Generation of Mice Deficient in both KLF3/BKLF and KLF8 Reveals a Genetic Interaction and a Role for These Factors in Embryonic Globin Gene Silencing


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Krüppel-like factors 3 and 8 (KLF3 and KLF8) are highly related transcriptional regulators that bind to similar sequences of DNA. We have previously shown that in erythroid cells there is a regulatory hierarchy within the KLF family, whereby KLF1 drives the expression of both the KLF3 and KLF8 genes and KLF3 in turn represses KLF8 expression. While the erythroid roles of KLF1 and KLF3 have been explored, the contribution of KLF8 to this regulatory network has been unknown. To investigate this, we have generated a mouse model with disrupted KLF8 expression. While these mice are viable, albeit with a reduced life span, mice lacking both KLF3 and KLF8 die at around embryonic day 14.5 (E14.5), indicative of a genetic interaction between these two factors. In the fetal liver, KLF3 KLF8 double mutant embryos exhibit greater dysregulation of gene expression than either of the two single mutants. In particular, we observe derepression of embryonic, but not adult, globin expression. Taken together, these results suggest that KLF3 and KLF8 have overlapping roles in vivo and participate in the silencing of embryonic globin expression during development.

Krüppel-like factors (KLFs) are DNA-binding transcriptional regulators that are involved in a wide range of biological processes (1, 2). The defining feature of KLFs is the presence of a conserved, tandem repeat of three Cys2-His2-type zinc fingers at their C termini, through which KLFs make sequence-specific contacts with GC-rich and CACCC-related elements of DNA (3–6). While as a whole this domain is highly conserved within the family, specific amino acid differences between the various KLFs result in different DNA-binding preferences (7). As such, individual KLFs have been shown to regulate overlapping, but also distinct, sets of target genes (8, 9). In addition, particular KLFs group together in their DNA-binding specificities. For instance, KLF1 and KLF3 recognize similar sequences and hence regulate overlapping sets of target genes in erythroid cells (8, 10, 11), while KLF4 and KLF5 together coregulate other genes in stem cells and other tissues (9,–16).

Outside of the DNA-binding domain, KLFs exhibit relatively little conservation and interact with a range of transcriptional coregulators and histone-modifying enzymes (2, 17). Some KLFs, such as KLF1, primarily function as activators of transcription (18), while other KLFs, for example KLF3, have typically been characterized as transcriptional repressors (19). It is also evident that some KLFs can act as either activators or repressors, depending on biological context and the gene regulatory region through which they are operating. This is indeed the case for KLF8 (20–24) and has also been reported in some instances for KLF1 (25–28) and KLF3 (29, 30).

KLF1, formerly known as erythroid Krüppel-like factor (EKLF), is primarily expressed in erythroid cells (18) and is a master regulator of multiple facets of erythropoietic differentiation (6, 28, 31–37). It is predominantly a transcriptional activator and binds to CACCC box motifs of the general consensus 5′-CCM CRC CCN-3′ (3, 6). Such motifs are present in the regulatory regions of many diverse genes but in particular have long been noted to be essential for the expression of various erythroid genes, such as those coding for the globins (38). A notable example is the CACCC box that resides within the promoter of the adult β-major globin (Hbb-b1) gene (3, 6, 18).

Hemoglobin is an oxygen-conveying metalloprotein that is expressed in erythrocytes and is composed of two α-like and two β-like globin peptide chains. Throughout ontogeny, there are different forms of both the α-like and β-like globins that are expressed to meet various developmental oxygen demands. This is known as globin switching, and it accompanies shifting sites of erythropoiesis during development. For instance, the embryonic α-like globin (Hba-x) and β-like globins (Hbb-y and Hbb-bh1) are expressed in primitive erythroid cells that are transiently produced in the yolk sac from around embryonic day 7.5 (E7.5) (39, 40). In contrast, the adult globin genes (Hba-a1 and Hbb-b1) are expressed in definitive cells produced in the fetal liver from...
around E9.5 up until birth, at which point erythropoiesis shifts to the bone marrow (39). In humans, the β-globin locus is subject to a similar yet distinct mechanism of switching: embryonic ε-globin is produced by primitive erythroid cells in the yolk sac; fetal γ-globin is expressed by definitive cells in the liver until birth, and transcription of the adult β- and δ-globin genes commences perinatally and continues throughout life.

KLF1 plays a crucial role in the switching of β-like globins. It directly binds to the β-major globin promoter in vivo and is required for its transcriptional activity in definitive erythroid cells (6, 28, 32, 41, 42). Consequently, Klf1 null mice have depleted levels of β-major globin and die at around E15 of severe anemia (43, 44). Consistent with this, mutations within the orthologous KLF1-binding site in the human β-globin promoter are associated with β-thalassemia (45). In addition to being essential for the normal expression of adult β-globin, KLF1 also plays an indirect role in the silencing of fetal γ-globin in human erythroid cells. It achieves this by driving the expression of BCL11A, an established repressor of γ-globin (46–49). Lastly, in addition to its role in definitive erythroid cells, KLF1 has recently been shown to directly activate transcription of embryonic globins in primitive erythroid cells and accordingly, Hbb-y mRNA and Hbb-bh1 mRNA are reduced in Klf1−/− embryos (50–52).

In addition to globin genes, KLF1 drives the expression of many genes involved in erythropoietic pathways, such as heme biosynthesis, cell cycle control, and the establishment of membrane integrity (6, 28, 31–37). KLF1 also activates the transcription of two other family members in erythroid cells, Klf3 and Klf8 (10, 11). KLF3 (previously, basic Krüppel-like factor [BKLF]) is a potent transcriptional repressor that silences gene expression by recruiting the corepressor C-terminal binding protein (CtBP) (19, 53). KLF3 is expressed widely (54) but in particular is found at high levels in erythroid tissue owing to an erythroid cell-specific promoter that is directly activated by KLF1 (6, 10). KLF3 exhibits similar DNA-binding preferences to KLF1 in vitro (29). Consistent with this, KLF3 represses many genes that are activated by KLF1 in erythroid cells in vivo and is thought to fine-tune their expression during erythropoiesis (8). In the absence of KLF3, a set of genes is abnormally derepressed in mature erythroid cells. This is thought to explain the multiple erythroid defects of the Klf3 null mice, namely, reticulocytosis, increased nuclear inclusions (Howe et al., 2001), and is thought to fine-tune their expression in vivo (8). In the absence of KLF3, a set of genes is abnormally derepressed in mature erythroid cells. This is thought to explain the multiple erythroid defects of the Klf3 null mice, namely, reticulocytosis, increased nuclear inclusions (Howe et al., 2001), and is thought to fine-tune their expression in vivo (8).

One such gene that is activated by KLF1 and repressed by KLF3 is Klf8 (11). KLF8 is highly related to KLF3, with the two proteins sharing 96% sequence similarity in their zinc finger domain (54). They recognize similar sequences of DNA that broadly fit the KLF DNA-binding consensus 5'-NCN CNC CCGN-3' (3, 20, 29). Moreover, both proteins are able to silence gene expression by recruiting CtBP corepressors via a conserved Pro-X-Asp-Leu-Ser type motif (19, 20). In addition, KLF8 has been shown to activate transcription of some gene promoters (21–24). KLF8 is not expressed at readily detectable levels in most cell and tissue types studied to date (55), but numerous studies have reported its up-regulation in various human cancers, including prostate (56), gastric (57, 58), hepatocellular (59, 60), glioma (61), breast (62, 63), renal (64), and ovarian (65). KLF8 has been shown to regulate oncogenesis by promoting cellular proliferation and tumor invasion and by inhibiting apoptosis (21, 65–68).

Despite a multitude of studies that have investigated the dys-regulation of KLF8 expression in various cancers, little is known about its role in normal physiology. Here we report the generation of the first animal model with a gene trap (gt) insertion in the Klf8 locus and no detectable KLF8 protein. Mice with homozygous disruption of Klf8 (Klf8gt/gt) are viable but have a shortened life span. Crossing these mice with Klf3 null mice results in embryonic lethality, indicative of a genetic interaction between these two factors. This interaction is pronounced in erythroid tissue, in which we observe considerable derepression of KLF8 expression in the absence of KLF3. A cohort of genes is deregulated upon disruption of this network in fetal liver cells, and in particular, the embryonic globin genes are derepressed in the absence of both KLF3 and KLF8.

MATERIALS AND METHODS

Klf3−/− and Klf8gt/gt mice. Murine embryonic stem (ES) cells (clone name AD0101; Sanger Institute Gene Trap Resource) containing a β-geo gene trap cassette in intron 2 of Klf8 were injected into C57BL/6 blastocysts by standard methods in order to generate chimeric mice. Germ line transmission of the Klf8 gene trap was confirmed, and mice were backcrossed for at least 10 generations to the FVB/NJ strain. Klf3−/− mice have previously been described and were also maintained on a pure FVB/NJ strain (69). Ethical approval for the use of these mice was obtained from the Animal Care and Ethics Committees at the University of Sydney (approval no. L2/26-2006/3/4344 and L2/27-2009/3/5079) and the University of New South Wales (approval no. 09/128A).

Histological examination of mice. Necropsies were conducted on four wild-type and four litter-matched Klf8gt/gt male mice, ages 12 to 15 weeks (Australian Phenomics Network, The University of Melbourne, Melbourne, Australia, and IMVS, Adelaide, Australia). A wide range of organs were sectioned and subjected to standard histological examination, including heart, liver, spleen, pancreas, kidney, brain, thyroid, thymus, lungs, thymus, skin, testis, epididymis, seminal vesicles, prostate gland, penis, preputial gland, bladder, gallbladder, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph node, adrenal gland, tail, eyes, Harderian glands, spinal cord, salivary glands, and regional lymph nodes. Paraffin embedding of five Klf3−/− Klf8gt/gt and three Klf3+/− Klf8gt/gt E13.5 embryos and subsequent staining with hematoxylin and eosin were performed by the Histology and Microscopy Unit, University of New South Wales.

Real-time qRT-PCR. Total RNA from murine tissues was extracted, DNase treated, and analyzed by real-time quantitative reverse transcription (qRT)-PCR as described previously (8, 10, 70). Adult tissues were from 3- to 4-month-old, sex-matched littermates. The sequences of primers used are as follows and as listed previously (10): Klf8, 5'-TGAGATGTC CGAATTAATYACGAAA-3' and 5'-GAAGAATCTTGCTGGCAACA G-3' or 5'-CCAAAGCTTCTCGATGCAAAGC-3' and 5'-AGCGAGCAA ATTTCAGGAAA-3'; ε-globin (Hbb-ε), 5'-GGCCCTGATGAGTAAAGGT CAA-3' and 5'-GCAAGAGGACAGTTCACCGAA-3'; β1-globin (Hbb-bh1), 5'-CTCAAGGAAGACCTGCTGCA-3' and 5'-GGAGAGGACAGGCAA TTCCAGGAAA-3'; γ-globin (Hbb-γ), 5'-GGGTTGTGAAGTAAAGGT CAA-3' and 5'-CCAAAATGCTCTCACCTGAAAGC-3'; β-major globin (Hbb-bh1), 5'-CAGCTGTGCAACCTGCTGCA-3' and 5'-CGAATGCTAAGGCGAGG AA-3'; β-2-globin promoter (Hbb-β2), 5'-AGGTCACGGCAAGAAGGTT TCA-3' and 5'-ATGCGGTTAAGAGCATCGAC-3'; ε-globin (Hba-ε), 5'-ATGCGGTTAAGAGCATCGAC-3' and 5'-GGGTTGTGAAGTAAAGGT CAA-3' and 5'-CCAAAATGCTCTCACCTGAAAGC-3'; β-2-globin promoter (Hbb-β2), 5'-CAGCTGTGCAACCTGCTGCA-3' and 5'-CGAATGCTAAGGCGAGG AA-3'; γ-globin (Hbb-γ), 5'-GTCAGCCGAAGAAGGTTTCA-3' and 5'-GGGTTGTGAAGTAAAGGT CAA-3'.

Protein overexpression in COS cells. COS cells were cultured and transfected as previously described (10) using 1 to 2 μg pMT3-mKlf8 (11), pMT2-Klf3 (29), or empty pMT3 vector.

Western blotting. Western blotting of nuclear extracts and the anti-KLF3/BKLF and KLF8 antibodies was performed according to Western blotting protocol described in the Materials and Methods section.
TBirds for 1 to 1.5 h. Secondary labeling was achieved using 1:15,000 horse-radish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Pharmacia Biotech) in TBST for 1 h at room temperature. Signals were visualized using Immobilon Western chemiluminescent HRP substrate (Millipore). Blots were stripped in a mixture of 62.5 mM Tris (pH 6.8), 2% SDS, and 0.7% β-mercaptoethanol at 70°C for 30 min and were subsequently washed six times with TBST over 30 min. Blots were then re-blocked and probed using anti-β-actin (1:10,000) (Sigma) and horseradish peroxidase-linked anti-mouse antibody (1:15,000) (Amersham Pharmacia Biotech) in TBST, each for 1 h at room temperature.

**Determination of the precise genomic location of the Klf8 gene trap.** Confirmation that the gene trap lies within intron 2 of Klf8 was achieved by RT-PCR analysis of AD0101 ES cells using a forward primer specific for Klf8 exon 2 (5′-TGGATGTCGAATTTAATCGGAAAGATCG-3′) and reverse primers specific for the β-gene gene trap (5′-AGTACCGCCTCAAGGAGTACG-3′ and 5′-ATTCCAGCCTGGCACAACGTGTGGG-3′). The precise site of integration was determined by conducting genomic PCR using these reverse primers together with 23 forward primers evenly spaced across the 11.1 kb of intron 2. A single forward primer (5′-GACCTGATTTGACACGGG-3′) yielded a strong PCR band with both of the reverse primers. Sequencing of the PCR products revealed that the site of integration lies 434 bp upstream of exon 3.

**Genotyping.** Genomic DNA was extracted from tail snips or embryonic tissue using DirectPCR lysis buffer (Viagen Biotech) as per the manufacturer’s instructions. Klf8 gene trap mice were genotyped by multiplex PCR using the primer pairs 5′-GACAAGCTTGATTTGACACGGG-3′ and 5′-GACTTTGTCAGTTACGACG-3′, which flank the site of gene trap insertion and generate a 210-bp product, indicative of an intact, wild-type allele, and 5′-CATGATCGACACGGGACTGAGCTTGGG-3′ and 5′-ATTCCAGCCTGGCACAACGTGTGGG-3′, which recognize the gene trap itself and produce a 348-bp product. PCRs were conducted using REDTag DNA polymerase (Roche Molecular Biochemicals) as recommended by the supplier and in the presence of 10% dimethyl sulf oxide (DMSO). The following PCR parameters were used: 1 cycle of 94°C for 2 min, 31 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and 1 cycle of 72°C for 5 min. Klf3 knockout line mice were genotyped as previously described (69). Klf8 Klf8 double mutant mice were genotyped by multiplex PCR using the Klf8 primers listed above together with primers specific for wild-type Klf3 (5′-CATCCCTGCGTATGGCAG-3′ and 5′-TTTCAGGAGGACCTTTGAG-3′). For the Klf8 Neo cassette (5′-CTTGATGTCATGGCAGCTCTG-3′ and 5′-ATTACGGGCTCCATGTTCC-3′) and reverse primers specific for wild-type Klf3 (5′-CATCCCTGCGTATGGCAG-3′ and 5′-TTTCAGGAGGACCTTTGAG-3′), microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo) under accession no. GSE45324.

**RESULTS**

**Generation of a Klf8 gene trap mutant mouse model.** While a KLF3 knockout mouse line has been previously characterized and shown to have mild erythropoietic, adipogenic, and B-cell defects (8, 69, 75, 76), a mutant mouse model for KLF8 has not been described. Therefore, to investigate the biological role of KLF8, we generated mice from ES cells (Sanger Institute Gene Trap Resource) containing a gene trap (gt) cassette within intron 2 of the Klf8 gene (Fig. 1A and B). Sequencing of genomic PCRs using a series of primers spread across Klf8 intron 2 revealed that the precise site of gene trap integration is 434 bp upstream of exon 3 (Fig. 1C) (data not shown). The gene trap contains a potent splice acceptor site to disrupt expression of KLF8 and instead generate a β-gene fusion protein. Such splicing was confirmed by RT-PCR analysis using a forward primer specific for Klf8 exon 2 and reverse primers specific for the gene trap (data not shown).

While KLF8 protein is normally expressed at very low levels in noncancerous tissues (55), we have been able to detect endogenous KLF8 in wild-type fetal brain and placental tissue (Fig. 1D and E). Mice that are homozygous for the gene trap have no detectable KLF8 protein in these tissues (Fig. 1D and E). Similarly, qRT-PCR analysis of a range of erythroid tissues (E11.5 yolk sac, E13.5, E14.5, and E16.5 fetal livers) using mass spectrometry (MS) columns and anti-Ter119 Microbeads (Miltenyi Biotech) as per the supplier’s protocol. Following elution, purified cells were centrifuged for 10 min at 300 g (4°C), the supernatant was removed, and pellets were harvested for RNA as described above.

**Cytopsins of peripheral blood and fetal livers.** Peripheral blood and liver was taken from individual E14.5 embryos by dissection in 1 ml filtered fluorescence-activated cell sorter (FACS) buffer (10 mM EDTA, 0.5% fetal calf serum, 0.05% NaN3 in phosphate-buffered saline [PBS]), and cells were counted using a Countess automated cell counter (Life Technologies). Cells were diluted to 4 × 10^5/ml in FACS buffer, and 100 to 150 μl was centrifuged for 5 min at 300 rpm in Shandon Cytofunsels (Thermo-scientific). Slides were subsequently stained using Diff-Quik (Lab Aids, Pty., Ltd.), and the cells were counted.

**ChIP analysis of MEL cells.** Murine erythroleukemia (MEL) cells were cultured and induced to differentiate for 72 h in 1.8% DMSO as described previously (70). Chromatin immunoprecipitation (ChIP) assays were conducted in triplicate as described previously (72, 73), using rabbit polyclonal anti-KLF3 serum (29) or Pierce anti-KLF3 antibody (PAS-18030) and preimmune serum or IgG (Santa Cruz sc-2028) as negative controls. The primer sequences are found elsewhere (11, 50, 74) and as follows: Hba-a1 promoter, 5′-GTTTGGGAGATTCTCTCCTGA-3′ and 5′-GCCCGGAGACACTTCTTACC-3′; Hba-x promoter, 5′-AGGCCATGGGACTGAGCT-3′ and 5′-CATTCGCTTGGGCACTCTTG-3′; and Hbb-y promoter, 5′-CAGGCGGCTTCCACCCATGAG-3′ and 5′-CTGGCTCGTAAGGTGTGGCCTT-3′.

**Microarrays.** Total RNA was extracted from Ter119 fetal liver cells purified from four wild-type, four Klf8^gt/gt, three Klf3^−/−, and four Klf3^−/− Klf8^gt/gt embryos (E13.5), litter matched where possible. RNA was prepared and analyzed by Affymetrix GeneChIP 1.0 ST arrays as previously described (8). Genes showing a greater than 2-fold change in expression and passing a false discovery rate (FDR) threshold of 0.3 to correct for multiple testing were considered to be significantly differentially expressed.

**Microarray data accession numbers.** Microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo) under accession no. GSE45324.
to wild-type males and females, who exhibited only 5% and 4% lethality, respectively, over the same period (Fig. 1f). Heterozygous females exhibited a partially penetrant phenotype, with 16% of animals dying within 6 months (\( P < 0.05 \) compared to wild-type females). This might be explained by decreased KLF8 expression in these animals, the extent of which may vary between individuals due to nonrandom lyonization (X inactivation).

Necropsies and extensive histological examination of \( Klf8^{gt/gt} \) mice have not yet revealed any phenotypic abnormalities that might explain the shortened life span of these animals (see Materials and Methods). Similarly, full blood counts indicated that erythroid parameters and platelet numbers are normal in \( Klf8^{gt/gt} \) mice (see Fig. S1 in the supplemental material). Moreover, phenylhydrazine treatment did not reveal any significant differences in blood count parameters between \( Klf8^{gt/gt} \) and wild-type mice (data not shown), suggesting that stress erythropoiesis is not appreciably modulated by KLF8.

Mice lacking both KLF3 and KLF8 are embryonic lethal. KLF3 and KLF8 are highly related proteins that recognize similar sequences of DNA, repress transcription via CtBP corepressors, and are directly upregulated by KLF1 in erythroid cells (11, 19, 20, 29). Given these similarities, we reasoned that they may have overlapping physiological roles in vivo and may be able to compensate functionally for each other’s absence. This hypothesis is made all the more salient by our previous observation that KLF8 expression is elevated in \( Klf3^{null} \) mice (8, 11).

Therefore, to explore the possibility of functional redundancy between these two factors and to elucidate their biological roles, we crossed the \( Klf3^{null} \) mouse line with the newly generated \( Klf8^{gt/gt} \) line. We have found that \( Klf3^{null} \) mice are essentially infertile, but using a series of different parental crosses involving \( Klf3^{null} \) heterozygous animals, we analyzed the frequency of the nine possible resulting genotypes (see Fig. S2 in the supplemental material) at weaning age (Table 1). The observed numbers...
do not adhere to Mendelian expectation, with no mice observed that were deficient in both KLF3 and KLF8 (Klf3\(^{-/-}\) Klf8\(^{0/0}\)) and an underrepresentation of female mice containing only a single allele of Klf8, that is, Klf3\(^{-/-}\) Klf8\(^{+/+}\) (observed at 10% of the expected number). Klf3 knockout mice (Klf3\(^{-/-}\) Klf8\(^{+/+}\)) were also observed at lower than expected numbers (43% of expected), in line with a reduced viability of Klf3-deficient animals that we have previously reported (69). Investigations in utero revealed that Klf3\(^{-/-}\) Klf8\(^{+/+}\) embryos are viable and found at expected Mendelian numbers at E12.5 (Table 2) but start to die thereafter, with our previous observation that the master erythroid regulator

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KLF1 directly activates
downstream gene expression than Klf3\(^{-/-}\) cells, with 112 genes being upregulated and 74 downregulated compared to the wild type (Fig. 3C and Fig. 4). Strikingly, almost all of the 64 genes that were derepressed in Klf3\(^{-/-}\) cells were also significantly derepressed in Klf3\(^{-/-}\) Klf8\(^{0/0}\) embryos and was subjected to Affymetrix microarray analysis. In addition, we confirmed that the Klf8 gene trap ablated KLF8 protein expression in Klf3\(^{-/-}\) Klf8\(^{0/0}\) compared to Klf3\(^{-/-}\) fetal liver (Fig. 2C).

Comparing Klf8\(^{0/0}\) embryos with wild-type embryos, there were very few genes that were significantly deregulated (>2-fold; FDR < 0.3), and only one of these encoded an annotated mRNA transcript (H2-Q6; Histo compatibility 2, Q region locus 6) (Fig. 3A). The lack of any considerable gene deregulation in Klf8\(^{0/0}\) embryos is not unexpected given that KLF8 protein is not readily detectable in wild-type fetal liver (Fig. 2B and C).

Next we compared Klf3\(^{-/-}\) samples with wild-type samples and observed considerable derepression of gene expression (Fig. 3B; see Fig. S4 and Table S1 in the supplemental material). In total, 64 genes were significantly derepressed, while only 4 genes were downregulated in the absence of KLF3, consistent with the notion that KLF3 is primarily a transcriptional repressor in erythroid cells. These results are highly concordant with our previous microarrays performed with E14.5 fetal liver cells, with 57 of these 68 genes being significantly deregulated in both studies (see Table S1). As we had previously observed, Klf8 was one of the most highly derepressed genes in these cells (10.2-fold) (Fig. 3B; see Table S1).

Klf3\(^{-/-}\) Klf8\(^{0/0}\) cells exhibited even greater deregulation of gene expression than Klf3\(^{-/-}\) cells, with 112 genes being upregulated and 74 downregulated compared to the wild type (Fig. 3C and Fig. 4). Strikingly, almost all of the 64 genes that were derepressed in Klf3\(^{-/-}\) cells were also significantly derepressed in Klf3\(^{-/-}\) Klf8\(^{0/0}\) tissue (see Fig. S4 and Table S1 in the supplemental material). Only four of the derepressed genes did not meet these criteria (Frrs1, Cmpk2, Acot9, and Treml2) but nonetheless displayed upregulation in Klf3\(^{-/-}\) Klf8\(^{0/0}\) cells (between 1.8- and 1.9-fold), albeit not significantly.

The genes that are derepressed in the absence of KLF3 are, by and large, not further derepressed upon ablation of KLF8 (Fig. 4; see Fig. S4 and Table S1 in the supplemental material). This suggests that these genes are largely regulated by KLF3 and that KLF8 may play little or no role in their regulation in the absence of KLF3. Nonetheless, Klf3\(^{-/-}\) Klf8\(^{0/0}\) cells did indeed display a greater derepression.
deregulation of gene expression than Klf3−/− cells (Fig. 3C and Fig. 4). These additional genes displayed both upregulation and downregulation consistent with the dual role of KLF3 as both an activator and a repressor of transcription. These genes represent potential target genes of KLF8, which KLF8 may redundantly regulate with KLF3 or which may be uniquely regulated by KLF8.

To identify likely KLF8 candidate target genes, we compared expression profiles between Klf3−/−/Klf8−/− and Klf3−/− cells and compiled a list of 30 significantly differentially expressed genes, 17 of which were upregulated and 13 of which were downregulated (Fig. 3D; see Fig. S5 in the supplemental material). Notably, several of these genes, including Itgb7 (78), Vegfa (79, 80), Periostin (81), Parml (82, 83), Gbp1 (84, 85), Tcf15 (86, 87), Rps3a (88), and Fpr1 (89, 90), are regulators of cell adhesion, migration, and invasiveness, consistent with the role of KLF8 in oncogenesis. In addition, the Klf8 gene itself is significantly downregulated in Klf3−/− Klf8−/− compared to Klf3−/− cells, as anticipated (Fig. 3D; see Fig. S5).

Embryonic globin genes are derepressed in the absence of KLF3 and KLF8. The most highly upregulated gene within this list was that encoding Hbb-bh1 (3.8-fold), an embryonic β-globin (Fig. 3D). This was of interest given that KLF1 plays a crucial role in the activation of adult β-globin and the silencing of embryonic and fetal globins in definitive erythroid cells. This raised the possibility of an elegant system whereby KLF1 drives adult β-globin and also upregulates KLF3 and KLF8, which then function to repress the expression of embryonic globins. Indeed, inspection of globin gene expression in Klf3−/− Klf8−/− cells compared to Klf3−/− cells revealed that the other murine embryonic genes, Hba-x and Hbb-y, are elevated 2.8-fold and 1.5-fold, respectively (Fig. 3D), while the adult globin genes, Hbb-b1 and Hba-a1, are unaltered.

We next sought to validate the derepression of embryonic globins by qRT-PCR analysis of Ter119+ E13.5 fetal liver cell samples independently purified from those used in the arrays. We consistently observed significant upregulation of all three embryonic globin genes in Klf3−/− cells compared to wild-type cells (4.07-, 2.66-, and 1.62-fold, respectively, for Hba-x, Hbb-y, and Hbb-bh1) (Fig. 5A to C). We also tested the hypothesis that these embryonic globin genes are further derepressed in Klf3−/− Klf8−/− cells and found this to be the case (1.60-, 1.68-, and 1.67-fold, respectively, compared to Klf3−/−; P = 0.04, P = 0.07, and P = 0.03, one-tailed t tests) (Fig. 5A to C). Adult globin expression was unaffected in both Klf3−/− and Klf3−/− Klf8−/− cells compared to wild-type cells (Fig. 5D and E).

The embryonic lethality of Klf3−/− Klf8−/− mice precluded analysis of later developmental stages; however, examination of Klf3−/− cells revealed elevated expression of embryonic but not adult globins at both E14.5 (see Fig. S6 in the supplemental material) and E16.5 (see Fig. S7 in the supplemental material). Cytosins of peripheral blood and fetal liver tissue at E14.5 revealed no difference in the numbers of primitive erythrocytes between wild-type and Klf3−/− animals that might explain the elevated expression of embryonic globins (see Fig. S8 in the supplemental material). Furthermore, we observed no deregulation of either embryonic or adult globin expression in Klf3−/− E10.5 yolk sac, a source of primitive erythroid cells (see Fig. S9A to E in the supplemental material). Similarly, both embryonic and adult globin transcript levels were comparable in Klf3−/− Klf8−/− and litter-matched Klf3−/− Klf8−/− yolk sacs (see Fig. S9F to J). Taken together, these results suggest that KLF3 in particular, represses the expression of the embryonic globin genes in definitive erythroid cells and that KLF8 partially compensates in its absence.
Other KLFs, namely, KLF1 and KLF2, have previously been shown to directly bind to the promoters of the embryonic globin genes in erythroid cells (50, 52). To explore the possibility that KLF3 represses embryonic globin expression by a similar mechanism, we conducted chromatin immunoprecipitation (ChIP) studies using differentiated MEL cells. These erythroid cells routinely yield robust immunoprecipitation using antibodies for KLF3, as evidenced by significant enrichment at a positive-control region (Klf8 promoter 1a) (8, 11) (Fig. 6). We observed no enrichment at the promoters of the embryonic globin genes (Hba-x and Hbb-y), but unexpectedly, we found significant KLF3 occupancy at the promoters of the adult globin genes, particularly at Hba-a1 and to a lesser extent at Hbb-b1. We also observed significant binding at upstream DNase-hypersensitive sites (HS26 and HS2) known to play a role in the regulation of the α- and β-globin loci, respectively. These data raise the possibility that KLF3 influences embryonic globin gene expression not by direct binding to their promoters but through distal sites within the globin loci.

DISCUSSION

Deregulation of KLF8 has been observed in a wide variety of cancers; however, the normal physiological roles of this transcription factor have remained largely unknown. To this end, we have generated a mouse line with disrupted KLF8 expression. Klf8<sup>gt/gt</sup> mice are viable and fertile but have a shortened life span, the cause of which could not be identified despite extensive histological characterization. Mice that are deficient in the related family member KLF3 are also viable, yet Klf3<sup>−/−</sup> Klf8<sup>gt/gt</sup> double mutant animals die at around E14.5. This suggests that KLF3 and KLF8 have overlapping roles in vivo and can at least partially compensate in each other’s absence.

Analysis of Klf3<sup>−/−</sup> mice revealed marked upregulation of KLF8 expression in several tissues, but particularly erythroid tissue. Given that both the Klf3 and Klf8 genes are activated by the erythroid factor KLF1, we hypothesized that these three factors operate in a regulatory network to control gene expression. Microarray analysis of Ter119<sup>+</sup> fetal liver cells from single mutant
and double mutant (Klf3−/− Klf8−/−) embryos revealed that this was indeed the case. We observed more extensive deregulation of gene expression in Klf3−/− Klf8−/− cells compared to either of the single knockouts. It appears that KLF3 plays a somewhat nonredundant role in this network in that 64 genes are upregulated in the absence of KLF3 alone, and these are not further significantly elevated in Klf3−/− Klf8−/− embryos. Klf3−/− Klf8−/− embryos did, however, display greater dysregulation of gene expression than Klf3−/− embryos, with 186 genes being significantly differentially expressed relative to the wild type. These genes were both up- and downregulated, consistent with the dual role of KLF8 as both an activator and a repressor of transcription. By comparing Klf3−/− Klf8−/− gene expression profiles

FIG 4 Heat map showing the relative expression of the genes that are deregulated in Klf3−/− Klf8−/− Ter119 E13.5 fetal liver cells compared to the wild type (WT). Genes that are significantly upregulated (group I) and downregulated (group II) in Klf3−/− Klf8−/− cells are represented, and their relative expression across the four genotypes (wild type, Klf8−/−, Klf3−/−, and Klf3−/− Klf8−/−) is shown.

FIG 5 Embryonic, but not adult, globin genes are derepressed in Klf3−/− and Klf8−/− Ter119 E13.5 fetal liver cells. Transcript levels for Hba-x (A), Hbb-y (B), Hbb-bh1 (C), Hba-a1 (D), and Hbb-b1 (E) were determined by qRT-PCR analysis of total RNA from three wild-type (WT), five Klf3−/−, seven Klf8−/−, and four Klf3−/− Klf8−/− embryos. Embryonic globin genes are shown in light gray, and adult globin genes are in dark gray. Expression has been normalized to 18S rRNA levels, and wild-type samples have been set to 1.0. Error bars indicate standard errors of the mean. *, P < 0.05; **, P < 0.005 (two-tailed t test compared to wild type).

FIG 6 Chromatin immunoprecipitation analysis of KLF3 occupancy at α- and β-globin loci in induced MEL cells. ChIP assays were conducted in triplicate, and enrichment has been determined by quantitative real-time PCR and has been normalized to input. The lowest values for both IgG and anti-KLF3 have been set to 1.0. Klf8 promoter 1a has been included as a positive control. Error bars represent standard errors of the mean. *, P < 0.05; **, P < 0.005 (one-tailed t test compared to IgG). HS, DNase-hypersensitive site.
with Klf3−/−, we refined a list of the most likely KLF8 targets. Included in this list were several genes involved in cell cycle regulation, adherence, and invasiveness, in agreement with the role of KLF8 in oncogenesis.

We also confirmed by qRT-PCR that embryonic globin expression is derepressed in Klf3−/− and Klf8−/− Klf8−/− cells, while adult globin expression is unchanged. This was observed not only at E13.5 but also at later stages of fetal liver development (E14.5 and E16.5) in Klf3−/− embryos. The simplest interpretation of these results is that KLF3, which is highly expressed in fetal liver, is the primary repressor of embryonic globin expression, but in its absence, KLF8 is able to partially compensate. While the effects on embryonic globin expression are indeed modest, we suggest that KLF3 and KLF8 together participate in the silencing of embryonic globins with other proposed repressors, such as SOX6, GATA1, YY1, COUP-TF, and DRED (91–94). The expression levels of these repressors were not significantly altered in the microarrays presented here in either Klf3−/− or Klf3−/− Klf8−/− cells (all less than 1.12- and 1.35-fold, respectively, compared to the wild type). Similarly, the expression of BCL11A, a transcriptional repressor of the murine embryonic and human fetal globin genes (48, 49), was found to be unchanged in Klf3−/− or Klf3−/− Klf8−/− cells compared to the wild type. Together, this suggests that the repression of embryonic globin genes by KLF3 and KLF8 is not indirectly achieved by altering the transcription of these known regulators in erythroid cells.

Interestingly, an analogous system of embryonic globin gene regulation and functional compensation has been observed for the activator KLFs, KLF1 and KLF2. The Klf1−/− and Klf2−/− single knockout mice die at around E14.5 and E12.5 to E14.5, respectively, whereas Klf1−/− Klf2−/− double knockout animals die before E11.5 (43, 44, 95–97). Expression of the β-like embryonic globins (Hbb-y and Hbb-bh1) is significantly reduced in the primitive erythroid cells of both Klf1−/− and Klf2−/− single knockout embryos (50, 51, 98) and is further depleted in Klf1−/− Klf2−/− double knockout embryos (97). Thus, in primitive erythroid cells, KLF1 and KLF2 drive transcription of embryonic globin genes, while in definitive erythroid cells, KLF3 and KLF8 serve to repress embryonic globin expression.

In addition to the β-like embryonic globins, we also observed upregulated Hba-x expression in Klf3−/− and Klf3−/− Klf8−/− fetal liver cells. The Hba-x gene has a functional CACCC box in its promoter (99) and like Hbb-y and Hbb-bh1 is also downregulated in Klf1−/− and Klf1−/− Klf2−/− embryos (97). Hba-x expression is also strongly upregulated, more so than Hbb-y and Hbb-bh1, upon inductive restoration of KLF1 activity induced in Klf1−/− erythroid blasts (32). Taken together, these results implicate the KLFs in the regulation of the α-globin locus in addition to the β-globin locus.

The concomitant upregulation of both α- and β-like embryonic globin genes has also been observed in other mouse models. Perhaps the best characterized repressor of embryonic globin expression is the SOX6 transcription factor (100). SOX6 directly binds to the Hbb-y promoter and silences its expression in definitive erythroid cells. As such, mice lacking SOX6 exhibit persistent expression of Hbb-y in the fetal liver. In addition, Hba-x and Hbb-bh1 transcript levels are also considerably elevated in Sox6−/− fetal liver cells, albeit not to the same extent (100). Thus, the coinciding deregulation of embryonic globin genes is a feature that is shared by the KLF3/8, KLF1/2, and SOX6 mouse models.

It is likely that this network of cross-regulation and functional compensation within the KLF family serves to fine-tune the expression of globin genes and other genes during development. Such regulatory circuitries have indeed been identified for other transcription factor families, such as the myogenic basic helix-loop-helix factors (MYOD, MYF5, myogenin, and MRF4) and the paired box (PAX) factors. These families are subject to transcriptional cross-regulation, and family members display overlapping physiological roles, such that single factor knockouts often exhibit mild or no phenotype, while combinatorial knockouts can result in severe physiological perturbations (101, 102). Correct temporal expression of globins is thus likely to be sensitive to the levels of both activating and repressing KLFs. Indeed a dose effect has previously been observed for both KLF1 and KLF2. Hbb-y and Hbb-bh1 are both downregulated in Klf2−/− primitive erythroid cells compared to wild-type cells (98), while Klf1−/− fetal livers show a reduction in β-globin transcript levels (103). In addition, haploinsufficiency of KLF1 results in delayed globin switching (103, 104), while overexpression of KLF1 causes premature switching (105).

The network discussed here, in which KLF1 activates the expression of Klf3 and Klf8, while KLF3 represses Klf8, is known as an incoherent type 1 feed forward network. Such networks are able to effect transient pulses in expression of their target genes and can accelerate the response of these genes to upstream signaling (106). They can also serve to sensitize transcriptional programs to various amplitudes of input signals from external stimuli (107). From the work presented here and previously (8, 32, 33, 36), it is evident that disruption of the KLF1/KLF3/KLF8 network, by removal of single factors or combinations thereof, results in altered transcriptional profiles, suggesting that the network operates to ensure the correct developmental control of gene expression required for normal erythropoiesis.

Lastly, it should be noted that although KLF1 and KLF2 have both been shown to directly bind to the promoters of the embryonic globin genes, we have thus far not detected KLF3 occupancy at these sites (Fig. 6). Unexpectedly, we observed binding at the promoters of the adult globin genes and also at upstream DNase-hypersensitive sites. The mechanism by which KLF3 regulates globin expression thus remains unclear and may involve distal binding and the formation or disruption of long-range interactions, as has been shown to be the case for BCL11A (48, 108). Alternatively, it is possible that KLF3 does bind the proximal promoter CACCC boxes of the embryonic globin genes during a particular window of development or stage of cellular maturation that we have not yet examined.

Taken together, these results establish KLF3 and KLF8 as a pair of transcriptional regulators that operate in an erythroid transcriptional network downstream of KLF1. Of these two factors, KLF3 plays the dominant role in regulating gene expression owing to its comparatively higher abundance. KLF8 is able to partially compensate at some loci, however, and indeed ablation of both KLF3 and KLF8 results in more widespread gene dysregulation than knockout of KLF3 alone. Among the most significantly affected genes, we identified the embryonic globin genes, suggesting that KLF3 and KLF8 participate in their developmental silencing together with other repressors, such as SOX6, GATA1, YY1, COUP-TF, and DRED. Thus, in addition to BCL11A, KLF3 and KLF8 represent two examples of transcriptional repressors down-
stream of KLF1 that cooperate to achieve normal globin regulation during ontogeny.

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We declare that no competing financial interests exist.

REFERENCES


