Developmental and species-divergent globin switching are driven by BCL11A

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Abstract

The contribution of changes in cis-regulatory elements or trans-acting factors to interspecies differences in gene expression is not well understood. The mammalian β-globin loci have served as a paradigm for gene regulation during development. Transgenic mice harboring the human β-globin locus, consisting of the linked embryonic (ε), fetal (γ) and adult (β) genes, have been used as a model system to study the temporal switch from fetal to adult hemoglobin, as occurs in humans. We show that the human *γ-globin* genes in these mice behave as murine embryonic globin genes, revealing a limitation of the model and demonstrating that critical differences in the trans-acting milieu have arisen during mammalian evolution. We show that the expression of BCL11A, a repressor of human *γ-globin* expression identified through genome-wide association studies, differs between mouse and human. Developmental silencing of the mouse embryonic globin and human *γ-globin* genes fails to occur in mice in the absence of BCL11A. Thus, BCL11A is a critical mediator of species-divergent globin switching. By comparing the ontogeny of β-globin gene regulation in mice and humans, we have shown that alterations in expression of a trans-acting factor constitute a critical driver of gene expression changes during evolution.

The extent to which changes in cis-regulatory elements or the trans-acting environment account for differences in gene expression in closely related species is the subject of debate[1,2]. Some studies suggest that changes in cis-regulatory elements are largely responsible for many interspecies differences in gene expression[3,4]. The contribution of alterations in the trans-acting milieu is less established. With their temporal switches of globin expression, mammalian β-globin loci serve as a paradigm for developmental gene regulation[5]. To study the regulation of human cis-elements in a mouse trans-acting environment, we employed human β-globin locus transgenic mice (β-locus mice). The regulation of the human β-globin locus has been widely studied using such mouse models[6–8]. It is generally accepted that these mice provide a valid system for evaluating human developmental globin gene regulation, though some differences have been noted between humans and these mice. For example, the onset of γ-globin expression occurs during the embryonic, yolk sac stage of erythropoiesis in the mouse, while high-level expression of this gene occurs during the fetal liver stage in man. Moreover, the switch from γ-globin to adult β-globin occurs during early fetal liver erythropoiesis in these mice[6–8], whereas it occurs around the time of birth in humans[9]. In addition, differences have been noted in the capacity of these mice to respond to fetal hemoglobin (HbF) eliciting responses that are active in humans[10,11]. We began by evaluating whether these mice respond to stimuli that consistently increase the level of HbF in humans[12]. We found that these mice have much lower basal levels of *γ-globin* expression than adult humans and fail to respond to stimuli that result in elevated levels of HbF in humans (Supplementary Fig. 1). Also, in a model of juvenile myelomonocytic leukemia created in these mice, no elevation in *γ-globin* levels was observed, in contrast to the high levels of *γ-globin* seen in humans with this syndrome[13].

Human fetal *γ-globin genes* behave as embryonic genes in the mouse

To pursue the underlying basis of these species differences, we reassessed the ontogeny of human *γ-globin* expression during mouse development. We first isolated circulating blood from embryos at a time when *γ-globin* expression is observed (E13.5)[6–8]. Using differences in cell size that permit separation of circulating primitive and definitive lineage cells using flow cytometry[14,15], we enriched the erythroid cells in blood from embryonic day 13.5 (E13.5) β-locus mice (Fig. 1a). As anticipated, expression of the mouse embryonic gene *εy globin*, a gene confined to the primitive erythroid lineage along with mouse *βh1 globin*[14,15], was enriched (approximately 5-fold) in the primitive population relative to the definitive population (Fig. 1b). Consistent with this distribution, human embryonic *ε-globin* transcripts were similarly enriched in the primitive population (Fig. 1b). Surprisingly, there was no difference observed between the relative enrichment of the embryonic genes and the degree of enrichment of human *γ-globin* transcripts in the primitive erythroid population compared to the definitive cells (Fig. 1b). This finding indicates that the human *γ-globin* genes are not robustly expressed in early definitive erythroid cells in β-locus mice.

We then used immunohistochemistry (IHC) of γ-globin in E13.5 embryos to examine its cellular distribution. IHC of human fetal liver (FL) revealed positive labeling of all erythroblasts (Fig. 1c). In contrast, the majority of erythroblasts present in the murine FL of β-locus mice failed to stain for γ-globin. We observed occasional large nucleated, megaloblastic cells in FL positive for γ-globin (Fig. 1d, e). Morphologically these cells resemble primitive cells that continue to circulate in substantial numbers during this period of gestation[5]. Consistent with this interpretation, the numerous γ-globin positive cells seen in the circulation were all megaloblastic primitive cells, whereas enucleate, smaller definitive cells were uniformly negative (Fig. 1e, f). To generalize these findings, we performed similar immunohistochemical staining in other independently-derived lines of β-locus mice (Fig. 1g, h)[8]. In all lines, γ-globin expression (as indicated by positive IHC) was

confined to circulating megaloblastic cells that were infrequent in FL parenchyma. As similar observations have been made in independently derived β-locus mice, our findings demonstrate a characteristic feature of β-locus mice.

Single cell analysis confirms the divergent behavior of human β-globin loci in mice

To gain additional insight at the single cell level, we employed primary transcript RNA fluorescence *in situ* hybridization (PT-FISH) to examine the relative expression of the endogenous mouse and human globin genes at different stages of ontogeny[16,17]. First, we examined the relative expression of human γ- and β-*globin* (with murine α-*globin* as a control) in E11.5 primitive erythroid cells from two independent transgenic lines (A20 and A85). Consistent with prior analyses demonstrating high-level expression of γ-*globin* at the primitive erythroid stage in β-locus mice, we noted relatively high expression of γ-*globin* by PT-FISH, with low or absent expression of human β-*globin* (Fig. 2a, b). Among circulating primitive cells from a later stage of development (E13.5) a similar pattern was observed, although more human β-*globin* expression was seen and an overall reduction in the percentage of cells with a PT-FISH signal (using the murine α-*globin* control) was noted, with only a fraction of cells (~1/3) showing transcriptionally active loci at a single time point (Fig. 2a, b). Examples of the cells used in this analysis are shown (Fig. 2e–g). An interesting observation made with concomitant PT-FISH analysis of human γ- and β-*globin* is the extent of cotranscription, which represents the concomitant presence of two primary transcript signals within the same gene locus (Supplementary Fig. 2 and Supplementary Text).

Comparison of mouse embryonic εy *globin* with γ-*globin* revealed similar expression of the mouse embryonic gene with human γ-*globin* in circulating primitive cells from E13.5 (Fig. 2c, d, h, i). This finding indicates that expression of the human γ-*globin* genes parallels that of mouse embryonic β-like genes in the mouse trans-acting environment. FL cells from E13.5 were analyzed in a similar manner, by examining the expression of mouse εy and human γ-*globin* by PT-FISH in these cells. Only a low percentage of cells showed staining for either εy or γ-*globin* (Fig. 2c, d), compared with robust transcription of human β-*globin* at the same stage (Fig. 2a, b). Consistent with prior developmental analyses in mice[14,17], cells positive for mouse εy represent circulating primitive cells present within the mouse fetal liver. The cells that are positive for human γ-*globin* expression are also likely to be primitive erythroid cells, and it is important to recognize that in these cells only a fraction (~1/3) of loci are active at any single time point, thereby limiting the degree of concomitant expression seen. Of note, 45 and 54% (in the A85 and A20 lines, respectively) of the primitive cells from E13.5 (PBC) with γ-*globin* transcript showed expression of εy *globin*, supporting the notion that γ-*globin* is treated as an embryonic gene in the mouse trans-acting environment. Interestingly, an early analysis of very low expressing transgenes lacking critical locus region regulatory sequences had suggested that γ-globin indeed behaved as an embryonic gene, as we have shown for mice containing the entire robustly expressed human β-locus[18].

BCL11A restricts mouse embryonic β-like globin expression to the primitive lineage

From these results we conclude that the homologous mouse erythroid trans-acting environment differs from that of the human, presumably with respect to the composition or regulation of critical transcriptional regulators. We have recently shown that the gene *BCL11A*, which harbors genetic variants that affect HbF levels in humans[19–22], encodes a

developmental stage-specific repressor of the human *γ-globin* genes[23]. Our prior findings were confined to an analysis of human erythroid cells, where we found that forms of full-length BCL11A were expressed robustly in adult bone marrow erythroblasts, at substantially lower levels in FL, and absent within primitive erythroblasts. Moreover, shorter variant forms of BCL11A are expressed in human primitive and FL erythroblasts, both of which express γ-globin. To investigate potential species differences in BCL11A protein expression, we examined stage-matched, FACS-sorted populations of mouse and human erythroid cells. Remarkably, comparison of BCL11A expression in mouse and human samples reveals striking differences (Fig. 3a, Supplementary Fig. 3). First, BCL11A protein and RNA transcripts are absent in primitive erythroid cells of mice. Second, full-length forms of BCL11A are expressed at similar levels in definitive erythroid cells of both mouse FL and bone marrow, whereas no shorter variant forms could be identified in mice (Fig. 3a). These results highlight important interspecies differences that could potentially play a role in mediating divergent globin gene regulation. A model based upon our findings of the developmental expression of the β-like globin genes in humans, mice, and β-locus mice is shown, along with a summary of BCL11A expression in these two species (Fig. 3b).

We have demonstrated that expression of the human *γ-globin* genes strictly parallels that of the mouse embryonic genes, *εy* and *βh1*, in the context of the mouse trans-acting environment. Moreover, the pattern of BCL11A expression suggests a role throughout definitive erythropoiesis in mice, as opposed to its predominant role after birth in humans. Thus we hypothesize that changes in expression of BCL11A may be responsible, at least in part, for the observed interspecies divergent expression of β-like globin genes. To test directly a potential role for BCL11A in silencing the endogenous embryonic genes in the definitive erythroid lineage, we examined BCL11A knockout mice. As described previously[24], *BCL11A −/−* mice die in the perinatal period from unknown causes. We examined *BCL11A −/−* mice at E14.5 and E18.5 during gestation when robust definitive erythropoiesis is taking place within the FL (Supplementary Fig. 4). By phenotypic and morphologic approaches[25,26], erythropoiesis appeared ostensibly normal within these embryos (Fig. 4a, Supplementary Figs. 5–7). We then assessed expression of the mouse globin genes. In strong support of our hypothesis, we observed that silencing of expression of mouse embryonic globin genes fails to occur in E14.5 and E18.5 FL erythroid cells (Fig. 4b–e, Supplementary Fig. 8). Restriction of embryonic globin expression to the primitive lineage is lost. Expression of the *εy* and *βh1* globin genes was up-regulated by 70 and 350-fold, respectively, at E14.5 (Fig. 4b). Together these embryonic globin genes account for 50 percent of the total β-like globin genes at this stage, compared with 0.4 percent in the controls. At E18.5, while the contribution of their transcripts to total β-like globin transcripts was somewhat reduced, *εy* and *βh1* globin transcripts were 2600 and 7600-fold elevated compared to controls (Fig. 4c). To determine the cellular distribution of the mouse embryonic globins, we performed immunohistochemistry. Using this approach we found that βh1 and εy globins were robustly expressed in definitive erythroid cells (Fig. 4d, e, Supplementary Fig. 9), whereas normally these embryonic globins are confined to the primitive erythroid lineage[5] (Fig. 3b).

Silencing of human γ–globin expression depends on BCL11A

We then examined the consequence of BCL11A loss on regulation of human globin genes in the β-locus mice. By introducing the β-locus transgene into the knockout environment, we found that in the absence of BCL11A developmental silencing of the *γ-globin* genes is markedly impaired in the definitive erythroid lineage (Fig 4f, Supplementary Fig. 10). In *BCL11A −/−, +/−*, and littermate control mice *γ-globin* RNA comprised 76, 20, and 0.24 percent of total β-like globin gene RNA at E18.5, respectively (Fig 4f, Supplementary Fig. 10). Relaxation of *γ-globin* gene silencing in BCL11A +/− heterozygotes is consistent with

the genetic association of BCL11A and HbF levels and extends our prior observations using knockdown approaches in human cells[23] that together point to BCL11A as a quantitative regulator of γ-globin expression. The failure of γ-globin gene silencing in the face of otherwise ostensibly normal erythropoiesis provides compelling evidence that BCL11A is a major regulator of the globin switches in mouse and human ontogeny.

Concluding Remarks

Taken together, our findings demonstrate how changes in expression of a single transacting factor over the course of evolution may lead to altered developmental gene expression. We have shown that cis-elements within the human β-globin locus are insufficient to recapitulate proper developmental regulation in a mouse context. Previously it has been postulated that the evolution of β-like globin gene expression is largely mediated through changes in cis-elements[27]. Our findings argue persuasively that changes in trans-acting factors may exert striking effects on gene switching during development. BCL11A serves to silence the embryonic genes in mouse definitive erythroid cells, in contrast to its role in humans where it acts to silence γ-globin expression after birth. Moreover, we show that BCL11A is a powerful regulator of the species divergent globin switches by demonstrating that the γ-globin gene escapes proper developmental silencing in a mouse trans-acting *BCL11A* −/− environment. Our findings suggest a model in which one (or more) trans-acting silencers of the embryonic globin genes, initially expressed throughout definitive erythropoiesis, have been altered during primate evolution, such that their expression is shifted to a later phase of definitive erythropoiesis, allowing for the evolution of a unique fetal hemoglobin expression stage. We have shown here that BCL11A represents one of the major factors regulating this switch. These findings allow for simplification of molecular models accounting for this critical developmental transition. This work provides not only unique insights into how alterations in gene expression occur in the course of evolution, but also reveals additional mechanistic clues to the clinically important fetal-to-adult hemoglobin switch in humans.

Methods

Experimental Animals

The wild-type β-globin locus YAC transgenic (β-YAC) mouse strains that were used in this study display a similar pattern of human globin gene expression and are representative of the various strains of transgenic mice harboring the entire human β-globin locus[28–33]. One transgenic mouse line was kindly provided by K. Peterson and was created with the insertion of a 213 kb YAC containing the entire intact human β-globin locus and has been described and characterized previously[28–30]. This β-YAC line contains three intact copies of the human β-globin locus integrated at a single genomic locus. Two β-YAC lines (A20 and A85) harboring a single copy of an ~150 kb β-globin locus YAC were also used in this study and have been described previously[31] (kindly provided by K. Gaensler). These transgenes were maintained in the hemizygous state. The animals were maintained on a pure C57Bl/6 background for all experiments involving adult hematopoietic analysis. A juvenile myelomonocytic leukemia-type myeloproliferative disorder was induced by crossing the Mx1-Cre line with the K-rasG12D conditional allele[34,35], along with the β-YAC transgene from K. Peterson. Congenic B6.SJL-PtprcaPep3b/BoyJ (Ptprca or CD45.1) mice were purchased from Taconic Farms or The Jackson Laboratory. Mice containing a BCL11A floxed allele (with loxP sites flanking exon 1) were created through gene targeting approaches and will be described in future work (G.C.I., S.D.M, and P.W.T., *unpublished*). To obtain the BCL11A null allele, these mice were crossed with GATA1-Cre mice and screened for germline deletion[36,37]. All experiments were performed with the approval of

the Children's Hospital Boston Animal Ethics Committee and the Ethics Committee of the Fred Hutchinson Cancer Research Center.

Adult Hematopoietic Analysis

Analyses of adult hematology, bone marrow transplants, and 5-fluorouracil (5-FU) induction were performed as described previously[38,39]. Whole PB was analyzed on a Beckman Coulter AcT[10] hematological analyzer. Recipient (CD45.1) mice were irradiated with a total of 10.5Gy γ radiation (5Gy and 5.5Gy, 3 hours apart) on the day of transplantation. Whole BM was isolated and pooled from β-YAC mice. A total of 2×10^6 cells/mouse were retro-orbitally injected into recipients. RNA was obtained from blood using the QiaAmp Blood Mini Kit (Qiagen Inc., Valencia, CA) and quantitative RT-PCR (qRT-PCR) was performed as described[38,40] using the human globin gene primers listed below or previously reported murine primers[41]. The human globin gene primers were *e-globin* exon 1 forward 5′-GAGAGGCAGCAGCACATATC-3′, *e-globin* exon 2 reverse 5′-CAGGGGTAAACAACGAGGAG-3′, *γ-globin* exon 2 forward 5′-TGGATGATCTCAAGGGCAC-3′, *γ-globin* exon 3 reverse 5′-TCAGTGGTATCTGGAGGACA-3′, *β-globin* exon 1 forward 5′-CTGAGGAGAAGTCTGCCGTTA-3′, and *β-globin* exon 2 reverse 5′-AGCATCAGGAGTGGACAGAT-3′. The mouse globin gene primers were *ey globin* exon 1 forward 5′-TGGCCTGTGGAGTAAGGTCAA-3′, *ey globin* exon 2 reverse 5′-GAAGCAGAGGACAAGTTCCCA-3′, *βh1 globin* exon 2 forward 5′-TGGACAACCTCAAGGAGACC-3′, *βh1 globin* exon 3 reverse 5′-ACCTCTGGGGTGAATTCCTT-3′, *βmajor/βminor globins* exon 2 forward 5′-TTTAACGATGGCCTGAATCACTT-3′, and *βmajor/βminor globins* exon 3 reverse 5′-CAGCACAATCACGATCATATTGC-3′. The mouse *BCL11A* qRT-PCR primers were forward 5′-AACCCCAGCACTTAAGCAAA-3′ and reverse 5′-ACAGGTGAGAAGGTCGTGGT-3′.

Developmental Hematopoietic Analysis

Embryos were obtained from timed matings, bled, and Ter119 positive cells were sorted based upon forward and side scatter similar to what has been previously described[41]. Cells were maintained in phosphate buffered saline (PBS) with 5% fetal calf serum (FCS). Unfractionated heparin in PBS was added to this solution to a final concentration of 12.5 µg/ml. Immunohistochemistry using an anti-HbF polyclonal antibody was performed on fixed paraffin-embedded sections as described[42]. The fetal livers of E13.5 murine embryos were dissected and a single cell suspension was created. Similarly, bone marrow cells were harvested as has been described previously from mice[38]. In both cases, the cells were labeled with Ter-119 and CD71, as well as 7-AAD. The Ter-119+/CD71+ populations were sorted as described previously[38] Stage-matched human samples were obtained and sorted as previously described[40]. These human samples were kindly provided by H. Mikkola and B. Van Handel.

Western Blot Analysis of BCL11A

Expression of BCL11A was performed using antibody 14B5 (Abcam Inc., ab19487), as described previously[40]. Expression of GAPDH was assessed as a standard using rabbit polyclonal antibody FL-335 (Santa Cruz Biotechnology Inc., sc-25778).

RNA Primary Transcript FISH

Primary transcript RNA FISH was largely performed as previously described[43,44] with some modifications. Prior to hybridization, the slides were equilibrated in 50% formamide/2X SSC, pH 7.0. Single-stranded DNA probes against the introns of the murine *a-* and *ey-* and

human *γ- and β-globin* genes were generated by *in vitro* transcription of cloned intron fragments followed by reverse transcription and inclusion of DIG-11-dUTP, biotin-16-dUTP (Roche) or DNP-11-dUTP (Perkin Elmer) in the reactions as described[45]. Labeled probes were hybridized to the cells in 50% formamide/10% dextran sulfate/2XSSC/5 mM ribonucleotide vanadate complex/0.05% BSA/0.1 mg/ml Cot-1 DNA/1μg/μl *E. coli* tRNA. The probes were heat denatured at 80°C for 5 minutes, preannealed at 37°C, and then hybridized overnight at 37°C in a humid chamber. Slides were washed in 50% formamide/ 2X SSC, pH 7 at 37°C, rinsed in 2X SSC and blocked in 145 mM NaCl/0.1M Tris pH 7.5/2% BSA/2 mM ribonucleotide vanadate complex. Primary transcript foci were detected by indirect immunofluorescence with Cy3-, Alexa Fluor 488- and 647-conjugated antibodies including one or two layers of signal amplification, as described[46].

FISH Image Acquisition and Analysis

Image stacks (Z sections spaced 0.25 μm apart) were captured on an Olympus IX71 microscope (Olympus objective 100X/1.40, UPLS Apo) equipped with a cooled CCD camera using Deltavision SoftWorx software (Applied Precision). The presence of the globin gene primary transcripts was determined in 2D projections of the Z stacks using Photoshop (Adobe). Between 100–200 nuclei were analyzed for each probe set and maturation stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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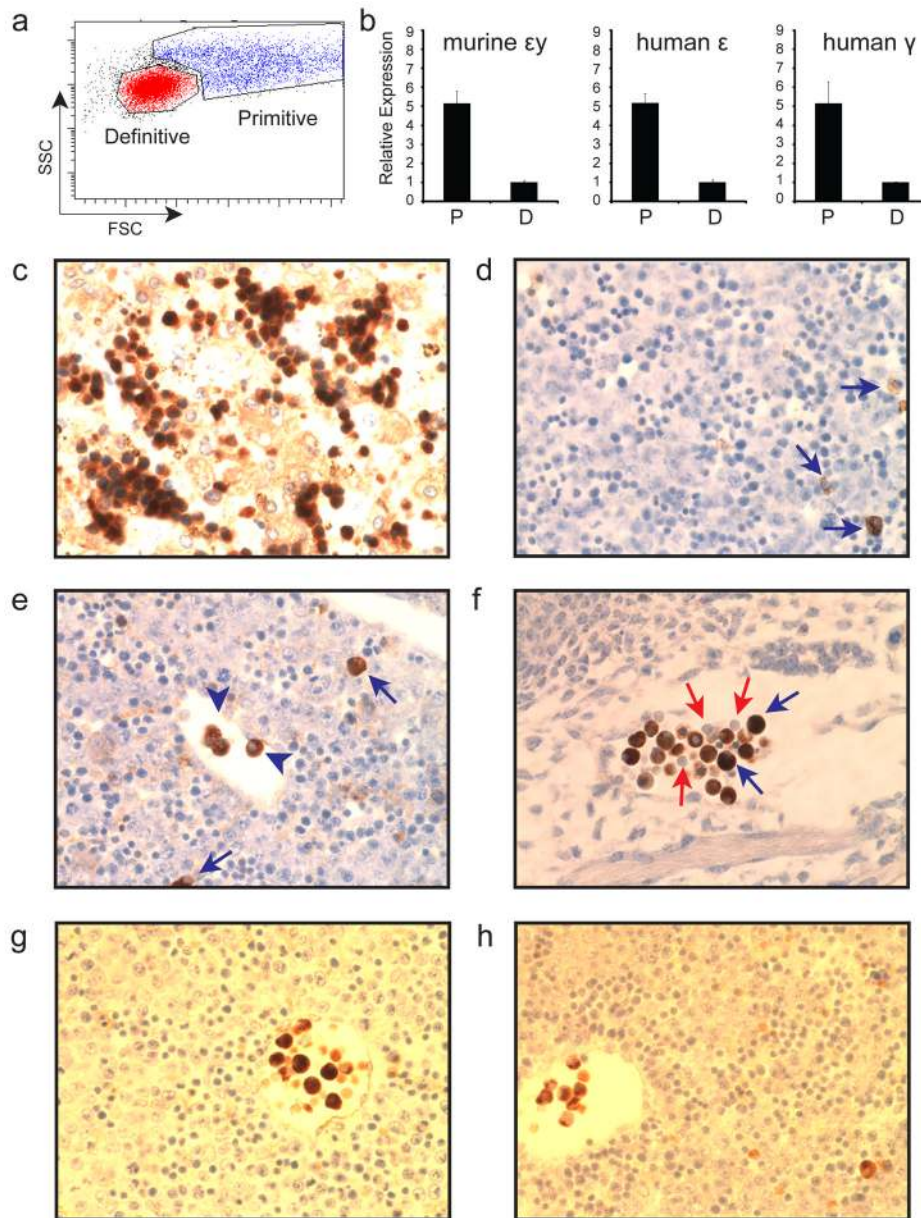
Figure 1. Human γ-globin is primarily expressed in primitive erythroid cells of β-locus mice
a, Representative FACS plot showing FSC (linear scale) versus SSC (log scale) for E13.5 embryonic blood. Gating is shown to allow for the enrichment of primitive (blue population) and definitive lineages (red population). **b,** Relative expression of murine *εy globin* gene, human embryonic *ε* gene, and human *γ-globin* genes showed similar relative enrichment levels in the primitive population (P), as compared with the definitive population (D). Results are shown as mean ± standard deviation (n ≳3 per group). P=0.98 for a two-sided t-test comparing the relative enrichment of *εy* with *γ-globin*. **c–h,** Representative immunohistochemical staining with an anti-HbF antibody from human and murine E13.5 fetal livers. All images are taken with a 60X objective. **c,** Human fetal livers contain numerous erythroblasts, which all stain positive for γ-globin expression. **d,e,** In contrast, murine fetal liver definitive erythroblasts do not show major γ-globin staining and only occasional cells with megaloblastic primitive morphology show staining (blue arrows). **e,f,**

Many megaloblastic primitive cells in the circulation show highly positive staining (**e,** blue arrowheads; **f,** blue arrows), while smaller definitive erythrocytes are negative (**f,** red arrows). **g, h,** Staining performed on the single copy YAC lines A20 and A85[8] showed similar staining patterns. Positive staining was determined in comparison with background staining from transgene negative littermate controls.
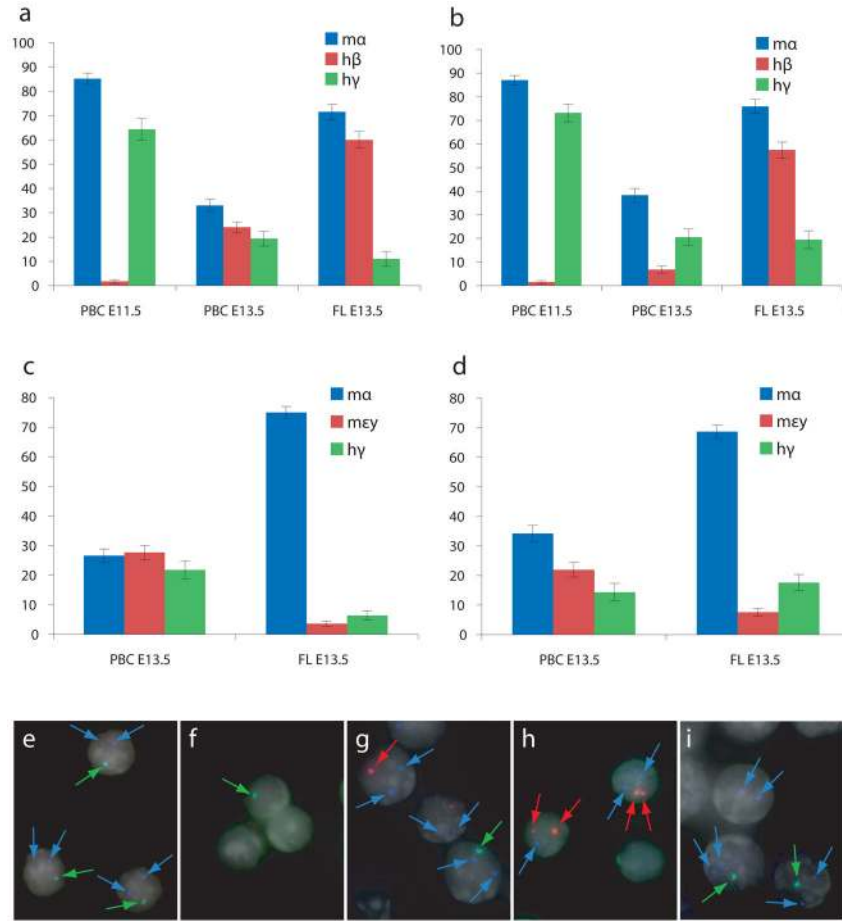
Figure 2. PT-FISH analysis reveals that γ-globin expression parallels the murine embryonic globins in primitive erythroid cells
Two independent lines of transgenic YAC mice, A85 (**a,c**) and A20 (**b,d**) were analysed using four color primary transcript RNA fluorescence *in situ* hybridization (PT-FISH). For the first set of experiments, probes were made to target murine *α-globin* (mα), human *β-globin* (hβ), and human *γ-globin* (hγ). Additionally DAPI was used to identify nuclei of cells. **a,b,** Expression of *γ-globin* predominates within the two lines in the primitive populations seen circulating in primitive blood cells (PBC) from embryos E11.5 and E13.5. Minor expression is seen in the mature definitive populations from fetal liver (FL) at E13.5. Many of these cells may represent primitive cells found within the FL parenchyma. **e–g,** Representative images with this staining pattern of each developmental time point are shown, respectively, for PBC at E11.5, PBC at E13.5, and FL at E13.5. **c,d,** Probes were made to target murine *α-globin* (mα), murine *εy globin* (mεy), and human *γ-globin* (hγ). These data reveal parallel expression of mεy and hγ. **h,i,** Representative images with this staining pattern are shown for PBC at E13.5 and FL at E13.5, respectively. The color of the bars in all of the graphs corresponds to the colors of the probes that were detected for each of these primary transcripts. The graphs depict the percentage of active loci and are measured for ≥100 nuclei per probe set at each time point.
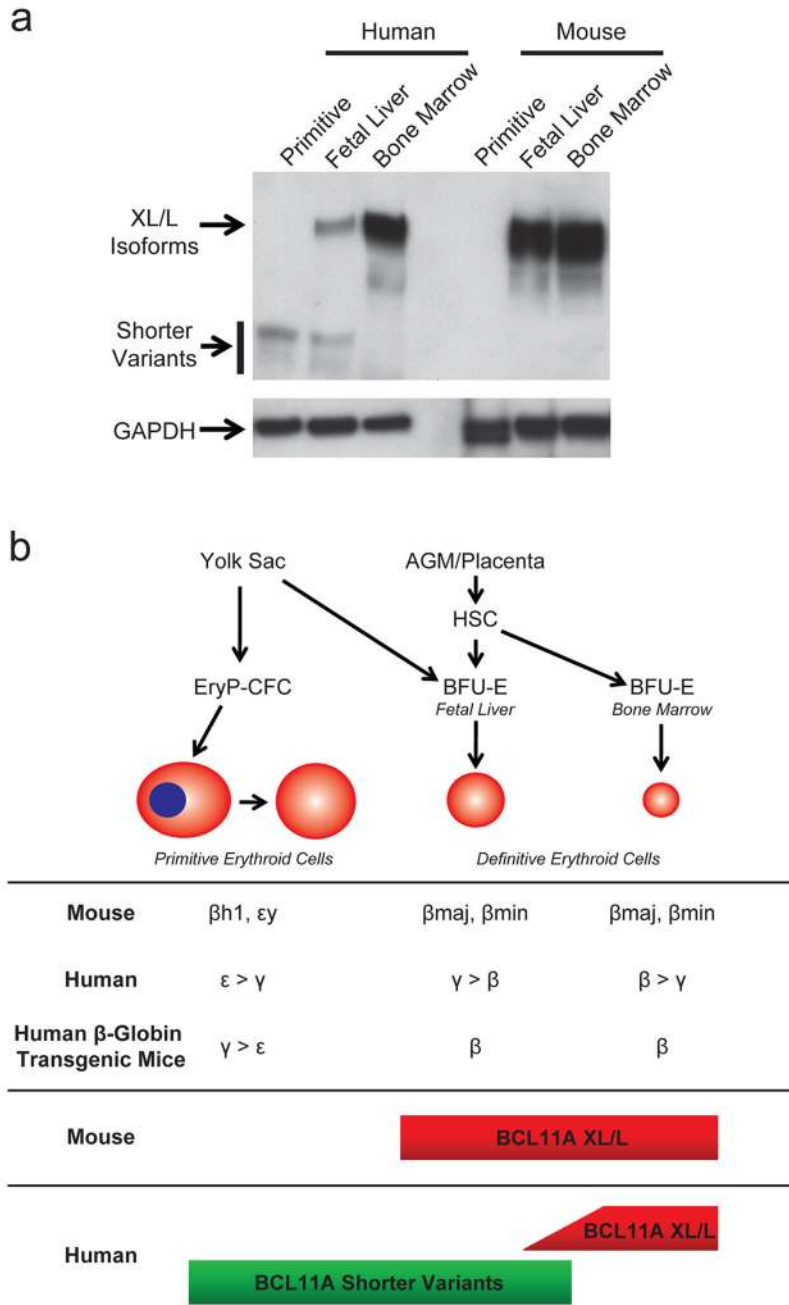
Figure 3. BCL11A expression varies between humans and mice, suggesting a model for trans-acting variation in β-globin gene expression

a, In human cells full-length proteins of BCL11A (XL/L isoforms) are reduced within cell populations that express high levels of γ-globin, including primitive and fetal liver cells[23]. Additionally, short variant forms are present at these earlier developmental stages. All human cells were sorted for CD235 and CD71 expression. In contrast, in murine cells, full-length BCL11A protein expression is evident in all definitive progenitor populations, including sorted stage-matched E13.5 fetal liver and bone marrow erythroid cells (all populations were sorted for Ter119+/CD71+). No expression of BCL11A within murine primitive cell populations was detected. **b,** This model summarizes the ontogeny of β-like globin gene regulation in humans, mice, and β-locus mice[5,9]. The ontogeny of mammalian

erythropoiesis and progenitor populations is shown at the top. Progenitor populations, including primitive erythroid populations (EryP-CFC), definitive hematopoietic stem cells (HSC), and definitive erythroid burst-forming unit cells (BFU-E) are depicted. The aorto-gonado-mesonephros (AGM) and placenta are sites of definitive hematopoiesis. The patterns of β-like globin and BCL11A expression seen in the two species are shown below.
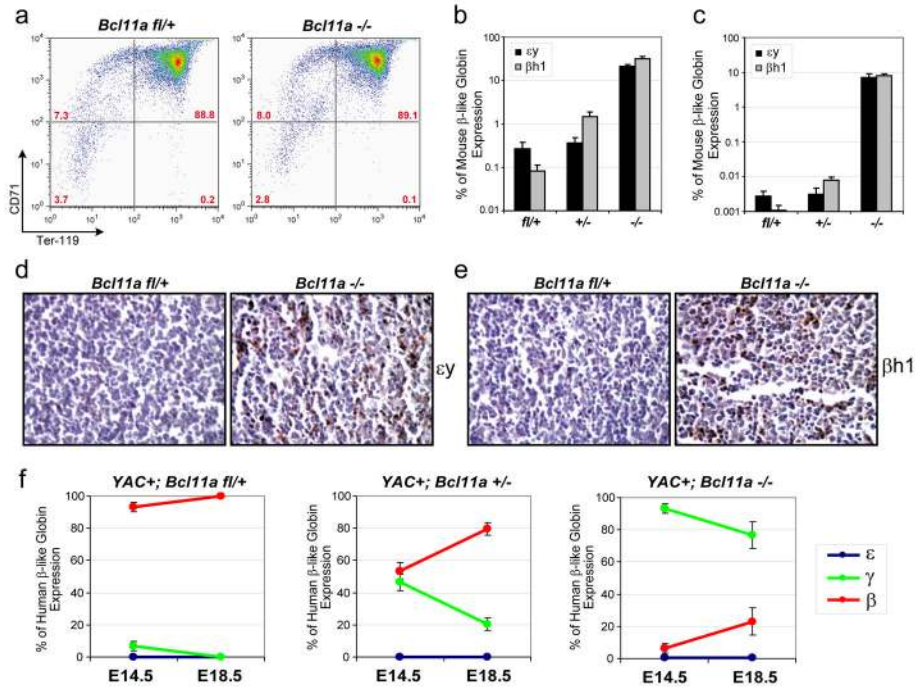
Figure 4. BCL11A −/− mice fail to silence expression of mouse embryonic β-like globins and human γ-globin genes

a, The CD71/Ter119 expression pattern is shown for fetal liver cells from E14.5 embryos, revealing grossly normal erythropoiesis with these phenotypic markers. The mean percentages for the populations in each quadrant are shown in red (n=6 for fl/+ controls and n=4 for −/− mutants). The P > 0.1 by a two-sided t-test for all gated populations analyzed. **b,** The expression of the embryonic globins is shown as a percentage of total mouse β-like globins for control mice (fl/+), BCL11A heterozygous (+/−), and null mice (−/−) at E14.5 (n=10,14,11 respectively). **c,** The expression of the embryonic globins is shown as a percentage of total mouse β-like globins at E18.5 (n=9,9,7 respectively) **d,** Immunohistochemistry was performed on E14.5 FLs from BCL11A fl/+ and −/− animals for the embryonic globin εy. Representative sections at 40X magnification with a 10X objective lens are shown. **e,** Similar IHC staining was performed for βh1 globin. In both cases robust expression is seen in the scattered erythroblasts of the FL in −/−, but not control mice. **f,** Expression of human β-globin locus genes is shown for animals with the various BCL11A genotypes in the presence of the β-locus YAC transgene (YAC+) at E14.5 (n=4,6,4 for the fl/+, +/−, and −/− animals, respectively) and E18.5 (n=4,7,4). All γ- and β-globin levels for the different genotypes are significantly different (P < 1×10^{-5} by a two-sided t-test). All data are plotted as the mean ± the standard deviation of the measurement.