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Author:
Funnell, Alister; Mak, Ka Sin; Twine, Natalie; Pelka, G; Norton, Laura; Radziewic, T; Power, M; Wilkins, Marc; Bell Anderson, Kim; ... Crossley, Merlin

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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

Running title: Overlapping roles of KLF3/BKLF and KLF8

Alister P. W. Funnell[1*], Ka Sin Mak[1*], Natalie A. Twine[1], Gregory J. Pelka[2], Laura J. Norton[1], Tania Radziewic[2], Melinda Power[2], Marc R. Wilkins[1], Kim S. Bell-Anderson[3], Stuart T. Fraser[4], Andrew C. Perkins[5,6], Patrick P. Tam[2], Richard C. M. Pearson[1] and Merlin Crossley[1#]

[1]School of Biotechnology and Biomolecular Sciences, University of New South Wales, NSW 2052, Australia

[2]Children's Medical Research Institute, Westmead, NSW 2145, Australia

[3]School of Molecular Bioscience, University of Sydney, NSW 2006, Australia

[4]Disciplines of Physiology, Anatomy and Histology, School of Medical Science, University of Sydney, NSW 2006, Australia

[5]Mater Medical Research Institute, Translational Research Institute, Woolloongabba, QLD 4102, Australia

[6]Division of Molecular Genetics and Development, Institute for Molecular Bioscience, University of Queensland, QLD 4072, Australia

[*]A. F. and K. S. M. contributed equally to this work.

[#]Address correspondence to: Merlin Crossley, School of Biotechnology and Biomolecular Sciences, University of New South Wales, NSW 2052, Australia. E-mail address: m.crossley@unsw.edu.au; Tel.: 61-2-9385-7916; Fax: 61-2-9385-7920.

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Abstract

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. Whilst these mice are viable, albeit with a reduced lifespan, mice lacking both KLF3 and KLF8 die at around 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.

Introduction

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 Cys_2His_2 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). Whilst as a whole this domain is highly conserved within the family, specific amino acid differences between the various KLFs result in differing 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 co-regulate other genes in stem cells and other tissues (9, 12-16).

Outside of the DNA-binding domain, KLFs exhibit relatively little conservation and interact with a range of transcriptional co-regulators and histone-modifying enzymes (2, 17). Some KLFs, such as KLF1, primarily function as activators of transcription (18) whilst 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 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 varying 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 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 adult *β*- and *δ-globin* 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 embryonic day 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* and *Hbb-bh1* mRNA are reduced in *Klf1*$^{-/-}$ embryos (50-52).

In addition to *globins*, 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 co-repressor C-terminal binding protein (CtBP) (19, 53). KLF3 is expressed widely (29), but in particular, is found at high levels in erythroid tissue owing to an erythroid 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 (Howell-Jolly bodies) in peripheral blood and mild, compensated anemia (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 CCN-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 from 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 upregulation 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 dysregulation 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 insertion in the *Klf8* locus and no detectable KLF8 protein. Mice with homozygous disruption of *Klf8* (*Klf8*$^{gt/gt}$) are viable but have a shortened lifespan. 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.

Methods

Klf3$^{-/-}$ and *Klf8*$^{gt/gt}$ mice

Murine embryonic stem 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. Germline transmission of the *Klf8* gene trap was confirmed and mice were backcrossed for at least ten 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 numbers L02/6-2006/3/4344 and L02/7-2009/3/5079) and the University of New South Wales (approval number 09/128A).

Histological examination of mice

Necropsies were conducted on four wildtype and four litter-matched *Klf8*^{gt} male mice, age 12-15 weeks (Australian Phenomics Network, The University of Melbourne and IMVS, Adelaide). A wide range of organs were sectioned and subject to standard histological examination, including:

heart, liver, spleen, pancreas, kidney, brain, thyroid, trachea, lungs, thymus, skin, testes, epididymis, seminal vesicles, prostate gland, penis, preputial gland, bladder, gall bladder, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph node, adrenal glands, tail, eyes, Harderian glands, spinal cord, salivary glands and regional lymph nodes. Paraffin embedding of five *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ and three *Klf3*$^{+/+}$ *Klf8*$^{gt/gt}$ E13.5 embryos and subsequent staining with hematoxylin and eosin was performed by the Histology and Microscopy Unit, University of New South Wales.

Quantitative real time RT-PCR

Total RNA from murine tissues was extracted, DNase-treated and analyzed by quantitative real time RT-PCR as described previously (8, 10, 70). Adult tissues were from three to four month old, sex-matched littermates. The sequences of primers used are as follows: *Klf8*, 5′-TGGATGTCCGAATTAAATCAGAAA-3′ and 5′-GAAGGATCTCTGGTCGGAACAG -3′ or 5′-CCAAAAGCTCTCACCTGAAAGC-3′ and 5′-AGCGAGCAAATTTCCAGGAA-3′; *εy-globin* (*Hbb-y*), 5′-GGCCTGTGGAGTAAGGTCAA-3′ and 5′-GCAGAGGACAAGTTCCCAAA-3′; *βh1-globin* (*Hbb-bh1*), 5′-CTCAAGGAGACCTTTGCTCA-3′ and 5′-AATCACCAGCTTCTGCCAGGC-3′; *β-major globin* (*Hbb-b1*), 5′-CACTGTGACAAGCTGCATGT-3′ and 5′-TAGTGGTACTTGTGAGCCAG-3′; *ζ-globin* (*Hba-x*), 5′-ATGCGGTTAAGAGCATCGAC-3′ and 5′-GGGACAGGAGCTTGAAGTTG-3′; *α-globin* (*Hba-a1*), 5′-GTCACGGCAAGAAGGTCGC-3′ and 5′-GGGGTGAAATCGGCAGGGT-3′; and as listed previously (10).

176

177 **Protein overexpression in COS cells**

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

180

181 **Western blotting**

182 Western blotting of nuclear extracts and anti-KLF3 and anti-KLF8 sera (αKLF3 and αKLF8)
183 have been described previously (11, 29, 71). To detect KLF8, 0.1% (v/v) αKLF8 in TBST (50
184 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20) with 5% (w/v) skim milk powder was
185 allowed to hybridize with the blot for 12-18 h at RT. KLF3 was detected using 0.03% αKLF3 in
186 TBST for 1-1.5 h. Secondary labeling was achieved using 1:15,000 horseradish peroxidase-
187 linked anti-rabbit antibody (Amersham Pharmacia Biotech) in TBST for 1 hour at RT. Signals
188 were visualized using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).
189 Blots were stripped in 62.5 mM Tris, pH 6.8, 2% SDS, 0.7% β-mercaptoethanol at 70°C for 30
190 min and were subsequently washed six times with TBST over 30 min. Blots were then
191 re-blocked and probed using anti-β-actin (1:10,000) (Sigma) and horseradish peroxidase-linked
192 anti-mouse antibody (1:15,000) (Amersham Pharmacia Biotech) in TBST each for 1 h at RT.

193

194 **Determining 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′-TGGATGTCCGAATTAAATCAGAAA-3′) and reverse primers specific for the *β-geo* gene trap (5′-AGTATCGGCCTCAGGAAGATCG-3′ and 5′-ATTCAGGCTGCGCAACTGTTGGG-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′-GGAACCTGTGACTGATTTGACTAGGC-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′-GGAACCTGTGACTGATTTGACTAGGC-3′ and 5′-GCATTGTGCTAAGTCCACTGACAGC-3′, which flank the site of gene trap insertion and generate a 210 bp product, indicative of an intact, wildtype allele, and; 5′-CAGTATCTGCAACCTCAAGCTAGCTTGG-3′ and 5′-ATTCAGGCTGCGCAACTGTTGGG-3′, which recognize the gene trap itself and produce a 348 bp product. PCRs were conducted using *REDTaq*® DNA polymerase (Roche Molecular Biochemicals) as recommended by the supplier and in the presence of 10% DMSO. PCR parameters used were: one cycle of 94°C for 2 min, 31 cycles of (94°C for 30 s, 60°C for 30 s,

72°C for 1 min) and one cycle of 72°C for 5 min. *Klf3* knockout line mice were genotyped as previously described (69). *Klf3 Klf8* double mutant mice were genotyped by multiplex PCR using the *Klf8* primers listed above together with primers specific for wildtype *Klf3* (5′-CATCCTTCCGTCATCGTGCAG-3′ and 5′-TTTCAAGTGCGAGCTCTTAGTGTAGACC-3′, 135 bp product) and for the *Klf3 Neo* cassette (5′-TCCATGTCTGTCTCCCCCTA-3′ and 5′-ATTAAGGGCCAGCTCATTCC-3′, 250 bp product). PCRs were prepared using Mango*Taq*™ DNA polymerase (Bioline) as per the manufacturer's instructions and using the following thermocycler parameters: one cycle of 94°C for 2 min, 29 cycles of (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) and one cycle of 72°C for 5 min.

Full blood count analysis

Full blood counts were performed for 21 *Klf8* mutant mice ($Klf8^{gt}$ males or $Klf8^{gt/gt}$ females) and 18 wildtype mice at 11-12 weeks of age as previously described (8). For phenylhydrazine treatment of mice, 7 wildtype and 9 $Klf8^{gt}$ males (18-22 weeks of age) were treated by sequential intraperitoneal injections of 1-acetyl-2-phenylhydrazine (0.5 % (w/v) in Hank's balanced salt solution) at a dose of 0.04 mg per gram body mass at t=0 and t=16 h. Peripheral blood was collected by cardiac puncture at t=120 h and was subjected to full blood count analysis as above.

Sorting of Ter119+ erythroid cells

Ter119+ cells were sorted from E13.5, E14.5 and E16.5 fetal livers using 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.

Cytospins of peripheral blood and fetal livers

Peripheral blood and livers from individual E14.5 embryos were disaggregated in 1 mL filtered FACS buffer (10 mM EDTA, 5% fetal calf serum, 0.05% NaN_3 in PBS) and were counted using a Countess® Automated Cell Counter (Life Technologies). Cells were diluted to 4 x 10^5/mL in FACS buffer and 100-150 uL was centrifuged for 5 min at 300 rpm in Shandon Cytofunnels (Thermoscientific). Slides were subsequently stained using Diff-Quik (Lab Aids Pty Ltd) and were counted.

Chromatin immunoprecipitation (ChIP) analysis of murine erythroleukemia (MEL) cells. MEL cells were cultured and induced to differentiate for 72 h in 1.8% DMSO as described previously (70). ChIP assays were conducted in triplicate as described previously (72, 73) using rabbit polyclonal anti-KLF3 serum (29) or Pierce anti-KLF3 antibody (PA5-18030) and pre-immune serum or IgG (Santa Cruz sc-2028) as negative controls. Primer sequences are found elsewhere (11, 50, 74) and as follows: *Hba-a1* promoter, 5′-GTTTGAGGGACTTGCTTCTCTGA-3′ and 5′-GCCCGGACACACTTCTTACC-3′; *Hba-x* promoter, 5′-AGCCCATTGGCACTGAGACT-3′ and 5′-CAATCCCTCTTCTGACCTGCTTA-

3′; and *Hbb-y* promoter, 5′-CATGACCTGGCTCCACCCATGAG-3′ and 5′-CTGCTGCTAGAAGTGGTGGCCTT-3′.

Microarrays

Total RNA was extracted from Ter119+ fetal liver cells purified from four wildtype, 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 two-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 the accession number GSE43524.

Results

Generation of a *Klf8* gene trap mutant mouse model

Whilst 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, 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 and *data not shown*). The gene trap contains a potent splice acceptor site to disrupt expression of KLF8 and instead generate a β-geo 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*).

Whilst KLF8 protein is normally expressed at very low levels in non-cancerous tissues (55), we have been able to detect endogenous KLF8 in wildtype fetal brain and placental tissue (Fig. 1d, e). Mice that are homozygous for the gene trap have no detectable KLF8 protein in these tissues (Fig. 1d, e). Similarly, qRT-PCR analysis of a range of erythroid tissues (E11.5 yolk sac, E14.5 fetal liver and adult peripheral blood) revealed that the gene trap reduced *Klf8* mRNA below the level of detection (*data not shown*).

The *Klf8* gene lies on the X-chromosome and hence males are hemizygous for the gene trap ($Klf8^{gt}$), whilst females can either be heterozygous ($Klf8^{gt/+}$) or homozygous ($Klf8^{gt/gt}$). For simplicity, hemizygous males and homozygous females will be collectively referred to as $Klf8^{gt/gt}$ throughout. $Klf8^{gt/gt}$ males and females are both viable and fertile. They have a significantly shortened lifespan, with approximately 30% dying by the age of six months ($P < 0.01$ and $P < 0.001$ for males and females respectively), compared to wildtype males and females which 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 six months ($P < 0.05$ compared to wildtype females). This might be explained by decreased KLF8 expression in these animals, the extent of which may vary between individuals due to non-random 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 lifespan of these animals (see Methods). Similarly, full blood counts indicated that erythroid parameters and platelet numbers are normal in *Klf8*$^{gt/gt}$ mice (Fig. S1). Moreover, phenylhydrazine treatment did not reveal any significant differences in blood count parameters between *Klf8*$^{gt/gt}$ and wildtype 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* knockout (*Klf3*$^{-/-}$) mouse line with the newly generated *Klf8*$^{gt/gt}$ line. We have found that *Klf3*$^{-/-}$ mice are essentially infertile, but using a series

of different parental crosses involving *Klf3* heterozygous animals, we analyzed the frequency of the nine possible resulting genotypes (Fig. S2) at weaning age (Table 1). The observed numbers do not adhere to Mendelian expectance, with no mice observed that were deficient in both KLF3 and KLF8 ($Klf3^{-/-}$ $Klf8^{gt/gt}$) and an underrepresentation of female mice containing only a single allele of *Klf8*, that is, $Klf3^{-/-}$ $Klf8^{gt/+}$ (observed at 10% of expected). *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 $Klf3^{-/-}$ $Klf8^{gt/gt}$ embryos are viable and found at expected Mendelian numbers at embryonic day E12.5 (Table 2) but start to die thereafter, with n = 10/15 embryos surviving at E13.5 and n = 1/5 surviving at E14.5. Lethality at this developmental stage is often caused by cardiovascular defects and/or disrupted liver hematopoiesis (77), however histological examination of sections of E13.5 $Klf3^{-/-}$ $Klf8^{gt/gt}$ embryos provided no conclusive explanations as to their cause of death. Taken together, the observation that the $Klf8^{gt/gt}$ and $Klf3^{-/-}$ single gene mutant mice are viable whilst the $Klf3^{-/-}$ $Klf8^{gt/gt}$ double mutants exhibit an embryonic lethal phenotype, indicates a genetic interaction between KLF3 and KLF8 and suggests that these factors have at least partial functional redundancy *in vivo*.

Derepression of KLF8 in *Klf3* null erythroid tissue

To further investigate the aforementioned repression of the *Klf8* gene by KLF3, we analyzed the levels of *Klf8* transcripts in a range of tissues from *Klf3* null mice (Fig. 2a and *data not shown*). In all tissues examined, apart from the brain, we found significant derepression of *Klf8* mRNA. However, despite this derepression, in some tissues, such as the liver and the intestine, the level

of *Klf8* expression remains relatively low. In contrast, we found that in erythroid tissues particularly (that is, the spleen and bone marrow), *Klf8* is expressed at comparatively high levels in the absence of KLF3 (Fig. 2a). This trend is observed not only in adult tissues but also in embryonic erythroid tissue: KLF8 protein is barely detected in wildtype and $Klf3^{+/-}$ fetal liver, but is considerably upregulated in *Klf3* null tissue (Fig. 2b and (11)). Taken together, the pronounced upregulation of *Klf8* expression in erythroid tissue is consistent with our previous observation that the master erythroid regulator KLF1 directly activates *Klf8* transcription in erythroblast cells, particularly in the absence of KLF3 (11).

Identification of KLF3 and KLF8 target genes in fetal liver

There is thus a regulatory network within the KLF family in erythroid cells, such that KLF1 drives the expression of KLF3 and KLF8, and KLF3 represses the *Klf8* gene. We have previously investigated the function of KLF3 in this network by microarray analysis of *Klf3* null E14.5 fetal liver cells (8). We next sought to further unravel the KLF1/KLF3/KLF8 network by analyzing and comparing tissue from single mutant ($Klf8^{gt/gt}$ and $Klf3^{-/-}$) and double mutant ($Klf3^{-/-}$ $Klf8^{gt/gt}$) animals. As $Klf3^{-/-}$ $Klf8^{gt/gt}$ embryos die at around E14.5, we analyzed $Ter119^{+}$ (erythroid) fetal liver cells at embryonic day E13.5. At this developmental stage, $Klf3^{-/-}$ $Klf8^{gt/gt}$ embryos are largely phenotypically normal (Fig. S3) and their livers display no gross morphological abnormalities by histological examination of sections. For this study, total RNA was extracted from cells from four wildtype, four $Klf8^{gt/gt}$, three $Klf3^{-/-}$ and four $Klf3^{-/-}$ $Klf8^{gt/gt}$ embryos and was subjected to Affymetrix microarray analysis. In addition, we confirmed that the

Klf8 gene trap ablated KLF8 protein expression in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ compared to *Klf3*$^{-/-}$ fetal liver (Fig. 2c).

Comparing *Klf8*$^{gt/gt}$ embryos with wildtype, 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*, *Histocompatibility 2, Q region locus 6*) (Fig. 3a). The lack of any considerable gene deregulation in *Klf8*$^{gt/gt}$ embryos is not unexpected given that KLF8 protein is not readily detectable in wildtype fetal liver (Fig. 2b, c).

Next we compared *Klf3*$^{-/-}$ samples with wildtype and observed considerable derepression of gene expression (Fig. 3b, Fig. S4 and Table S1). In total, 64 genes were significantly derepressed while only four 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 (Table S1). As we had previously observed, *Klf8* was one of the most highly derepressed genes in these cells (10.2-fold) (Fig. 3b and Table S1).

Klf3$^{-/-}$ *Klf8*$^{gt/gt}$ cells exhibited even greater deregulation of gene expression than *Klf3*$^{-/-}$ cells, with 112 genes being upregulated and 74 downregulated compared to wildtype (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*$^{gt/gt}$ tissue (Fig. S4 and Table S1). Only four of the derepressed genes did not meet these criteria (*Frrs1*, *Cmpk2*, *Acot9* and *Treml2*), but nonetheless displayed upregulation in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ 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, Fig. S4 and Table S1). 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*$^{gt/gt}$ cells did indeed display a greater 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 KLF8 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*$^{gt/gt}$ and *Klf3*$^{-/-}$ cells and compiled a list of 30 significantly differentially expressed genes, 17 of which were upregulated and 13 downregulated (Fig. 3d and Fig. S5). Notably, several of these genes, including *Itgb7* (78), *Vegfa* (79, 80), *Periostin* (81), *Parm1* (82, 83), *Gbp1* (84, 85), *Tcfl5* (86, 87), *Rps3a* (88) and *Fpr1* (89, 90), are regulators of cell adherence, migration and invasiveness, consistent with the role of KLF8 in oncogenesis. In addition, the *Klf8* gene itself is significantly downregulated in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ compared to *Klf3*$^{-/-}$ cells as anticipated (Fig. 3d and 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*$^{gt/gt}$ cells compared to *Klf3*$^{-/-}$ revealed that the other murine embryonic genes, *Hba-x* and *Hbb-y*, are elevated 2.8-fold and 1.5-fold respectively (Fig. 3d) whilst 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 wildtype (4.07-, 2.66- and 1.62-fold respectively for *Hba-x*, *Hbb-y* and *Hbb-bh1*) (Fig. 5a-c). We also tested the hypothesis that these embryonic globin genes are further derepressed in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ 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-c). Adult globin expression was unaffected in both *Klf3*$^{-/-}$ and *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ cells compared to wildtype (Fig. 5d, e).

The embryonic lethality of *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ 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 (Figure S6) and E16.5 (Figure S7). Cytospins of peripheral blood and fetal liver tissue at E14.5 revealed no difference in the number of primitive erythrocytes between wildtype and *Klf3*$^{-/-}$ animals that might explain the elevated expression of embryonic globins (Figure S8). Furthermore, we observed no deregulation of either embryonic or adult globin expression in *Klf3*$^{-/-}$ E10.5 yolk sac, a source of primitive erythroid cells (Figure S9a-e). Similarly, both embryonic and adult globin transcript levels were comparable in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ and litter-matched *Klf3*$^{+/+}$ *Klf8*$^{gt/gt}$ yolk sacs (Figure S9f-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 might repress 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 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

452 embryonic *globin* gene expression not by direct binding to their promoters, but through distal
453 sites within the globin loci.

454

455 **Discussion**

456 Deregulation of KLF8 has been observed in a wide variety of cancers, however, the normal
457 physiological roles of this transcription factor have remained largely unknown. To this end, we
458 have generated a mouse line with disrupted KLF8 expression. *Klf8*$^{gt/gt}$ mice are viable and fertile
459 but have a shortened lifespan, the cause of which could not be identified despite extensive
460 histological characterization. Mice that are deficient in the related family member KLF3 are also
461 viable, yet *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ double mutant animals die at around embryonic day E14.5. This
462 suggests that KLF3 and KLF8 have overlapping roles *in vivo* and can at least partially
463 compensate in each other's absence.

464

465 Analysis of *Klf3*$^{-/-}$ mice revealed marked upregulation of KLF8 expression in several tissues, but
466 particularly erythroid tissue. Given that both the *Klf3* and *Klf8* genes are activated by the
467 erythroid factor KLF1, we hypothesized that these three factors operate in a regulatory network
468 to control gene expression. Microarray analysis of Ter119^{+} fetal liver cells from single mutant
469 (*Klf3*$^{-/-}$ and *Klf8*$^{gt/gt}$) and double mutant (*Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$) embryos revealed that this was indeed
470 the case. We observed more extensive deregulation of gene expression in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ cells
471 compared to either of the single knockouts. It appears that KLF3 plays a somewhat
472 non-redundant role in this network in that 64 genes are upregulated in the absence of KLF3 alone
473 and these are not further significantly elevated in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ embryos. *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ embryos

did, however, display greater dysregulation of gene expression than *Klf3*$^{-/-}$ embryos, with 186 genes being significantly differentially expressed relative to wildtype. 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*$^{gt/gt}$ gene expression profiles 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 *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ cells whilst 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 that in its absence, KLF8 is able to partially compensate. Whilst 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*$^{gt/gt}$ cells (all less than 1.12- and 1.35-fold respectively compared to wildtype). 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*$^{-/-}$ and *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ cells compared to wildtype. 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-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 whilst 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*$^{gt/gt}$ 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 inducible restoration of KLF1 activity induced in *Klf1*$^{-/-}$ erythroblast cells (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-like 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, 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 whilst 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 wildtype (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), whilst 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 varying 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. 7). 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. Amongst the most significantly affected genes we identified the embryonic *globins*, 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 downstream of KLF1 that cooperate to achieve normal globin regulation during ontogeny.

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Authorship

Contributions are as follows: AF designed and performed research, analyzed data, and wrote the paper; KSM, LN, GP, TR and MP performed research and analyzed data; NT, MW and KB-A analyzed data; SF, AP and PT designed research and analyzed data; RP designed research, analyzed data and wrote the paper; MC devised concept and research, analyzed data and wrote the paper.

Conflict-of-interest disclosure

The authors declare that no competing financial interests exist.

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Figure legends

Figure 1. The *Klf8* gene trap disrupts normal KLF8 expression and results in increased mortality. (A and B) Schematic of the wildtype murine *Klf8* locus (A) and the location of the *β-geo* gene trap (B). *Klf8* exons are shown as lightly shaded boxes. The gene trap contains a splice acceptor site (SA), *β-galactosidase Neomycin resistance* fusion gene (*β-geo*) and a poly(A) signal. The β-geo fusion protein contains a short (24 amino acid) N-terminal portion encoded by exon 2 of *Klf8*. (C) Multiplex genotyping PCRs confirming the integration site of the gene trap in the *Klf8* locus. Primer pairs were used that flank the site of gene trap insertion (210 bp product for a wildtype, intact allele) and that recognize the gene trap (348 bp product). (D) Western blot of KLF8 expression (top panel) in the fetal brain (E14.5) for the genotypes indicated. KLF8 expressed in COS cells has been included as a positive control while β-actin levels are shown as loading controls (bottom panel). (E) Western blots of KLF8 (top panel) and β-actin (bottom panel) in E10.5 placenta. In (D and E) the band corresponding to KLF8 is marked with an asterisk. (F) Percentage of animals surviving for the genotypes indicated over a six month period. n = 40 wildtype males, n = 45 $Klf8^{gt}$ males, n = 52 wildtype females, n = 87 $Klf8^{gt/+}$ females, n = 16 $Klf8^{gt/gt}$ females. *, $P < 0.05$ and ***, $P < 0.001$ compared to wildtype females, and **, $P < 0.01$ compared to wildtype males (log-rank tests). WT, wildtype.

Figure 2. *Klf8* expression is elevated in erythroid tissue in the absence of KLF3. (A) *Klf8* transcript levels were quantified by real-time RT-PCR in whole tissues from adult wildtype (WT, n = 3) and $Klf3^{-/-}$ (Klf3 KO, n = 3) mice. Expression has been normalized to *18S* rRNA levels and the lowest level (wildtype liver) has been set to 1.0. Error bars represent standard error of the

mean. *, $P < 0.04$ (two-tailed *t* test for *Klf3*$^{-/-}$ compared to wildtype). (B and C) Western blots of E14.5 (B) and E13.5 (C) fetal liver nuclear extracts using anti-KLF8 (αKLF8), anti-KLF3 (αKLF3) and anti-β-actin sera. Genotypes are indicated and nuclear extracts from COS cells (mock transfected or overexpressing KLF3 or KLF8) have been included as controls. In (C), the band corresponding to KLF8 is marked with an asterisk.

Figure 3. Volcano plots demonstrating gene expression changes in *Klf8*$^{gt/gt}$, *Klf3*$^{-/-}$ and *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ Ter119^{+} E13.5 fetal liver cells. (A) *Klf8*$^{gt/gt}$ versus wildtype (WT). (B) *Klf3*$^{-/-}$ versus WT. (C) *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ versus WT. (D) *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ versus *Klf3*$^{-/-}$. Significance thresholds are shown (>2-fold deregulation, FDR < 0.3) and significantly deregulated genes are represented by red dots (derepressed relative to WT (A-C) or *Klf3*$^{-/-}$ (D)) or green dots (downregulated relative to WT (A-C) or *Klf3*$^{-/-}$ (D)).

Figure 4. Heat map showing the relative expression of the genes that are deregulated in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ Ter119^{+} E13.5 fetal liver cells compared to wildtype. Genes that are significantly upregulated (group I) and downregulated (group II) in *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ cells are represented and their relative expression across the four genotypes (wildtype, *Klf8*$^{gt/gt}$, *Klf3*$^{-/-}$ and *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$) is shown.

Figure 5. Embryonic, but not adult, globin genes are derepressed in *Klf3*$^{-/-}$ and *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ 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

wildtype (WT), five *Klf3*$^{-/-}$, seven *Klf8*$^{gt/gt}$ and four *Klf3*$^{-/-}$ *Klf8*$^{gt/gt}$ embryos. Embryonic globin genes are shown in light grey and adult globin genes are dark grey. Expression has been normalized to *18S* rRNA levels and wildtype samples have been set to 1.0 for each gene. Error bars indicate standard error of the mean. *, $P \leq 0.05$ (two-tailed *t* test compared to wildtype).

Figure 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 error of the mean. *, $P < 0.05$, **, $P < 0.005$ (one-tailed *t* test compared to IgG). HS, DNase-hypersensitive site.

Table footnotes

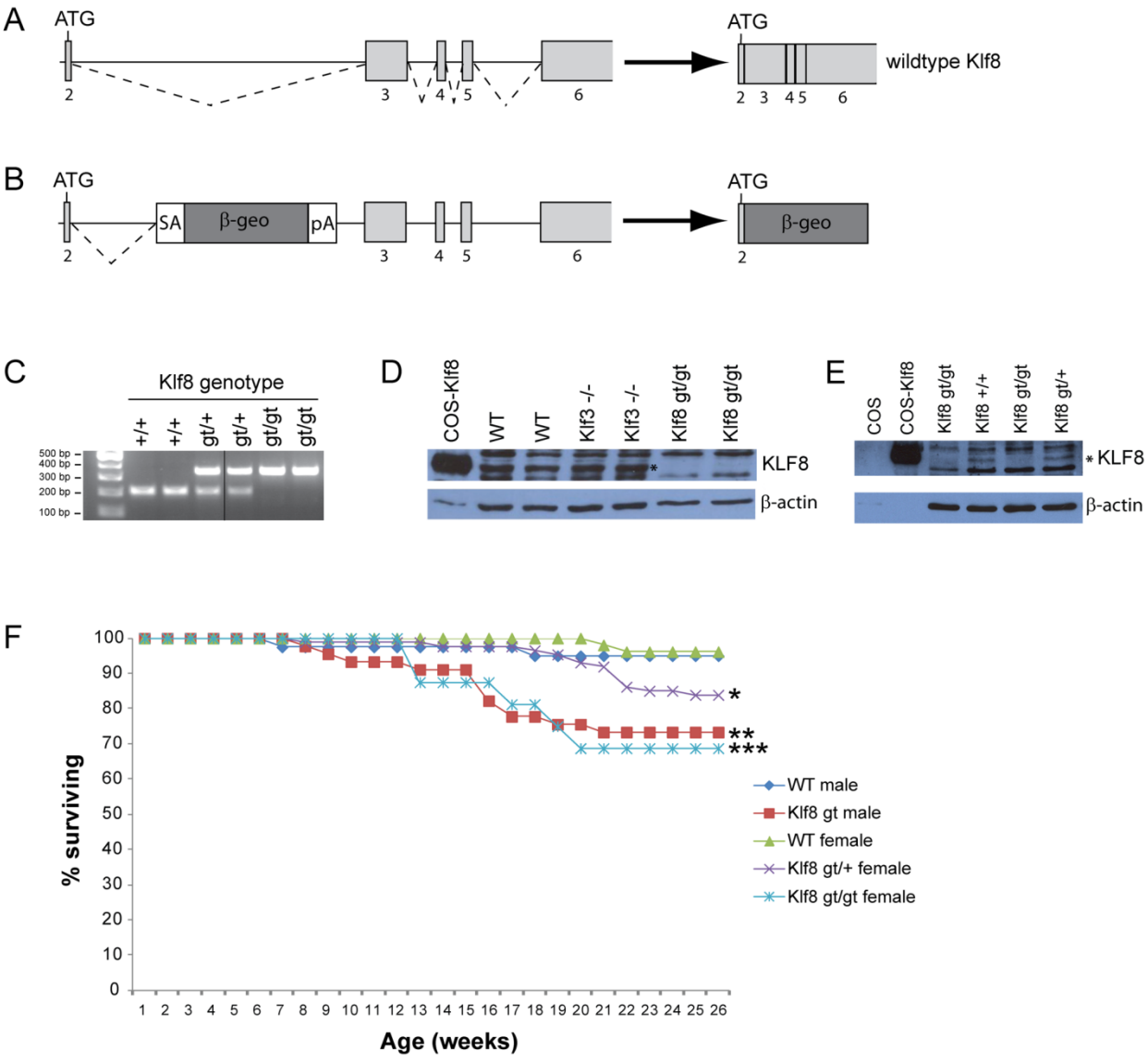
Table 1. Observed and expected numbers of mice from crosses between the *Klf3*$^{-/-}$ and *Klf8*$^{gt/gt}$ lines. 887 mice were genotyped at three to four weeks of age. *Klf8*^{gt} males and *Klf8*$^{gt/gt}$ females have been collectively grouped as *Klf8*$^{gt/gt}$ while *Klf8*$^{+}$ males and *Klf8*$^{+/+}$ females are denoted together as *Klf8*$^{+/+}$. Observed (O) numbers do not adhere to Mendelian expectance (E), $P < 0.001$, Chi-square χ^2 test.

Klf8 \ Klf3	+/+	+/-	-/-
+/+	E: 81 O: 109	E: 161 O: 226	E: 81 O: 35

gt/+	E: 59 O: 90	E: 118 O: 154	E: 59 O: 6
gt/gt	E: 82 O: 92	E: 165 O: 175	E: 82 O: 0

Table 2. Observed and expected numbers of embryos from crosses between the *Klf3*$^{-/-}$ and *Klf8*$^{gt/gt}$ mouse lines. Counts represent genotyping results of embryos up to E12.5 (n = 94). Observed (O) numbers were not found to significantly deviate from Mendelian expectance (E), P = 0.61, Chi-square χ^2 test.

Klf8 \ Klf3	+/+	+/-	-/-
+/+	E: 7 O: 10	E: 13 O: 18	E: 7 O: 5
gt/+	E: 6 O: 7	E: 12 O: 9	E: 6 O: 4
gt/gt	E: 11 O: 8	E: 22 O: 21	E: 11 O: 12


A
ATG
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wildtype Klf8
B
ATG
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5
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ATG
β-geo
2
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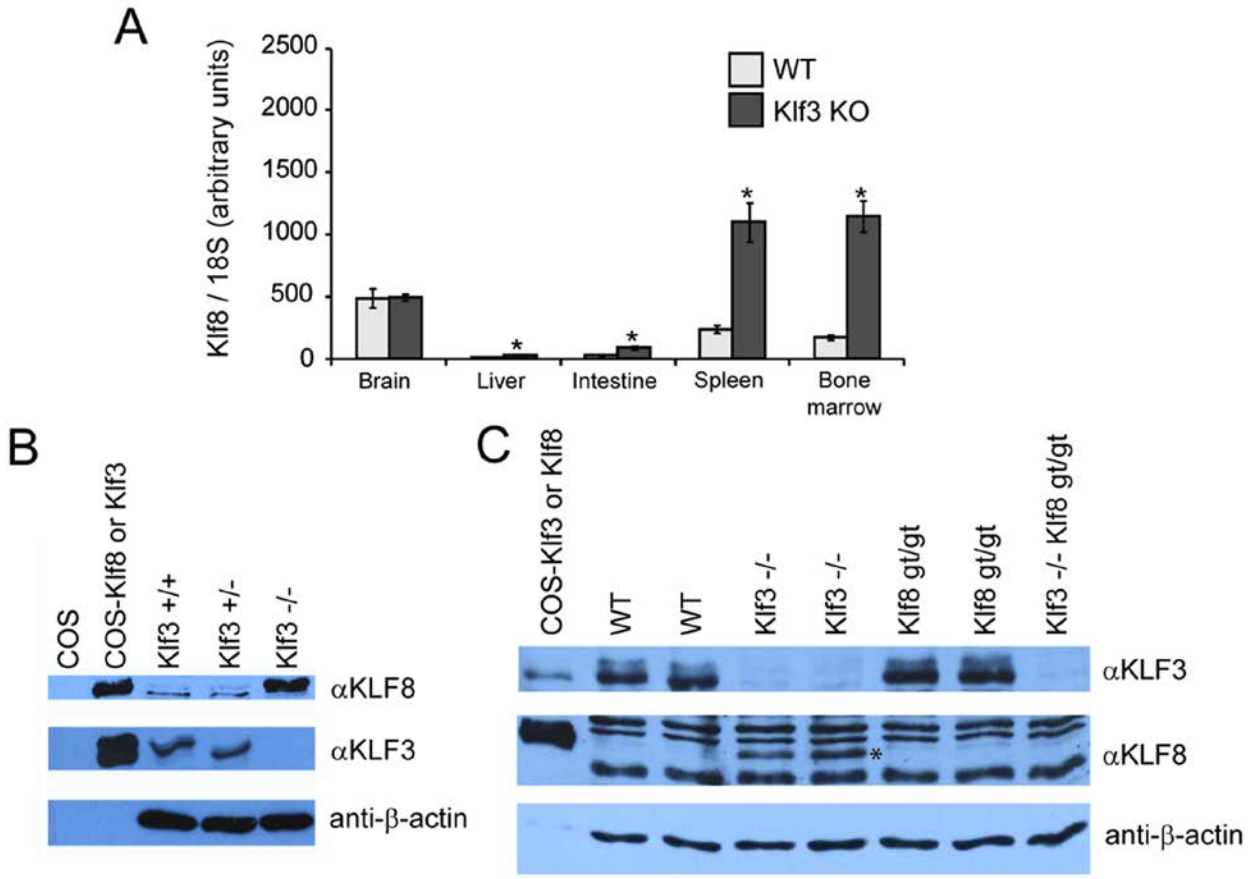
+/+
+/+
gt/+
gt/+
gt/gt
gt/gt
500 bp
400 bp
300 bp
200 bp
100 bp
D
COS-Klf8
WT
WT
Klf3 -/-
Klf3 -/-
Klf8 gt/gt
Klf8 gt/gt
KLF8
β-actin
E
COS
COS-Klf8
Klf8 gt/gt
Klf8 +/+
Klf8 gt/gt
Klf8 gt/+
* KLF8
β-actin
F
% surviving
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Age (weeks)
*
**
***
WT male
Klf8 gt male
WT female
Klf8 gt/+ female
Klf8 gt/gt female

1013

1014


A
Klf8 / 18S (arbitrary units)
2500
2000
1500
1000
500
0
WT
Klf3 KO
*
*
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Brain
Liver
Intestine
Spleen
Bone marrow
B
COS
COS-Klf8 or Klf3
Klf3 +/+
Klf3 +/-
Klf3 -/-
αKLF8
αKLF3
anti-β-actin
C
COS-Klf3 or Klf8
WT
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Klf3 -/-
Klf3 -/-
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αKLF8
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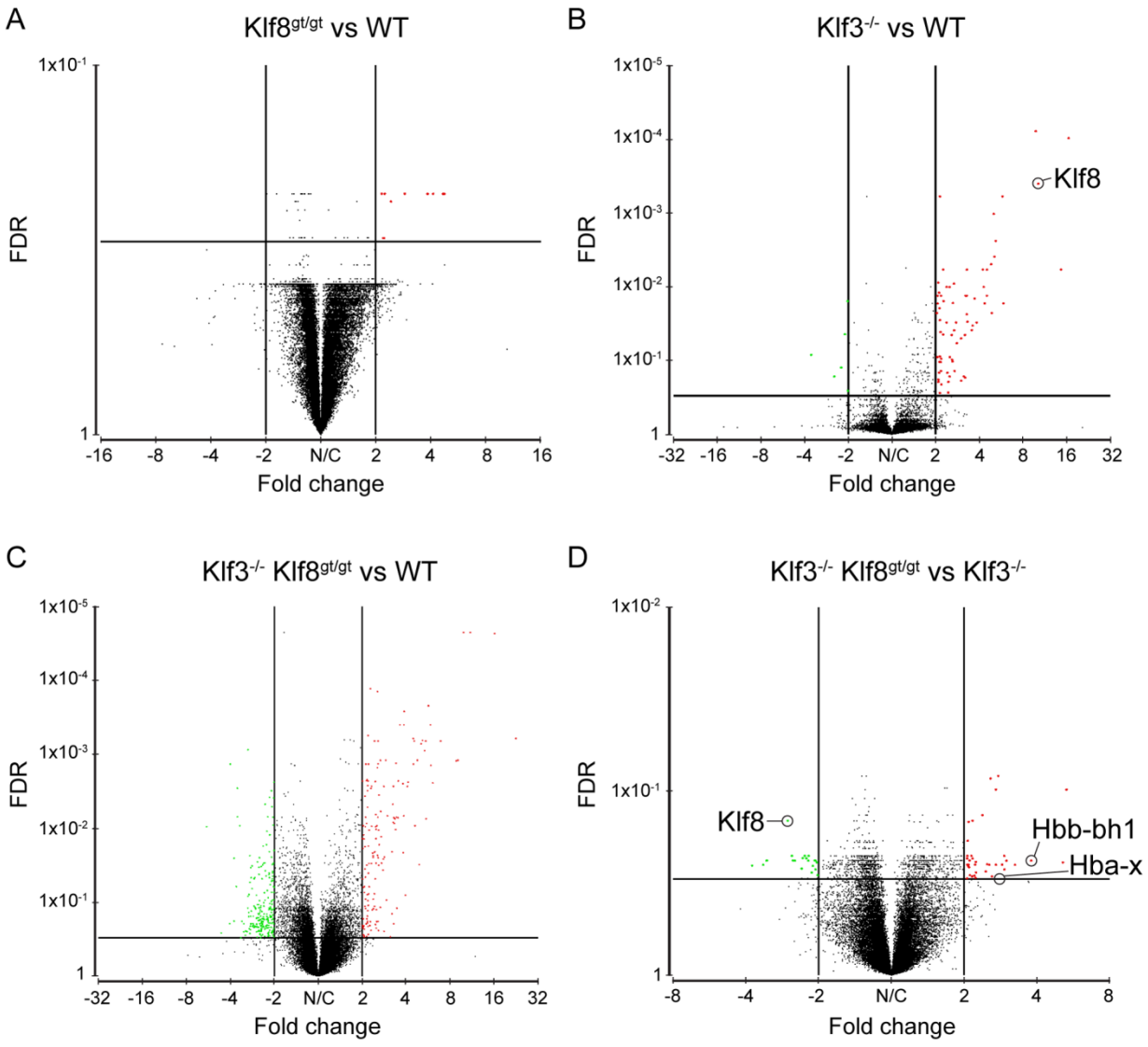
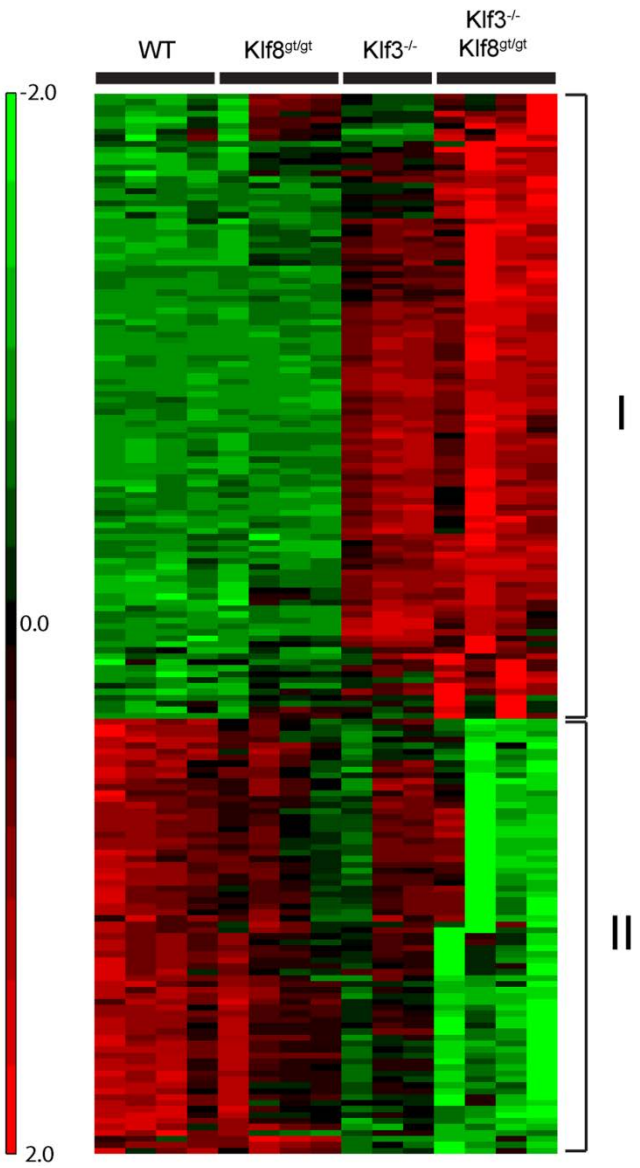
anti-β-actin

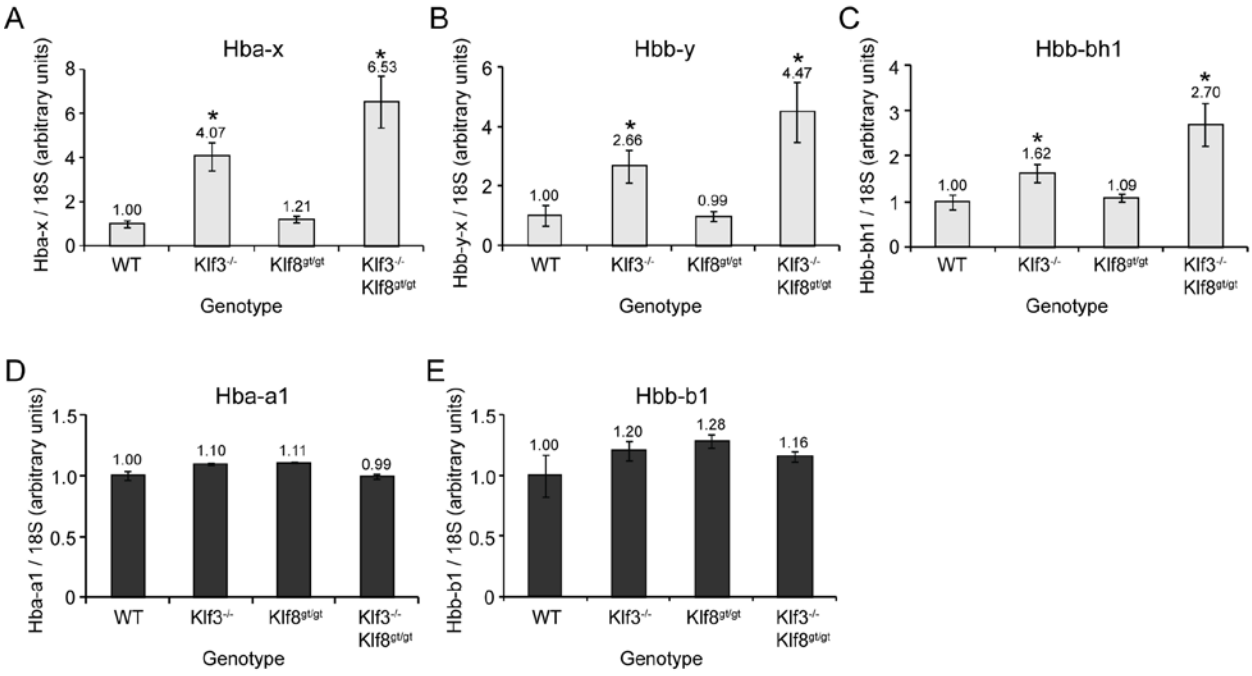
1015


A
Klf8gt/gt vs WT
FDR
Fold change
B
Klf3-/- vs WT
Klf8
C
Klf3-/- Klf8gt/gt vs WT
D
Klf3-/- Klf8gt/gt vs Klf3-/-
Klf8
Hbb-bh1
Hba-x

1016

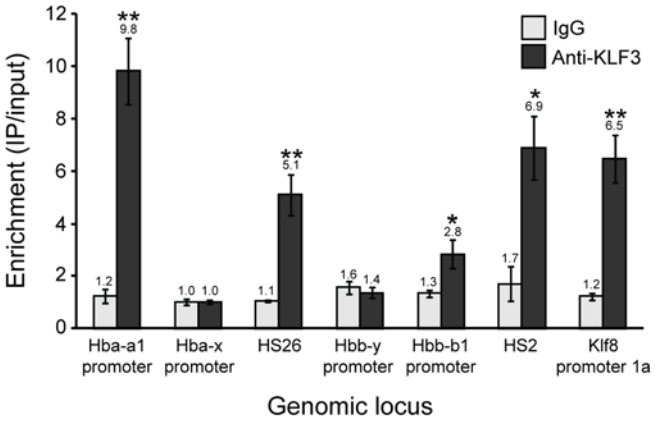


1017


A
Hba-x
Hba-x / 18S (arbitrary units)
1.00
4.07
1.21
6.53
WT
Klf3-/-
Klf8gt/gt
Klf3-/- Klf8gt/gt
Genotype
B
Hbb-y
Hbb-y-x / 18S (arbitrary units)
1.00
2.66
0.99
4.47
C
Hbb-bh1
Hbb-bh1 / 18S (arbitrary units)
1.00
1.62
1.09
2.70
D
Hba-a1
Hba-a1 / 18S (arbitrary units)
1.00
1.10
1.11
0.99
E
Hbb-b1
Hbb-b1 / 18S (arbitrary units)
1.00
1.20
1.28
1.16

1018

1019


Enrichment (IP/input)
12
10
8
6
4
2
0
IgG
Anti-KLF3
**
9.8
1.2
1.0
1.0
**
5.1
1.1
1.6
1.4
*
2.8
1.3
*
6.9
1.7
**
6.5
1.2
Hba-a1 promoter
Hba-x promoter
HS26
Hbb-y promoter
Hbb-b1 promoter
HS2
Klf8 promoter 1a
Genomic locus

1020