Mild dyserythropoiesis and β-like globin gene expression imbalance due to the loss of histone chaperone ASF1B

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Abstract

The expression of the human β-like globin genes follows a well-orchestrated developmental pattern, undergoing two essential switches, the first one during the first weeks of gestation (ε to γ), and the second one during the perinatal period (γ to β). The γ to β globin gene switching mechanism includes suppression of fetal (γ-globin, HbF) and activation of adult (β-globin, HbA) globin gene transcription. In Hereditary Persistence of Fetal Hemoglobin (HPFH), the γ-globin suppression mechanism is impaired leaving these individuals with unusual elevated levels of fetal hemoglobin (HbF) in adulthood. Recently, the transcription factors KLF1 and BCL11A have been established as master regulators of the γ to β globin switch. Previously a genomic variant in the *KLF1* gene, identified by linkage analysis performed on twenty-seven members of a Maltese family, was found to be associated with HPFH. However, variation in the levels of HbF amongst family members, and those from other reported families carrying genetic variants in *KLF1,* suggest additional contributors to globin switching. ASF1B was downregulated in family members with HPFH.

Here, we investigate the role of ASF1B in γ to β globin switching and erythropoiesis *in vivo*. Mouse-human interspecies ASF1B protein identity is 91.6%. By means of knockdown functional assays in human primary erythroid cultures and analysis of the erythroid lineage in *Asf1b* knockout mice, we provide evidence that ASF1B is a novel contributor to steady-state erythroid differentiation, and while its loss affects the balance of globin expression, it has no major role in hemoglobin switching.

Background

The expression of the β-like globin genes follows a well-orchestrated developmental pattern, undergoing two essential switches, the first one during the first weeks of gestation (ε to γ), and the second one around birth (γ to β).[1-4] Therefore, fetal hemoglobin (HbF), composed of two α-globin and two γ-globin chains, is the dominant type of hemoglobin during the fetal stages of development. Around birth, HbF is gradually replaced by adult hemoglobin (HbA), consisting of two α-globin and two β-globin chains. Already at 6 months of age the major hemoglobin in the circulation is HbA.[5] However, residual amounts of HbF continue to be synthesized throughout adult life, and the amounts vary considerably, with the large majority of adults having less than 1% of HbF. Our understanding of β-like globin transcriptional control is historically based on Mendelian models of inheritance of natural mutants. Indeed, a series of genetic variants of the β-globin cluster have been discovered that impair the fetal-to-adult hemoglobin switch, leading to persistent γ-globin expression and elevated HbF throughout adult life. This condition is termed Hereditary Persistence of Fetal Hemoglobin (HPFH).[6] There are two types of HPFH variants in the β–globin locus: point mutations in the promoter of the γ-globin genes (*HBG1* or *HBG2*) and deletions removing substantial regions of the β-globin cluster, often including the β-globin gene (*HBB*).[7] A range of conditions characterized by HbF levels that do not fit clear Mendelian inheritance models or the typical HPFH phenotype, *i.e.* high HbF levels accompanied by concomitant lower HbA_2 ($\alpha_2\delta_2$) levels, have been previously reported. Although some of this variability can be explained by the β-globin cluster haplotype, a substantial proportion of the HbF increase is not linked to the β-globin cluster, suggesting multifactorial genetic players in the fine tuning of β-like globin gene expression regulation.

It is now evident that common HbF variation is a quantitative genetic trait, shaped by common polymorphisms in genes that are not related to the human β-like globin gene cluster. There are only a few examples of such SNPs in genes, affecting for example *BCL11A* residing on

chromosome 2[8-11] and the intergenic region between the *MYB*[12] and *HBS1L* genes that resides on chromosome 6q22.3.[7, 13-15] These genes have been identified by genome-wide association or traditional linkage analysis approaches. In addition, it has been previously reported that genetic variants in the *KLF1* gene are causative for HPFH, in part by rendering the expression of BCL11A low.[16-18] The identification of some of the direct transcriptional regulatory regions within the β-like globin gene cluster targeted by these transcription factors has been reported.[15, 19-21]

The extremely high variation of HbF levels observed in carriers of the *KLF1* p.K288X variant led us to further examine other potential contributors to the fine-tuning of the γ- to β-globin gene expression balance.[22]

Whole transcriptome analysis performed on mRNA from primary erythroid cultures revealed that expression levels of *ASF1B,* located in chromosome 19, close to the region that showed the highest LOD scores when using multi-point parametric analysis at a penetrance of 90%,[16] were downregulated to 70% in HPFH family members. *ASF1B*, anti-silencing function 1B histone chaperone gene, is a member of the evolutionary conserved family of H3/H4 histone chaperone proteins. In human and mouse there are two paralogues of the Asf1 protein in yeast, namely ASF1A and ASF1B.[23-26] However, ASF1A and ASF1B are distinct in expression pattern and function. While ASF1A is ubiquitously expressed, ASF1B is expressed in highly proliferating tissues, and its expression levels decrease in terminally differentiated and quiescent cells.[27] It was described previously that ASF1B and histone H3.3 promote pancreatic β-cell proliferation in a synergic manner.[28] The ASF1B protein is the substrate of the tousled-like kinase family of cell cycle-regulated kinases,[29-31] and is thought to play a key role in modulating the nucleosome structure of chromatin by ensuring a constant supply of histones at sites of nucleosome assembly,[32] while interacting directly with transcription regulators.[23, 24, 33, 34] ASF1B also cooperates with chromatin assembly factor 1 (CAF-1) to promote replication-

dependent chromatin assembly,[35, 36] but it does not participate in replication-independent nucleosome deposition mediated by ASF1A and HIRA.[27, 37] Mouse-human interspecies ASF1B protein identity is 91.6%.

In the present study we have employed lentivirus-mediated knockdown in primary human erythroblast cultures and analyzed the *Asf1b* KO mice [38] to demonstrate that ASF1B histone chaperone is functionally linked to steady-state erythropoiesis. Our data shows that loss of ASF1B compromises steady-state erythropoiesis with modest effects on the regulation of the β-like globin genes.

Results

ASF1B is downregulated in human primary erythroid progenitors carrying the p.K288X KLF1 variant

A SNP in the *KLF1* gene resulting in p.K288X protein variant, causative of the inherited HPFH in 10 out of 27 recruited members of a Maltese family, was reported previously.[16] The identification of the point mutation in the Maltese family was achieved through SNP linkage analysis, which pointed to a region in chromosome 19p13.12-13 that showed the highest LOD scores when using multi-point parametric analysis at a penetrance of 90%.[16] No evidence of linkage was observed to other regions, including the *HBB* locus and the HPFH-linked loci of chromosomes 2q33 and 6q22.3. However, HbF levels ranged from ~3% to almost 20%, suggesting other contributing factors to the variation of HbF levels amongst HPFH family members.[16]

Examining in more detail the region in chromosome 19 that showed the highest LOD scores and the expression in erythroid primary cultures of the neighboring genes in close proximity (GSE22109), we identified *ASF1B*, which was found to be downregulated in HPFH family members (Figure 1A).[16] As shown in Figure 1A, and as previously reported, KLF1 targets *BCL11A*, *CD44*, *E2F2*, *E2F4* and *HBB* were significantly downregulated and the γ-globin vs total γ+β globin expression ratio was increased in primary erythroid cells derived from HPFH individuals.[16] We confirmed that *ASF1B* mRNA levels were significantly reduced to 70% in HPFH family members presenting with the highest HbF levels. Since we could not exclude that the transcription of *ASF1B* is dependent on KLF1 or BCL11A activities, we performed knockdown of either KLF1 or BCL11A in healthy human primary erythroid progenitors and analyzed *ASF1B* expression levels accordingly. BCL11A downregulation did not affect the expression of ASF1B at all, while KLF1 knockdown resulted in a small reduction of ASF1B expression which did not reach statistical significance (Figure 1B). This suggests that the significant reduction of ASF1B expression levels in primary erythroid cells cultured from the

HPFH family members might be partially, but not exclusively, due to transcription regulation by KLF1.

Knockdown of ASF1B in human primary erythroid cells induces γ-globin expression

To functionally assess the consequences of ASF1B downregulation on human β–globin gene expression, we performed knockdown experiments on primary human erythroblasts derived from healthy donors. We used three shRNA lentiviral constructs against ASF1B, of which two (#23 and #24, Supplementary Table 1) resulted in efficient knockdown as measured by RT-qPCR. Therefore, we used those for further analysis. We included BCL11A and KLF1 lentiviral shRNA-transduced cells as positive controls for γ-globin reactivation and a non-specific scrambled (Scr) shRNA as negative control. After lentiviral transduction and 24hr selection with puromycin, cells were induced to differentiate (+Epo and holo-transferrin), and after 24hrs, cells were collected for analysis. We confirmed shRNA-mediated downregulation of BCL11A, KLF1 and ASF1B transcripts, respectively, by RT-qPCR (Figure 2A). We then investigated the impact of ASF1B knockdown on BCL11A and KLF1 expression. We found that knockdown of ASF1B did not affect the expression of KLF1 and BCL11A levels significantly (Figure 2B). It is known that one of the principal targets of KLF1 is the *HBB* gene (encoding β-globin),[39, 40] and we could verify that by knocking down *KLF1* gene expression. Indirect down-regulation of *HBB* gene transcription, due to the upregulation of γ-globin expression, has been reported upon BCL11A knockdown in human proerythroblasts. [41] We also observed this in our human erythroid cultures (Figure 2C). In contrast, ASF1B knockdown did not reduce the levels of *HBB* mRNA expression (Figure 2C). *HBA* expression levels were also not altered significantly in the ASF1B KD experiments. When measuring hemoglobin composition from the lentiviral transduced HEPs by qRT-PCR and HPLC, we observed that ASF1B knockdown increased γ-globin expression, as shown by the percentage of HbF of total hemoglobin in the samples (Figure 2D).

Asf1b KO mice display mild dyserythropoiesis

To investigate the potential contribution of ASF1B in erythropoiesis and globin gene expression regulation, we set out to study these processes in *Asf1b* knockout (KO) mice. Complete blood count was performed on blood samples from adult *Asf1b* KO mice and wildtype (WT) littermates (Figure 3A). Interestingly, *Asf1b* KO mice present with a reduced red blood cell (RBC) count (8.62 versus 9.34 x 10^9 cells/mL; $p < 0.05$), an increased mean cell volume (MCV 49.15 versus 46.10 fL; $p < 10^{-7}$]) and an increased mean corpuscular hemoglobin (MCH 15.62 versus 14.80; $p > 10^{-6}$), suggestive of compensated anemia. Their spleen was significantly enlarged (116.91 versus 90.20 mg; $p < 0.05$), supporting this notion, considering that the body size of *Asf1b* KO mice had a tendency to an increase that did not reach significance (Table 1).

We next set out to study the erythroid compartment in the bone marrow and spleen of *Asf1b* KO mice (Figure 3B-C). The bone marrow presented an accumulation of early erythroid progenitors ($CD71^+Ter119^{neg}$) and a significant reduction of the Ter119 positive cells, "A" ($CD71^+FSC^{high}$), "B" ($CD71^+FSC^{low}$) and "C" ($CD71^{neg}FSC^{low}$) corresponding to more mature erythroid cells (Figure 3B, bar graph), based on the gating strategy by Socolovsky.[42] When the megakaryocyte-erythroid-progenitor (MEP) fraction was analyzed, we observed an increase of these progenitors in *Asf1b* KO bone marrows (Figure 3B, dot plot). Analysis of the splenic erythroid compartment revealed a significant increase of early erythroid progenitors (Proerythroblasts; $CD71^+Ter119^{neg}$), accompanied a significant reduction of more mature cells, specially population "C", indicative of moderate stress erythropoiesis (Figure 3C). Altogether this data suggest that *Asf1b* KO mice display mild dyserythropoiesis and have engaged stress erythropoiesis in the spleen.

Persistence of embryonic globin expression in adult Asf1b KO mice

We next set out to analyze the expression of the β-like globin genes in blood, bone marrow and spleen of *Asf1b* KO mice and WT littermates. In the mouse, the developmental progression of

β-like globin gene expression is marked by the expression of εy and βh1 in the embryonic stage, which is replaced by βmaj + βmin at the fetal stage. RT-qPCR revealed significant upregulation of the expression of εy-globin in blood and bone marrow, with a modest upregulation in the spleen which did not reach statistical significance (Figure 4). The upregulation of εy-globin hemoglobin occurred without major alterations in the expression levels of other embryonic globins, *i.e.* βh1- and ζ-globin. A significant reduction, although very mild, was identified in the bone marrow in the total amount of α-globin expression compared to the total amount of β-globin (Figure 4B). This imbalance was not detected in the blood of *Asf1b* KO mice (Figure 4A). This suggests that the normal α/β-globin ratio is restored via a post-transcriptional mechanism, reminiscent of erythropoiesis in individuals with thalassemia trait. Thereof we conclude that the increased expression of εy-globin is not due to hampered expression of the other globins. Collectively, these data indicate that although not to the same extent as when knocking down BCL11A (direct regulator) or KLF1 (indirect regulator via *BCL11A* and *HBB*), lower expression levels of ASF1B promote γ-globin expression, apparently without reducing *HBB* gene expression. Our data show that ASF1B loss of function in mice causes mild dyserythropoiesis with persistence of εy globin in adult hematopoietic tissues, and thus supporting the notion that the imbalance of β-like globin expression might be due to dysregulated erythroid differentiation, rather than to a direct role of ASF1B in globin switching.

Discussion

In this study, we report on the role of ASF1B in erythropoiesis and globin gene expression. ASF1B is known to function as a histone chaperone protein important in chromatin remodeling,[32] nucleosome assembly and disassembly, and when knocked down it is also known to influence gene expression.[43] It has been recently reported that ASF1B might play a role in epigenetic reprogramming,[44, 45] and furthermore, in alternative telomere lengthening.[46] However, the complete spectrum of ASF1B functions has not yet been asserted, and especially interesting is the reported cooperation between ASF1B and general transcription factors such as the TFIID.[24, 33] Microarray analysis on erythroid cells from a family carrying KLF1 p.K288X variant leading to HPFH showed that family members with high HbF levels had reduced *ASF1B* gene expression levels compared to family members with normal (low) HbF levels. We studied the possibility of KLF1 directly regulating ASF1B expression by performing knockdown experiments on primary HEPs. However, we did not observe significant downregulation of ASF1B as a direct consequence of KLF1 downregulation, possibly due to the short-term nature of the knockdown assays. It has been reported that *ASF1B* gene expression is regulated by E2F factors.[47, 48] Since E2F2 and E2F4 are direct targets of KLF1, and also downregulated in HPFH family members (Figure 1A), we cannot exclude an indirect contribution of the *KLF1* variant to the downregulation of ASF1B expression via E2F2/E2F4.

Importantly, the microarray data shows a significant decrease of *HBB* gene transcription in those family members with high HbF levels, similarly to what has been documented for cis-acting genetic variants or SNPs in the *HBB* gene promoter CACCC box (see also http://globin.bx.psu.edu/hbvar)[20, 21] to which KLF1 binds. In addition, γ-globin gene expression is upregulated. Thus, it is the balance β- vs γ-globin expression that results in the high percentage of HbF, in this case by directly affecting the expression of γ- and β-globin in an opposite manner.

Since microarrays are not suitable for measuring globin expression due to saturation of the probe sets, we confirmed these dynamics with RT-qPCR analysis in knockdown assays. The results of knockdown experiments of KLF1 and BCL11A in adult erythroid progenitors from healthy donors, agreed with such an observation, *i.e.* increase of HbF levels compared to the control shRNA, with accompanying decrease in β-globin expression (Figure 2). This effect on β-globin expression downregulation has been seen in single and compound knockout mouse models for *Klf1* and *Bcl11a*. [49] Interestingly, knockdown of ASF1B resulted in an increase of HbF, without significantly altering KLF1, BCL11A or β-globin expression (Figure 2).

It has been recently reported that KLF1 recruits histone chaperones HIRA and ASF1A to maintain an open β-globin locus, and that this recruitment is necessary for proper β-globin transcription.[40] Although ASF1A and ASF1B are paralogues, it is known that ASF1B does not cooperate with the ASF1A/HIRA complex and that it performs separate actions on nucleosome assembly.[27, 37] Our results are therefore in agreement with this notion, since depletion of ASF1B did not affect β-globin expression, neither in the *Asf1b* KO mouse model, nor in human erythroid progenitors where ASF1B had been knocked down.

The analogy between KLF1 and ASF1B goes further regarding general erythropoiesis. It has been recognized that a special case of genetic variant of KLF1 is causative of congenital dyserythropoietic anemia (CDA) variant (type IV) in addition to the accompanying HPFH already reported. Interestingly, the two genes involved in the development of CDA type I are CDAN1 and C15ORF41, both of which have been reported to physically interact with ASF1B.[50-54] This suggests that shared functions by the interacting proteins ASF1B, CDAN1 and C15ORF41 might be crucial during erythropoiesis and for β-like globin expression regulation.

Indeed, loss of function studies in *Asf1b* KO mice reveal mild dyserythropoiesis and persistence of εy-globin expression in adult hematopoietic tissues, without altering the expression levels of

other β-like globins. While there was a statistically significant reduction of the total β-like globins compared to α-globins, this was only identified in the bone marrow, and it was very mild.

These results, which are in total concordance with our observations in human cellular models of erythropoiesis, indicate that ASF1B fine-tunes erythropoiesis *in vivo*.

Conclusions

In essence, our data indicate that ASF1B cooperates to adult steady-state erythroid differentiation and, most probably associated with this mechanism, has an accessory function in silencing of the embryonic globin genes. At present, the exact mechanism by which ASF1B exerts its function related to globin gene expression remains unclear. It has been suggested that ASF1B deficiency results in cell cycle defects due to impaired nucleosome assembly.[26, 35, 36, 54, 55] In this regard, a defective shorter cell cycle during erythropoiesis might result in the production of immature “skipping division” erythroid cells that contain higher levels of HbF. In fact, hemolytic treatment in mice or baboons, results in an increase of erythroid cells containing fetal/embryonic globin.[56, 57] It has also been described that ASF1B interacts with TFIID (24) and transcription regulators, that we have previously showed to be important for mouse erythropoiesis.[58] Potential loss of coordination between chromatin remodeling, chromatin accessibility and transcription regulation, might affect gene expression of the β-like globin genes.[24, 33] How ASF1B is involved in these processes (cell cycle/replication, transcription, epigenetic regulation) and its role in the molecular mechanism behind the transcription/silencing of the γ-globin genes should be the subject of future investigations.

Methods

Mice

Asf1b knockout mice were generated as described previously.[38] All animal studies were performed in compliance with the European Community regulation for laboratory animal care and use (Directive 2010/63/UE). The mouse facility of the CEA at Fontenay-aux-roses received the agreement delivered by the French Ministry of Agriculture (n° D92032-02). Mice were sacrificed by cervical dislocation. All efforts were made to minimize animal stress and suffering. Mice were housed in controlled 12h light:12h darkness conditions (lights on from 08:00 to 20:00 h) and were supplied with commercial food and tap water *ad libitum*. The colony of mice was maintained by breeding heterozygous mice, so knockout mice could be compared with wildtype littermates, and all mating pairs in the same mouse facility. Mouse were collected for analysis at 3-8 months age.

Flow cytometry

Bone marrow and spleen single cell suspensions were labelled with respective antibody cocktails. For the analysis of the erythroid compartment, CD71-APCCy7 and Ter119-Pacific Blue antibodies were used (BD), and successive stages of erythroid maturation were calculated as previously described.[42] For MEP analyses, the BM cells were first treated with a 0.75% NH_4Cl solution (Sigma-Aldrich, St Louis, MO, USA) to lyse red blood cells and labeled with a biotinylated lineage cell detection cocktail (Miltenyi Biotec) and revealed by streptavidineFluor450 (eBioscience). The cells were then labeled with PE-conjugated anti-CD34, APCCy7-conjugated anti-c-Kit, APC-conjugated anti-Sca-1 and PECy7-conjugated anti-CD16/32 (eBioscience) antibodies, and MEPs were identified as Lin−, Sca-1−, c-Kit+, CD16/32−, CD34−.[59] At least 300,000 cells were acquired.

The cells were analyzed using a FACSCanto II and a SORP LSRII (BD) equipped with blue (488nm), violet (405nm) and red (638nm) lasers. Data were analyzed with the FlowJo software.

Cell cultures

Human erythroid progenitor cells (HEPs) were cultured as described [16, 60] in the presence of recombinant human Epo (1 U/ml), recombinant human SCF (100 ng/ml) and dexamethasone (10^{-6} M, Sigma) from processed buffy coats of active blood donors (Sanquin Blood Bank), according to the guidelines of the Declaration of Helsinki and after approval of the local ethical committee. Cells were cultured at 1.5 - 3 × 10^6/ml through daily dilutions or medium replacement. Cells were counted with an electronic cell counter (CASY-1, Schärfe-System, Germany). To induce terminal differentiation, cells were washed and transferred to medium with recombinant human Epo (10 U/ml), and a high concentration of iron-loaded transferrin (0.5 mg/ml). Cells were harvested for further analysis (RNA extraction and HPLC) after 24hr in differentiation medium.

Transduction of human proerythroblasts with shRNA lentiviral constructs

Lentivirus was produced by transient transfection of HEK 293T cells according to standard protocols.[16, 61] Two days after transfection, the supernatant was collected, filtered and concentrated by centrifugation at 20 krpm for 2h at 4°C. HEPs cultured for one week from fresh buffy coats from healthy volunteers (Sanquin Blood Supply) were transduced in 24-well plates. We used 0.5×10^6 cells per well and sufficient amounts of virus to transduce ~80% of the cells at day 1. Puromycin (1 μg/ml final concentration) was added to the cells at day 2, and selection was performed for 24 hours. At day 3, cells were induced to differentiate and were harvested at day 4 for RNA extraction and HPLC analysis as described above. The clones from The RNAi Consortium (TRC[62]; Sigma) used were: The non-target SHC002 vector (Scrambled "Scr" control; SHC002: 5'-CAACAAGATGAAGAGCACCAA-3'); KLF1 shRNA clone TRCN0000016276;[16] BCL11A shRNA clone TRCN0000033449 (validated by us), and ASF1B shRNA clones TRCN0000074225, TRCN0000074226 and TRCN0000074227, of which only the last two gave significant knockdown. Sequences are listed in Supplementary Table 1.

High Performance Liquid Chromatography (HPLC)

Separation and quantification of Hb fractions was performed by high-performance cation-exchange liquid chromatography (CE-HPLC) on Waters Alliance 2690 equipment (Waters, Milford, MA, USA). The protocol consisted of a 30-min elution over a combined 20-200 mM NaCl and pH 7.0-6.6 gradient in 20 mM BisTris/HCl, 2 mM KCN. The column, a PolyCAT A 100/4.6-mm, 3-μm, 1500-Å column, was purchased from PolyLC (Columbia, MD, USA).[63]

RNA extraction and RT-qPCR analysis

Total RNA (1 μg) from each harvested erythroid cell sample after extraction by Trizol (Invitrogen) was converted to cDNA with SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Paisley, UK). For mouse tissues, the RNeasy Mini Kit (Quiagen) was used to isolate total RNA according to the manufacturer's instructions. Primers used to amplify *BCL11A*, *ASF1B*, *E2F2*, *E2F4*, *HBB* and *HBG1/HBG2* human genes and mouse globin genes plus controls are listed in Supplementary Table 1.

All amplifications took place with SYBR Green PCR Master Mix (Applied Biosystems). RT-qPCR was performed on the Bio-Rad Optical IQ Thermal Cycler (Bio-Rad) under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 62°C for 30 seconds. All reactions were performed in triplicate.

Target gene expression was normalized to GAPDH expression. Gene expression levels were calculated with the 2(−DeltaDeltaC(T)) method.[64]

DECLARATIONS

Ethics approval and consent to participate

The buffy coats of active blood donors (Sanquin Blood Bank), were processed according to the guidelines of the Declaration of Helsinki and after approval of the local ethical committee.

All animal studies were performed in compliance with the European Community regulation for laboratory animal care and use (Directive 2010/63/UE). The mouse facility of the CEA at Fontenay-aux-roses received the agreement delivered by the French Ministry of Agriculture (n° D92032-02).

Consent for publication

Not applicable.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

PP designed/performed experiments, analyzed data and wrote the manuscript; AK performed experiments and analyzed data; IMDC, MV, JB, NG, VB, DL and ZK performed experiments; FGG, CMBC and RvZ designed experiments and participated in discussions; MV generated the *Asf1b* KO mouse model, designed experiments, analyzed data and reviewed the paper; SP, LG and GPP designed/performed experiments, analyzed data and wrote the paper. All authors read and approved the final manuscript.

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Authors' information

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Figure Legends

Figure 1: ASF1B is downregulated in primary erythroid progenitors carrying the p.K288X KLF1 variant.

A. Expression analysis from publicly available microarray data (GSE22109)[16] derived from cultured primary erythroid cells (HEPs) of Maltese family members with HPFH (green bars, n=3) and normal family members (grey bars, n=3). Family members with HPFH display reduction of BCL11A, ASF1B, E2F2, E2F4, CD44, HBB and increased HBG/(HBB+HBG) ratio. Mean and standard deviation are depicted. T-test *p* values are indicated. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

B. Analysis of KLF1, BCL11A and ASF1B expression by RT-qPCR on HEPs derived from buffy coats from healthy individuals and transduced with shRNA lentiviruses targeting KLF1 and BCL11A. Knockdown of either factor does not influence significantly ASF1B expression. Expression levels are normalized setting expression levels of mock knockdown controls at 100. Mean and standard deviation are depicted. Shadowed box at 100 corresponds to expression levels in mock knockdown controls. T-test *p* values are indicated. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Figure 2: Knockdown of ASF1B in human primary erythroid cells induces γ-globin expression.

A. Expression analysis by RT-qPCR of ASF1B, BCL11A and KLF1 (last two taken from Fig. 1B) on shRNA lentivirus-transduced HEP cultures. Expression levels are normalized setting expression n levels of mock knockdown controls (Scr) at 100. Mean and standard deviation are depicted. Shadowed box at 100 corresponds to expression levels in mock knockdown controls. T-test *p* values are indicated.

B. Expression of BCL11A and KLF1 is not significantly affected in ASF1B knockdown HEPs as analyzed by RT-qPCR. Expression levels are normalized setting expression levels of

mock knockdown controls (Scr) at 100. Mean and standard deviation are depicted. Shadowed box at 100 corresponds to expression levels in mock knockdown controls. T-test *p* values are indicated.

C. *HBB* and *HBA* expression analysis by RT-qPCR. Knockdown of either KLF1 or BCL11A has an effect on *HBB* expression, whereas ASF1B knockdown does not, while overall, *HBA* expression is not affected. Expression levels are normalized setting expression levels of mock knockdown controls (Scr) at 100. Mean and standard deviation are depicted. Shadowed box at 100 corresponds to expression levels in mock knockdown controls. T-test *p* values are indicated.

D. RT-qPCR of *HBG* expression and HPLC analysis of hemoglobin composition shows the increase in HbF after knockdown of ASF1B in HEPs. Values are normalized to those measured in mock knockdown controls (Scr). Mean and standard deviation are depicted. Shadowed box at 100 corresponds to expression levels in mock knockdown controls. T-test *p* values are indicated.

Figure 3: *Asf1b* KO mice display mild dyserythropoiesis.

A. Complete blood count of blood samples from *Asf1b* KO mice and WT littermates. The Red Blood Cell (RBC) counts, the Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) are depicted on individual animals. The black bar is the mean. T-test *p* value is indicated.

B. Left, bar graph depicting flow cytometry analysis of the erythroid compartment in the bone marrow of *Asf1b* KO and WT littermates following the gating strategy of Socolovsky. [42] Right, dot plot depicting the basal percentage of megakaryocyte-erythroid progenitors in the bone marrow of *Asf1B* KO mice and WT littermates. The black bar is the mean. T-test *p* value is indicated.

C. Bar graph depicting flow cytometry analysis of the erythroid compartment in the bone marrow of *Asf1b* KO and WT littermates following the gating strategy of Socolovsky.[42]

Figure 4: Persistence of embryonic globin expression in adult Asf1b KO mice.

Expression analysis by RT-qPCR of β-like and α-like globin genes in blood (A), bone marrow (B), and spleen (C) samples from *Asf1b* KO mice and WT littermates. Ratios of relative fold enrichment (RFE) of a given β-like or α-like globin vs total β- or α-globin respectively are depicted. Mean and standard deviation is represented, and T-test *p* values are indicated. * $p < 0.05$; *** $p < 0.0005$.

Genotype	Body Weight (g)	Spleen Weight (mg)	Spleen/body weight
WT (N = 10)	25.93 ± 2.67	90.20 ± 13.54	3.50 ± 0.54
Asf1b KO (N = 8)	28.38 ± 4.26	124.13 ± 28.09	4.56 ± 1.77
p value	NS	<0.05	NS

Table 1: Body and spleen weight of *Asf1b* KO mice and WT littermates. Mice included in this analysis are female littermates from heterozygous crossings. The body weight, spleen weight and spleen weight/body weight ratio are shown. The mean and standard deviation are indicated. T-test *p* values are given. NS, not significant.

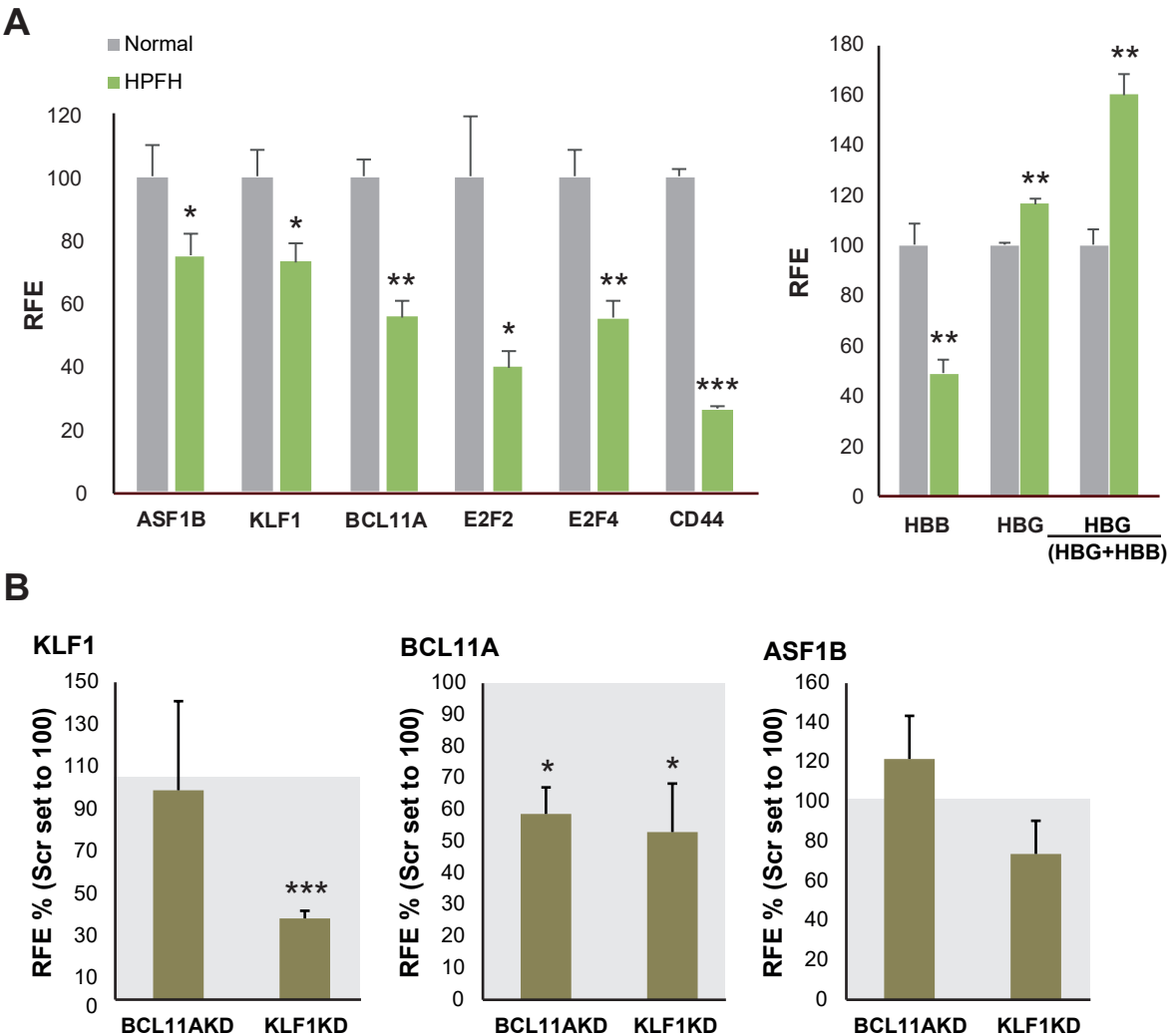
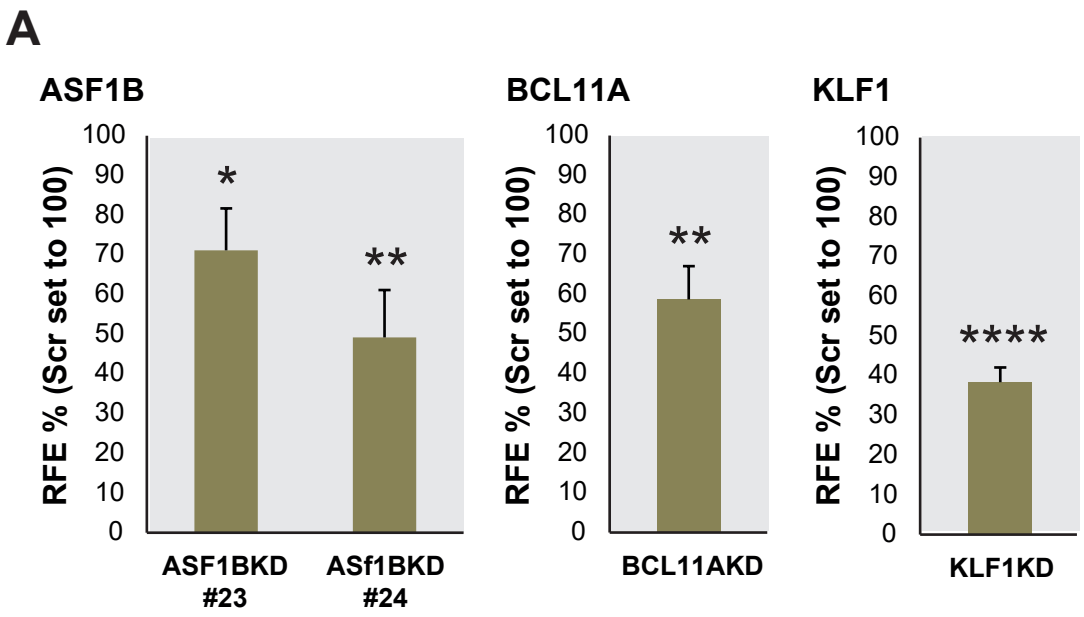
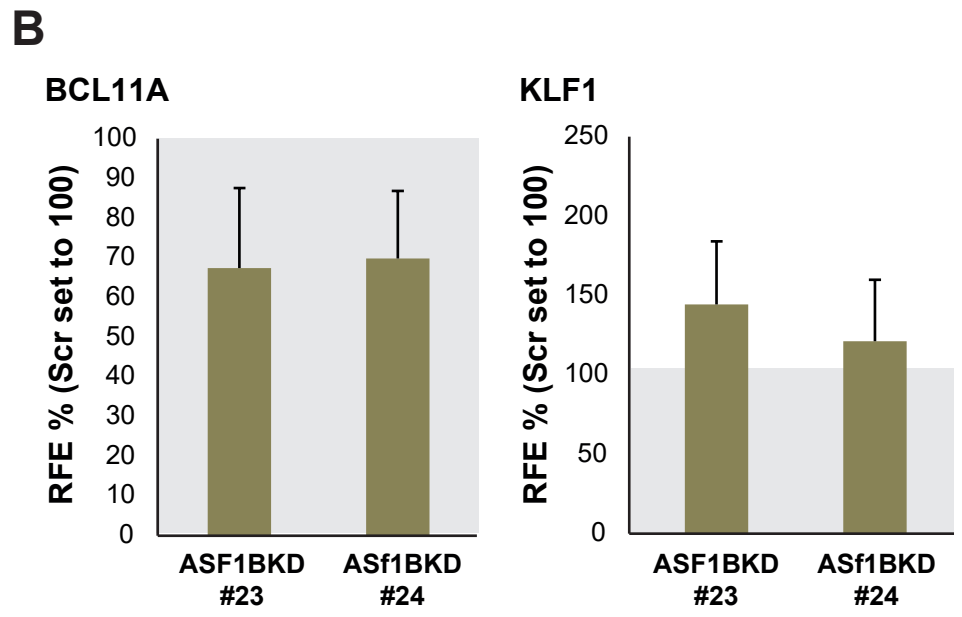
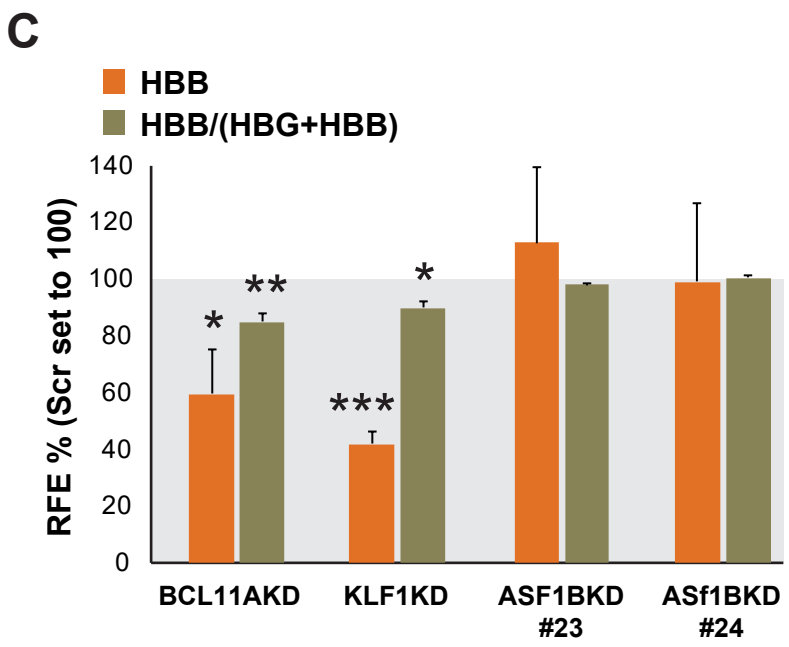
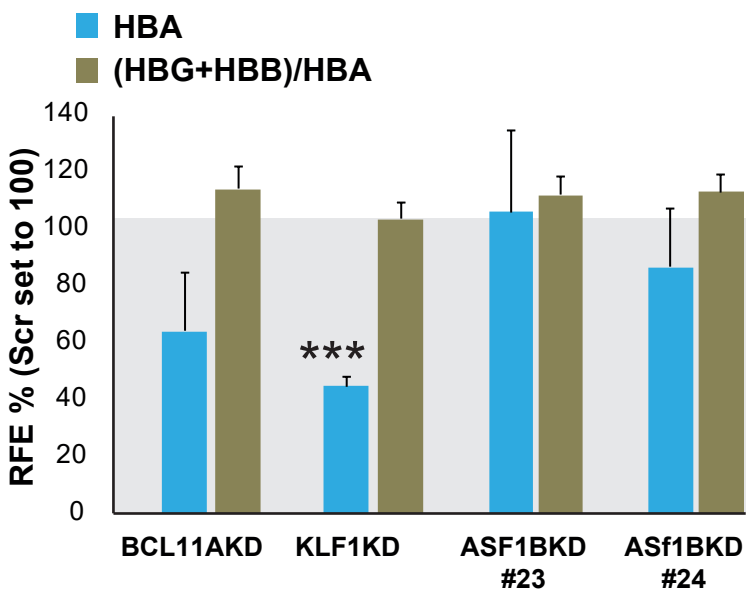

A
Normal
HPFH
RFE
120
100
80
60
40
20
0
*
*
**
*
**
***
ASF1B
KLF1
BCL11A
E2F2
E2F4
CD44
RFE
180
160
140
120
100
80
60
40
20
0
**
**
**
HBB
HBG
HBG
(HBG+HBB)
B
KLF1
RFE % (Scr set to 100)
150
130
110
90
70
50
30
10
0
***
BCL11AKD
KLF1KD
BCL11A
RFE % (Scr set to 100)
100
90
80
70
60
50
40
30
20
10
0
*
*
BCL11AKD
KLF1KD
ASF1B
RFE % (Scr set to 100)
160
140
120
100
80
60
40
20
0
BCL11AKD
KLF1KD

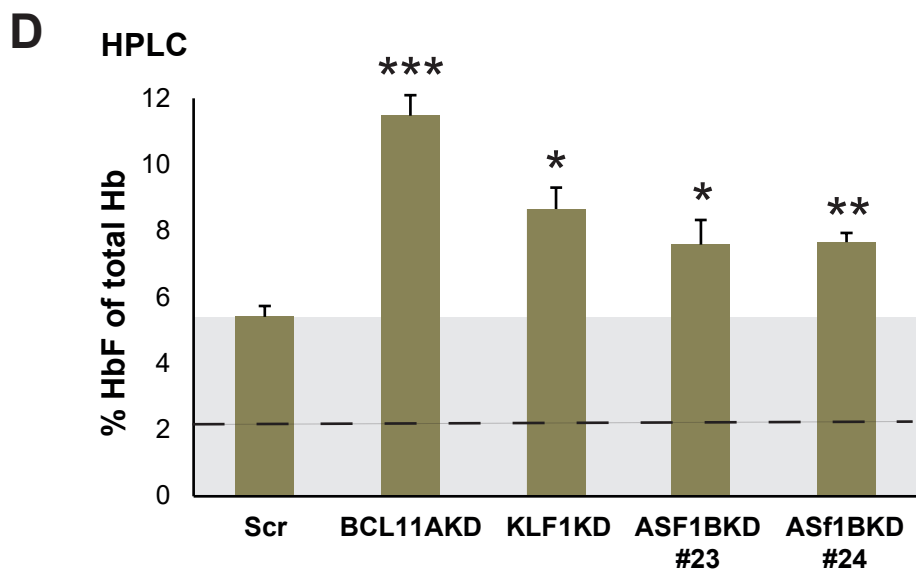
Figure 1


A
ASF1B
BCL11A
KLF1
RFE % (Scr set to 100)
100
90
80
70
60
50
40
30
20
10
0
*
**
****
ASF1BKD #23
ASf1BKD #24
BCL11AKD
KLF1KD


B
BCL11A
KLF1
RFE % (Scr set to 100)
250
200
150
100
50
0
ASF1BKD #23
ASf1BKD #24


C
HBB
HBB/(HBG+HBB)
RFE % (Scr set to 100)
140
120
100
80
60
40
20
0
*
**
***
BCL11AKD
KLF1KD
ASF1BKD #23
ASf1BKD #24


HBA
(HBG+HBB)/HBA
RFE % (Scr set to 100)
140
120
100
80
60
40
20
0
***
BCL11AKD
KLF1KD
ASF1BKD #23
ASf1BKD #24


D
HPLC
% HbF of total Hb
12
10
8
6
4
2
0
***
*
*
**
Scr
BCL11AKD
KLF1KD
ASF1BKD #23
ASf1BKD #24

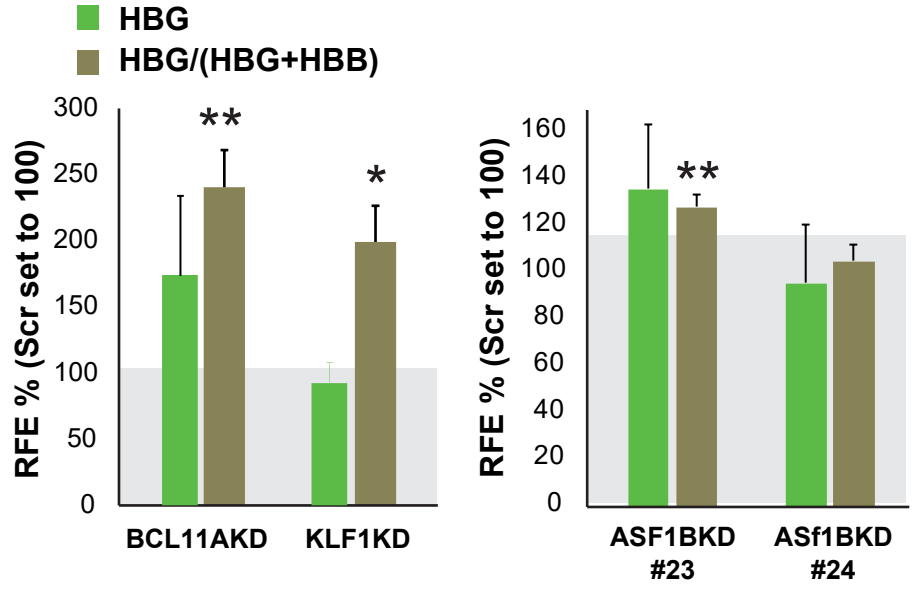

HBG
HBG/(HBG+HBB)
RFE % (Scr set to 100)
300
250
200
150
100
50
0
**
*
BCL11AKD
KLF1KD
160
140
120
100
80
60
40
20
0
**
ASF1BKD #23
ASf1BKD #24

Figure 2

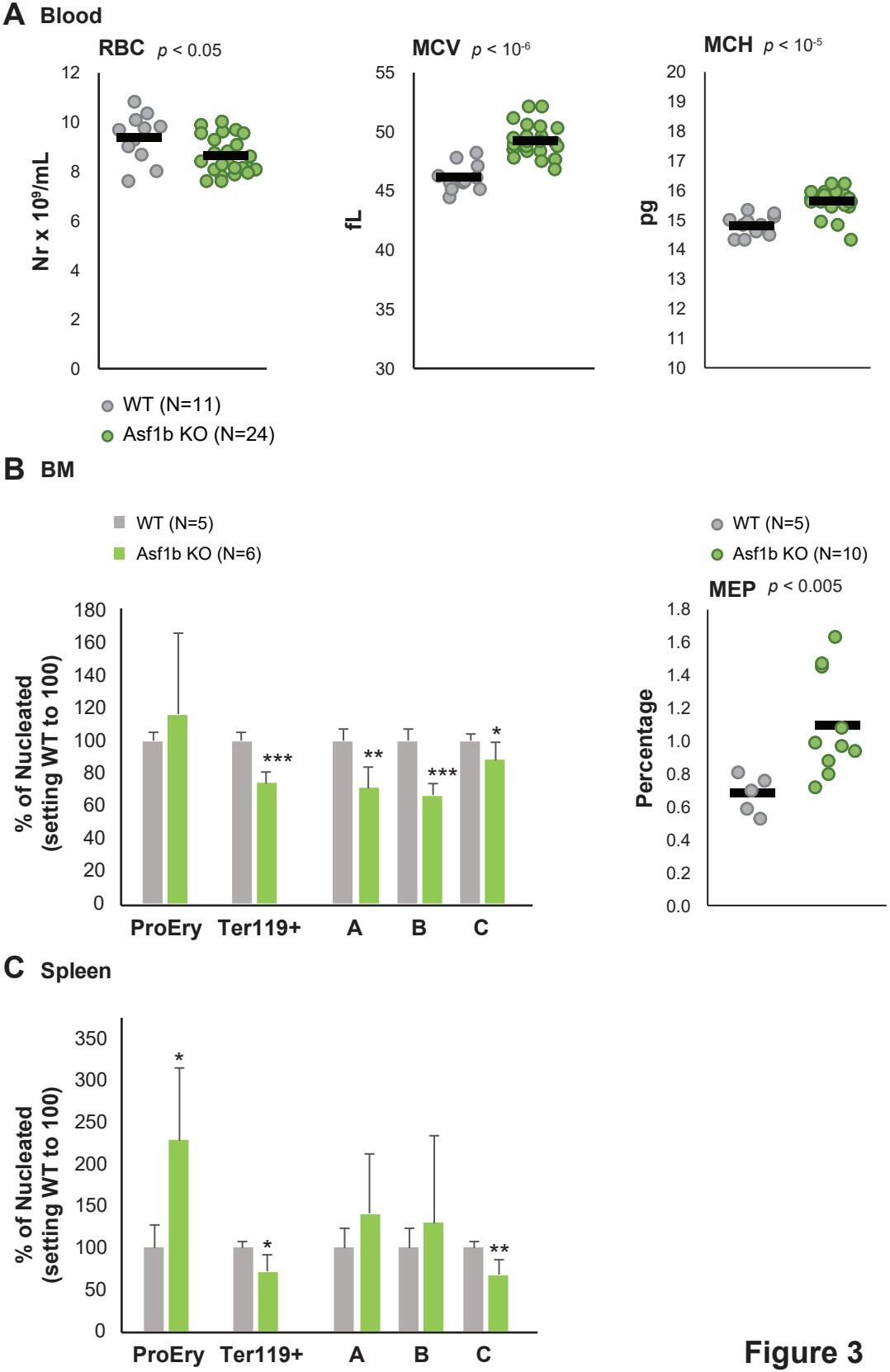

A Blood
RBC p < 0.05
MCV p < 10-6
MCH p < 10-5
Nr x 10⁹/mL
fL
pg
WT (N=11)
Asf1b KO (N=24)
B BM
WT (N=5)
Asf1b KO (N=6)
% of Nucleated (setting WT to 100)
ProEry
Ter119+
A
B
C
WT (N=5)
Asf1b KO (N=10)
MEP p < 0.005
Percentage
C Spleen
% of Nucleated (setting WT to 100)
ProEry
Ter119+
A
B
C

Figure 3

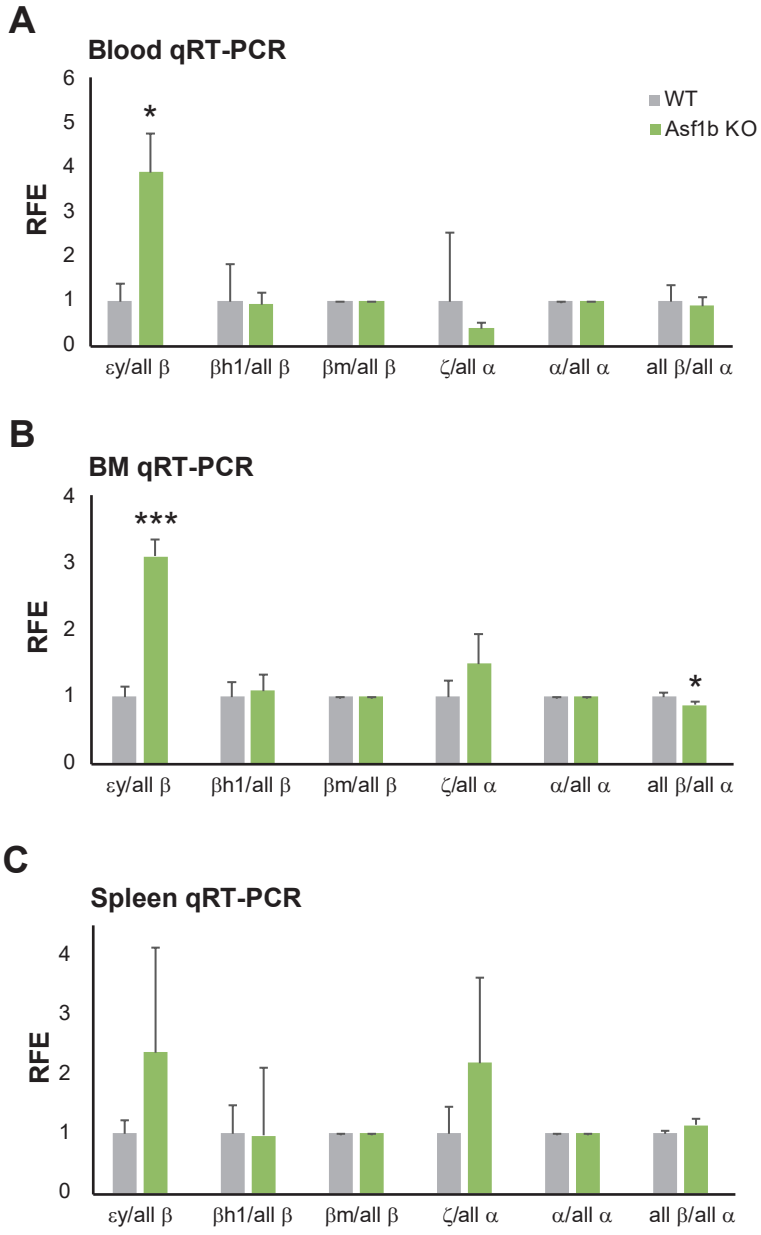

A
Blood qRT-PCR
RFE
WT
Asf1b KO
*
εy/all β
βh1/all β
βm/all β
ζ/all α
α/all α
all β/all α
B
BM qRT-PCR
RFE
***
*
εy/all β
βh1/all β
βm/all β
ζ/all α
α/all α
all β/all α
C
Spleen qRT-PCR
RFE
εy/all β
βh1/all β
βm/all β
ζ/all α
α/all α
all β/all α

Figure 4

Name	Sequence	Purpose
TRCN0000016276	GCAGAGGATCCAGGTGTGATA	shRNA KLF1
TRCN0000033449	CGCACAGAACACTCATGGATT	shRNA BCL11A
TRCN0000074225	GAGTGGAAGATCATTTATGTT	shRNA ASF1B #22
TRCN0000074226	CCCACTCAACTGCACTCCTAT	shRNA ASF1B #23
TRCN0000074227	AGTGGGCTACTACGTCAACAA	shRNA ASF1B #24
Hs ASF1B Forward	GGTTCGAGATCAGCTTCGAG	qRT-PCR
Hs ASF1B Reverse	ACCAGCACCGAGTCTAGGAT	qRT-PCR
Hs BCL11A Forward	GCCCCAAACAGGAACACATA	qRT-PCR
Hs BCL11A Reverse	TTGCAAGAGAAACCATGCAC	qRT-PCR
Hs E2F2 Forward	AGCTGGAACCGAGAGAACATG	qRT-PCR
Hs E2F2 Reverse	ACACGACCAGGCGAAACC	qRT-PCR
Hs E2F4 Forward	GGGCAGAAGAAGTACCAGATTCA	qRT-PCR
Hs E2F4 Reverse	GCTCCATGCCTCCTTGTTCA	qRT-PCR
Hs HBB Forward	ACAACTGTGTTCACTAGCAACC	qRT-PCR
Hs HBB Reverse	GTTGCCCATAACAGCATCAGG	qRT-PCR
Hs HBG1/HBG2 Forward	AGGTGCTGACTTCCTTGGG	qRT-PCR
Hs HBG1/HBG2 Reverse	GGGTGAATTCTTTGCCGAA	qRT-PCR
Hs KLF1 Forward	AACACGGACCCATAGACAGC	qRT-PCR
Hs KLF1 Reverse	TTGCACGACAGTTTGGACAT	qRT-PCR
Hs GAPDH Forward	GCCAAAAGGGTCATCATCTC	qRT-PCR
Hs GAPDH Reverse	GGTGCTAAGCAGTTGGTGGT	qRT-PCR
Mm Hbb-b Forward	GCTGCATGTGGATCCTGAGA	qRT-PCR
Mm Hbb-b Reverse	CTTCTGGAAGGCAGCCTGTG	qRT-PCR
Mm Hbb-bh1 Forward	TGGGCTTGGGGGTTAAGAAC	qRT-PCR
Mm Hbb-bh1 Reverse	AACATGTTGCCCAGGAGCTT	qRT-PCR
Mm Hbb-y Forward	TTGGCTAGTCACTTCGGCAAT	qRT-PCR
Mm Hbb-y Reverse	GCATAGCGGACACACAGGAT	qRT-PCR
Mm Hba-a Forward	GCTGAAGCCCTGGAAAGGAT	qRT-PCR
Mm Hba-a Reverse	CAGAGCCGTGGCTTACATCA	qRT-PCR
Mm Hba-x Forward	CCGGTCAACTTCAAGCTCCT	qRT-PCR
Mm Hba-x Reverse	TGAACTTGTCCCAGGCTTCG	qRT-PCR
Mm Actb Forward	ACAGCAGTTGGTTGGAGCAAA	qRT-PCR
Mm Actb Reverse	GGGTGAGGGACTTCCTGTAA	qRT-PCR
Mm Gapdh Forward	TGGTGAAGCAGGCATCTGAG	qRT-PCR
Mm Gapdh Reverse	TGAAGTCGCAGGAGACAACC	qRT-PCR

Supplementary Table 1: List of shRNA clones and primer sets used.