyme in the protection against oxidative stress. Notably, erythroid cells deficient in Prdx2 age prematurely. As a result, Prdx2-deficient mice suffer from hemolytic anemia (17).

The Gardos channel, encoded by *Kcnn4*, has been related to cell size reduction in erythroid differentiation (10). Lack of upregulation of Kcnn4 may be an important cause for the lack of cell size reduction in differentiating EKLF$^{-/-}$ progenitors observed in the culture system (Fig. 1).

Some of the differentially expressed genes are expressed at increased levels in EKLF$^{-/-}$ erythroblasts such as carbonic anhydrase 1 (Car1) (Table 1). The ubiquitously expressed Car1 gene is downregulated during erythroid differentiation, when it is replaced by the erythroid cell-specific isoform Car2. This suggests that EKLF may function in transcriptional repression. Indeed, it has recently been reported that EKLF may function as a developmental stage-specific repressor through interaction with Sin3A and HDAC1 (4). This is interesting, because by default genes are shut down during terminal erythroid differentiation owing to progressing general heterochromatization of the genome. However, the observation that downregulation of Car1 does not occur when de novo protein synthesis is inhibited by CHX (Fig. 3) strongly indicates that this is not a direct effect of EKLF. Therefore, it appears that the general shut down of transcription is counteracted by EKLF at loci that are of particular importance for erythroid maturation.

Synopsis. Our data show that EKLF coordinates the expression of a panel of proteins involved in differentiation of erythroid progenitors, and the maturation of erythrocytes (Fig. 6). This is reminiscent of other members of the KLF family of transcription factors that also play an important role in terminal differentiation of specific cell types. Klf4 is required for the terminal differentiation of epithelial cells in the skin and gut (12, 31), and Klf5 is a key regulator of adipocyte differentiation (24). Our results explain why expression of exogenous γ-globin only partially restores the observed shortened life span of EKLF$^{-/-}$ adult erythrocytes and fails to rescue definitive erythropoiesis in EKLF$^{-/-}$ mice (18, 25). These experiments show that EKLF$^{-/-}$ erythroid cells still have major defects even if the ratio of α- and β-like globin chains has been corrected. Our data suggest that mutations affecting the function of the EKLF transcription factor would have a major impact on the severity of disease in thalassemia and sickle cell anemia

patients. It is interesting to note that β-thalassemia resulting from dysfunction of the EKLF gene has not been found in the human population yet (37). We suggest that a complete abrogation of EKLF function would not be compatible with postembryonic life, because fetal liver erythropoiesis would be dysfunctional, even though γ-globin expression levels should be normal and the severe β-thalassemia observed in EKLF$^{-/-}$ mice should not occur during human fetal development.

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