Growth Factor Independence 1b (Gfi1b) Is Important for the Maturation of Erythroid Cells and the Regulation of Embryonic Globin Expression

Lothar Vassen¹, Hugues Beauchemin¹, Wafaa Lemsaddek¹, Joseph Krongold¹,³, Marie Trudel¹, Tarik Möröy¹,²,³

¹ Institut de Recherches Cliniques de Montréal, IRCM, Montréal, Québec, Canada, ² Département de Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montréal, Québec, Canada, ³ Division of Experimental Medicine, McGill University, Montréal, Québec, Canada

Abstract

Growth factor independence 1b (GFI1B) is a DNA binding repressor of transcription with vital functions in hematopoiesis. Gfi1b-null embryos die at midgestation very likely due to defects in erythroid- and megakaryopoiesis. To analyze the full functionality of Gfi1b, we used conditionally deficient mice that harbor floxed Gfi1b alleles and inducible (Mx-Cre, Cre-ERT) or erythroid specific (EpoR-Cre) Cre expressing transgenes. In contrast to the germline knockout, EpoR-Cre mediated erythroid specific ablation of Gfi1b allows full gestation, but causes perinatal lethality with very few mice surviving to adulthood. Both the embryonic deletion of Gfi1b by EpoR-Cre and the deletion in adult mice by Mx-Cre or Cre-ERT leads to reduced numbers of erythroid precursors, perturbed and delayed erythroid maturation, anemia and extramedullary erythropoiesis. Global expression analyses showed that the Hba-x, Hbb-bh1 and Hbb-y embryonic globin genes were upregulated in Gfi1b deficient TER119⁺ fetal liver cells over the gestation period from day 12.5–17.5 p.c. and an increased level of Hbb-bh1 and Hbb-y embryonic globin gene expression was even maintained in adult Gfi1b deficient mice. While the expression of Bcl11a, a regulator of embryonic globin expression was not affected by Gfi1b deficiency, the expression of Gata1 was reduced and the expression of Sox6, also involved in globin switch, was almost entirely lost when Gfi1b was absent. These findings establish Gfi1b as a regulator of embryonic globin expression and embryonic and adult erythroid maturation.

Introduction

The continuous process of hematopoiesis initiating from pluripotent hematopoietic stem cells and giving rise to all hematopoietic lineages compensates for the restricted life span of mature blood cells. Each terminally differentiated blood cell is the result of chronological steps of proliferation and differentiation, which are stringently controlled by underlying lineage specific and ubiquitously expressed transcription factors. The DNA binding repressors of transcription growth factor independence 1b (GFI1B) and its paralogue GFI1 are expressed in a complementary and crossrepression by Gfi1 [27,28]. Expression of Gfi1b is subject to autoregulation and its paralogue GFI1 is expressed in hematopoietic stem and several hematopoietic lineages as well as cells of the sensory and nervous systems [1–3]. Although knockout mutants for both proteins in mice resulted in different hematopoietic phenotypes [4–11], GFI1B can functionally replace GFI1 throughout the hematopoietic system, but not in sensory cells such as the inner ear hair cells [2].

Both GFI1 and GFI1B are considered to be proto-oncogenes and have been linked to several hematologic malignancies [5,12–23], underscoring the importance of their adequate regulation during blood cell differentiation. Gfi1b is expressed in hematopoietic stem cells (HSC), myeloid/erythroid precursors (MEP), megakaryocytes and to varying levels during erythrocyte maturation [1]. Accordingly, these are the cell-types with the most obvious phenotype in Gfi1b knockout mice and GFI1B has been described as an essential factor in embryonic erythroid and megakaryocytic development [6,24–26]. The expression of Gfi1b is subject to autoregulation and crossrepression by Gfi1 [27,28]. Expression of Gfi1b in the erythroid lineage is controlled by GATA1, to which GFI1B can bind, by NF-Y in K562 cells [29] and by HMGB2 in human erythroid differentiation [30]. The GFI1B/GATA1 complex is also involved in the auto-regulation of Gfi1b [31–33]. The expression of Gfi1b is downregulated by Oct1 and upon erythropoietin signaling in a Stat5 dependent manner [34,35].

Repression of transcription by Gfi1 or Gfi1b fully depends on its N-terminal Snail/Gfi (SNAG) domain, which enables the recruitment of the GFI1/GFI1B cofactors Lysine (K)-specific demethylase 1A (LSD1/KDM1A) and GoREST/Rcor1. Consequently, a knockdown of LSD1 has been shown to cause a phenotype reminiscent of Gfi1b or Gfi1 knockout phenotypes affecting HSCs, granulopoiesis, erythropoiesis and platelet production [36]. The function of the GFI1B/LSD1/CoREST complex in erythroid proliferation and differentiation was intensively studied [37,38]. Interestingly, the GFI1B/LSD1/CoREST complex binds to the
Meis1 promoter in erythroid cells, but not in megakaryocytes, despite the fact that it is highly expressed in both cell types, suggesting a functional difference of Gfi1b between the two lineages.

Germline deletion of Gfi1b in mice causes lethality at around day 14.5 of embryonic development, probably due to a combined phenotype of inappropriate erythropoiesis and severe bleeding caused by a failure to produce platelet-generating megakaryocytes [1,6]. However, other not yet discovered mechanisms may also play a role. This early lethality of Gfi1b deficient mice restricted all analyses to either prenatal hematopoiesis or to cell culture systems. The recent generation of conditional Gfi1b knockout mice [9] allowed us to perform a more specific analysis of pre- and postnatal function of Gfi1b in erythropoiesis. We inactivated the Gfi1b gene by crossing conditional Gfi1b knock-out mice with EpoR-Cre knock-in mice to delete specifically in the erythroid lineage or by inducibly ablating it in adult mice using Mx-Cre and Rosa-Cre-ERT mouse lines. Our results show that Gfi1b is required for the differentiation from pro-erythroblasts to mature erythrocytes and for the silencing of globin genes during embryonic development and at adult stages.

Methods

Ethics Statement

The protocols for the in vivo experiments described here were reviewed and approved by the IRCM Animal Care Committee.

Figure 1. The erythroid specific knockout of Gfi1b causes perinatal lethality and a delayed maturation of fetal erythroid cells. A: Typical situs of either wild type (wt) or specific knockout of Gfi1b in the erythroid lineage (EpoR-Cre, Gfi1b<sup>fl/fl</sup>). While germline deletion of Gfi1b results in anemic, pale embryos with severe hemorrhage, the erythroid lineage specific knockout of Gfi1b results in pale embryos but not in hemorrhage (right). B: Bar graph illustrating the percentage of expected EpoR-Cre transgenic Gfi1b<sup>fl/fl</sup> embryos found viable at different stages of development as indicated. The erythroid specific ablation of Gfi1b does not reduce the survival rates of mouse embryos until birth, but leads to perinatal lethality with few exceptions (two out of 129 at age 6-weeks). The total numbers of embryos (N) analyzed at each stage are indicated at the bottom. Pregnant females were humanely euthanized according to procedures approved by the Canadian Council on Animal Care (CCAC) at indicated gestational time points and embryos were taken for analysis. New born pups that survived until a few hours after birth were examined as soon as possible for signs of anemia (paleness), weakness and difficulty breathing (endpoints) and those showing such signs were humanely euthanized for analysis following procedures approved by the Canadian Council on Animal Care (CCAC). The two mice that survived to adulthood were monitored semiweekly but never showed any sign of distress and were perfectly healthy. These mice were humanely euthanized after 6 weeks for analysis. C: Flow cytometry of isolated fetal liver cells at 14.5 dpc from wt and erythroid specific Gfi1b-KO embryos using antibodies against TER119 in combination with the developmental markers CD71, cKIT and CD9 as well as GFP for the detection of the GFP-CRE fusion protein. GFP-Cre is expressed early in erythroid development before cells become TER119<sup>+</sup> (TER119/GFP-CRE panel). Gfi1b-KO embryos show an accumulation of TER119-low, CD71-low, cKIT-high, CD9-high erythroid precursors indicating a delayed maturation of erythrocytes. FACS plots are representative for at least 6 or more independent samples analyzed.

doi:10.1371/journal.pone.0096636.g001
Figure 2. Gfi1b regulates maturation of erythroid cells in adult mice. A: Gfi1b<sup>fl/fl</sup> mice either carrying (Gfi1b-KO) the Mx-Cre transgene or not (wild type) were treated five times with pIpC (500 µg each) every other day and sacrificed for analysis 42 days after the first injection. B: Flow cytometric analysis of progenitors (middle panel) and maturing erythrocytes from the bone marrow (upper and lower panel) and spleen (lower panel) of wild type and Gfi1b-KO mice. TER119<sup>+</sup> bone marrow cells from pIpC induced wild type and Gfi1b knockout animals were isolated by flow cytometry and RNA was prepared for microarray analysis of gene expression as indicated (lower left panel). FACS plots are representative for at least four individual samples from each genotype. C: Peripheral blood of wild type and conditional Gfi1b-KO mice was analyzed using an ADVIA hematology system and the comparison results are presented as box-whisker plots showing the central location and distribution of the indicated measures. Red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), macrocytic RBCs (Macro), mean corpuscular volume (MCV), reticulocytes (Retic), red cell size and shape (RDW), immature reticulocytes fraction high (IRF-H) and white blood cell count (WBC).

doi:10.1371/journal.pone.0096636.g002
Mice

The generation of Gfi1b{GFP} knock-in mice and Gfi1b{fl/fl} conditional knockout mice has been described previously [1], [9]. All mice were housed under specific pathogen-free conditions and institutional animal ethics committees reviewed animal experimentation protocols and certified animal technicians regularly observed the mice in sign of distress. Adult mice were sacrificed by carbon dioxide inhalation whereas newborn pups were euthanized by decapitation following anesthesia by carbon dioxide inhalation as per standard operating procedure approved by the IRCM ACC and the CCAC. All efforts were made to minimize the number of animals used and to reduce their suffering. All mice were backcrossed with C57BL/6 mice for at least 8 generations. No phenotype or differences in number of cells was observed for Gfi1b{fl/fl} or Gfi1b{KO/fl} mice. Rosa-Cre-ERT mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA), strain B6;129-Gt(Rosa)26Sortm1(cre/ERT)Nat/J Stock Nr. 004847. The generation of EpoR-Cre knock-in mice used for erythroid specific ablation of Gfi1b expression has been described previously [39].

Flow cytometry, cell sorting, microarray analysis and Q-PCR

Hematopoietic cell populations were analyzed by flow cytometry using an LSR (BD Biosciences) and sorted using a MoFlo (Cytomation). Cells were passed through a 23-gauge needle, filtered through a cell strainer and resuspended in PBS (1% FCS, 10 mM EDTA). 1-5 X 10^6 cells were stained with antibodies at a 1:200 concentration for 20 min, washed with PBS and measured or sorted immediately. Antibodies used were ordered from BD-Biosciences (Missisauga, ON, Canada) or Bio-Legend (San Diego, CA, USA).

TRIzol (Invitrogen) was applied to isolate RNA/DNA/protein from sorted cells according to the manufacturers protocol. Quantitative RT-PCR was performed in a 20 µl reaction volume containing 900 nM of each primer, 250 nM TaqMan probe, and 1 µl TaqMan Universal PCR Master Mix (ABI, Germany) according to the manufacturer’s instructions. The relative expression of genes of interest was calculated relative to the GAPDH mRNA levels. Primers used for quantitative analysis of mRNA were: m_alpha-F: ggtgctagggcaaatgc; m_alpha-R: tgtctca-cagagcagaatt; b_min-maj-ex2-F: tttaacgatggcctgaatcactt; b_min-maj-ex3-R: cagcacaatcacgatcatattgc; ey-ex1-F: tggcctgtggagtaaggt-caa; ey-ex2-R: gaagcagaggacaagttccca; b_h1-ex2-F: tggacaacctcaaggagacc; b_h1-ex3-R: acctctggggtgaattcctt; Hba-x-F: cgggcccacggcttcaagat; Hba-x-R: caggggtgaagtcggcggga; mBcl11a-F: gcacttaagcaaacgggaat; mBcl11a-R: caggtga-gaaggtcgtggtc; mSox6-F: aatttggacccctctgaaca; mSox6LS: agctgagcggcatagagc; Gfi1b-ex3-4-F: ccagacctgtggactggaaca; Gfi1b-ex3-4-R: ggagaagctgggcttgtaga; mGata1-F: gaatcctctgcatcaacaagc; mGata1-R: gggcaagggttctgaggt;

Primers used for genotyping were: Gfi1b allele: LP5-3s: ggtttctaccagtctggccctgaactc; LP5-3r: ctcacctctctgtggcagtttcctatc; LP5-4r: tacattcatgcttagaaacttgagtc; product length of the different alleles is: wt, 256 bp; floxed, 295 bp and deleted, 540 bp. Internal control: mRag1.1: gctgatgggaagtcaagcgac; mRag1.3: gggaactgctgaactttctgtg.

EpoR-Cre: 06-44: gtgtggctgccttcctgcca; 06-45: ggacacaatcctggaccttcac; 06-46: caggaattcaagctcaacctcaFor whole
genome gene expression analysis of TER119+ wild type or Mx-Cre induced Gfi1b-KO erythroid cells from adult mice, a total of 10 µg cRNA from sorted cells was hybridized on Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) Mouse Genome 430A 2.0 arrays (GPL1261). For analysis of wild type or EpoR-Cre induced TER119+ Gfi1b-KO fetal liver cells at 14.5 dpc, cRNA was hybridized to Affymetrix mouse Gene 1.0 ST arrays (GPL6246). Microarray data have been deposited in the public database Gene Expression Omnibus (National Center for Biotechnology Information; GEO accession number: GSE54206). Data were analyzed with AltAnalyze [40] software using the default settings. Where given in the figures or text, the rawp is a one-way analysis of variance (ANOVA) p-value calculated for each pairwise comparison (two groups only). The log-fold is the log2 fold calculated by geometric subtraction of the experimental from the control groups for each pairwise comparison. Gene set enrichment analysis was performed using the GSEA software (www.broadinstitute.org/gsea) [41]. The nominal p value (n. p-val) given for most analyses estimates the statistical significance of the enrichment score for a single gene set. Given 1000 gene-set permutations we chose for each analysis, a p value of zero (0.0) indicates an actual p value of less than 0.0001. Hierarchical clustering analysis and scatter plots were generated using Spotfire Decision Site for functional genomics software (www.spotfire.tibco.com). Statistical analysis: The unpaired Student’s t-test was chosen for analyzing data distribution.

Results

Erythroid specific ablation of Gfi1b by EpoR-Cre causes accumulation of immature erythroid cells and perinatal lethality

We generated a cell type specific knockout of Gfi1b using EpoR-Cre mice, which express a GFP-Cre recombinase fusion protein in immature erythroid cells (Figure 1A, Figure S1), which also allows monitoring the expression of the Cre-transgene by measuring green fluorescence. EpoR-Cre Gfi1bfl/fl mice did not show internal bleeding at stage E14.5 (Figure 1A) or at birth, but appeared pale compared to controls (Figure 1A, Figure S1A). Most EpoR-Cre Gfi1bfl/fl mice died within minutes after birth, but a few survived to adulthood (Figure 1B, Figure S1A). EpoR-Cre Gfi1bfl/fl fetal livers (E14.5) appeared similar to wild type controls (Figure 1C), but showed an accumulation of CD71+ erythroid precursor cells that are c-Kit+ and GFP+ (i.e. express the EpoR-Cre transgene) and a decrease of CD71+ TER119+ erythroblasts (Figure 1D), suggesting that deletion of Gfi1b delays the differentiation of embryonic erythroid cells.

Acute disruption of Gfi1b in adult mice affects erythroid differentiation and causes anemia

To investigate the role of Gfi1b in adult erythroid development, we used Gfi1bfl/fl mice expressing the inducible Mx-Cre transgene

Figure 4. Loss of Gfi1b affects the expression of STAT5 target genes and megakaryocyte/platelet genes in bone marrow derived TER119 cells. A: Pie chart of the statistical analysis of the numbers of protein coding genes regulated up or down more than two-fold in erythroid cells of Mx-Cre induced adult Gfi1b knockout mice. B: Gene set enrichment analysis (GSEA) comparing expression profiles of wild type and Gfi1b deficient TER119 cells reveals a significant enrichment of genes up-regulated in Gfi1b deficient cells that are targets of Stat5 signaling, marker genes for megakaryocytes/platelets, marker genes for AML or show a high expression in hematopoietic stem cells (HSC). C: Tables are showing the 10 most significant results of GSEA as in (B) using curated genesets from the Molecular Signatures Database (MSigDB) either for genes that are upregulated (upper table) or downregulated (lower table) in Gfi1b deficient mouse bone marrow derived TER119 cells.

doi:10.1371/journal.pone.0096636.g004
Figure 5. Deregulation of embryonic globin genes in Gfi1b deficient TER119+ cells. A: Scatter plot comparison of gene expression levels (log2 of normalized signal intensities) in TER119+ pIpc induced Mc-Cre, Gfi1bfl/fl bone marrow cells compared to pIpc induced cells from control mice. Dots represent probesets and are jittered for better visualization. Probesets were classified as indicated and probesets for hemoglobin genes and important regulators of hemoglobin gene expression and globin switch were labeled (red dots). Two RNA samples for each genotype were pooled and analyzed on single arrays. B: Scatter plot comparing the changes in gene expression induced by inactivation of Gfi1b in erythroid cells of adult mice (y-axis) as in (A) with genes regulated in fetal liver cells during development of the mouse embryo from 11 dpc (E11) compared to 16 dpc (E16). Raw data for fetal liver development was taken from GEO data series GSE13149 and reanalyzed. Embryonic globin genes (red), megakaryocyte/platelet specific genes (blue) and integrins known to be targets of Gfi1b in hematopoietic stem cells (yellow) are indicated. Genes that are downregulated in fetal liver cells during mouse development but upregulated in Gfi1b KO cells are likely to be direct targets of the transcriptional repressor Gfi1b and were subjected to GSEA analysis (green frame and table to the right) and show a high enrichment in megakaryocytic/coagulation related genes and globin genes.

doi:10.1371/journal.pone.0096636.g005

[42] and injected them with pIpc (Figure 2A) to induce the recombination of the Gfi1b alleles throughout the hematopoietic system. Deletion of the floxed Gfi1b alleles in bone marrow, spleen and in FACS sorted TER119+ cells was substantial but still incomplete (Figure S2). Nonetheless, Gfi1b knockout mice showed a relative increase of MEP percentage over GMPs and CMPs in bone marrow compared to controls (Figure 2B, middle panel). Also, the proportion of CD71+, TER119+ pro-erythroblasts and of more mature CD71+TER119+ erythroblasts was decreased (Figure 2B, upper panel), indicating a delay in erythroid maturation similar as seen in EpoR-Cre, Gfi1bgfp/+ mice. Splenectomy of Mc-Cre, Gfi1bgfp/+ mice were larger in size and showed a significant increase of TER119+ cells, suggesting ongoing extramedullary erythropoiesis (Figure 2B, lower panel and Figure S3). Peripheral blood analysis revealed a significant decrease in red blood cell count (RBC), hematocrit (HCT) and hemoglobin (Hgb) in Gfi1b deficient mice compared to wild type controls (Figure 2C). Consequently, numbers of reticulocytes (Retic), immature reticulocytes (IRF-H) and macrocytic RBCs (Macro) were increased in TER119+ erythrocytes (Figure 2B, lower panel and Figure S3). Gfi1b deficient mice suffer from anemia. To further confirm these findings, we used mice carrying a tamoxifen inducible Cre recombinase integrated into the Rosa26 locus for the ablation of Gfi1b expression in adult mice. In these mice the recombination of one or both conditional Gfi1b alleles was still incomplete when both Gfi1b alleles were floxed, but was efficient when only one floxed allele was present (Figure S4). We therefore used mice in which one floxed allele was replaced by a Gfi1b-GFP knock-in allele, which disrupts the Gfi1b coding region and allows to measure Gfi1b mRNA expression by monitoring green fluorescence [1–3], Rosa-Cre-ERT, Gfi1bgfp/+ mice were sacrificed between 8 and 15 days after the last of four treatments with tamoxifen (Figure 3A). Flow cytometric analysis of bone marrow cells from these mice showed strongly reduced percentages of late erythroblasts (TER119+, CD71+ and TER119+, CD71+), a slight increase of pro-erythroblasts (TER119+, CD71+ cells) and a marked increase of the percentage of MEPs at the expense of GMPs compared to controls (Figure 3B). The spleen in Rosa-Cre-ERT, Gfi1bgfp/+ mice was enlarged about twofold (not shown) and showed a significant accumulation of TER119+ cells (Figure 3C), which is indicative for ongoing extramedullary erythropoiesis as was also seen in Mc-Cre, Gfi1bgfp/+ mice. This suggests that Gfi1b deficiency initiates a mechanism to compensate for marrow insufficiency. In addition, Rosa-Cre-ERT, Gfi1bgfp/+ mice showed similar alterations in their blood parameters as Gfi1bgfp/+ Mc-Cre animals, namely reduced red blood cell count (RBC), hematocrit (HCT) and hemoglobin (Hgb) and increased numbers of reticulocytes, indicating again an anemic state in the absence of Gfi1b (not shown).

To gain more insight into the effects of tamoxifen on more differentiated erythroblast populations, we analyzed bone marrow of Rosa-Cre-ERT, Gfi1bgfp/+ and Rosa-Cre-EERT, Gfi1bgfp/+ "animals from day 2 to day 8 after two tamoxifen injections using gates that divide TER119+ cells into proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatophilic erythroblasts. Between day 2 and day 5 after tamoxifen injection, both basophilic and polychromatophilic erythroblasts almost entirely disappeared regardless whether a Gfi1b allele remained intact or not (Figure S5). Between day 6 and day 8 after tamoxifen injection, basophilic erythroblasts became detectable in the control mice but not or only to a lower extent in the Gfi1b deleted animals (Figure S5). This suggests that Cre-ERT has a detrimental effect on TER119+ cells upon tamoxifen treatment and that these cells require Gfi1b for the differentiation of the TER119+ precursor population.

To test the long term effect of Gfi1b ablation by activating CreERT, Rosa-Cre-ERT, Gfi1bgfp/+ and Rosa-Cre-EERT, Gfi1bgfp/+ mice were analyzed two or nine months after tamoxifen-induced deletion of Gfi1b. TER119+ cells were gated again into four erythroblast populations. While a decrease of basophilic erythroblasts was still maintained, all other TER119+ cells were present at wt frequencies at both nine months (data not shown) and two months after tamoxifen induced Gfi1b ablation (Figure S6A, B). In animals with floxed Gfi1b alleles, only TER119+ cells showed efficient Cre mediated excision two months after tamoxifen induction, whereas the TER119+ erythroblast population only contained floxed or GFP alleles (Figure S6C). This indicated that these TER119+ erythroblasts very likely emerged from few non-deleted precursors in the TER119+ population, which supersede those with efficient excision of the Gfi1b allele and develop into TER119+ erythrocytes.

Gfi1b deficient cells shows defects in the regulation of globin gene expression

To gain more insight into the maturation defect caused by Gfi1b deficiency and to avoid any non-specific effects seen with tamoxifen, we performed two independent genome wide expression profiling experiments with FACS-sorted TER119+ bone marrow cells from Gfi1bfl/fl, Mc-Cre mice (see Figure 2B, lower panel for sorting gate) and from TER119+ fetal liver cells from EpoR-Cre Gfi1bgfp/+ mice. Most of the significantly regulated protein coding genes were upregulated in Mc-Cre/pIpc induced Gfi1b deficient cells compared to controls, which is in agreement with the role for Gfi1b as a transcriptional repressor (Figure 4A). Gene set enrichment analysis (GSEA) revealed targets of Gata2 and genes negatively regulated by Stat5 to be most affected by Gfi1b deficiency (Figure 4B, C). Platelet, HSC and AML specific genes were also significantly enriched among Gfi1b effector genes, which were up regulated in Gfi1b deficient cells (Figure 4B). Analysis of
Factors known to be associated with erythropoiesis showed that the expression of Gata2, Klf2, Bcl11a, Sox6 and the embryonic globin genes Hba-x, Hbb-bh1 and Hbb-y were affected by the deletion of Gfi1b (Figure 5A). A comparison of genes up-regulated in Gfi1b deficient TER119 embryoid bodies with those down-regulated during embryonic development revealed that expression of the embryonic globin genes Hba-x, Hbb-bh1 and Hbb-y was strongly affected by Gfi1b ablation (Figure 5B). In addition, integrins alphaf6 and alphab2/b3 (CD41/61), already described as Gfi1b effectors in HSCs [9] and several other megakaryocyte/platelet specific genes such as Gp1bb (CD42C), Timp3 or Puf4 showed increased expression in Gfi1b deficient cells (Figure 5B).

The second expression profiling experiment comparing mRNA prepared from TER119 fetal liver cells from EpoR-Cre, Gfi1bfl/fl mice and wild type littersmates isolated at 14.5 dpc also demonstrated that over 60% of protein coding genes whose expression changed more than two-fold were up-regulated in Gfi1b knockout cells, which was again in agreement with a repressor function of Gfi1b (Figure 6A, pie diagram). Exon analysis confirmed deletion of the targeted Gfi1b exons in the TER119 fetal liver cells used for the analysis (Figure S7). GSEA analysis revealed that gene sets related to coagulation and immune response, hypoxia and CBFA2T3 (Eso2) targets were enriched among up-regulated genes, whereas gene sets related to cell cycle regulation, targets of E2F and DNA synthesis were enriched among down-regulated genes (Figure 6B, C). Similar to the analysis of TER119 bone marrow cells from Mx-Cre, Gfi1bfl/fl mice, a deregulated expression of genes encoding GATA2, SOX6 and of the Hba-x and Hbb-bh1 embryonic globin genes was observed (Figure 6D).

Gfi1b is required for the developmental repression of embryonic globin gene expression

To validate a potential regulation of embryonic globin genes by Gfi1b, we FACS-sorted CD71+, TER119+/−/− (proerythroblast) and CD71+, TER119+ (late erythroblast) fractions of E15.5 fetal liver cells from wt and EpoR-Cre Gfi1bfl/fl mice for RT-PCR expression analysis. The expression of the genes for Hba-x, Hbb-bh1 and Hbb-y were up-regulated about 10 fold in Gfi1b deficient cells compared to their wt counterparts (Figure 7A). A developmental expression analysis of the Hba-x, Hbb-y and Hbb-bh1 genes showed significantly higher levels in Gfi1b deficient fetal liver cells than in wild type controls throughout development from stages E12.5 to E16.5 (Figure 7B). A similarly enhanced expression was found in constitutive Gfi1b deficient (Gfi1bfl/fl/Gata1−/−) fetal liver cells (stage E 13.5) where the expression of Hbb-bh1, Hbb-y and Hba-x was induced up to over 25 fold over controls (Figure 7C). The expression of Bcl11a, a target of KLF1, remained similarly regulated during development in Gfi1b knockout fetal liver cells and wt controls (Figure 8A). In contrast, Sox6 was almost absent in Gfi1b deficient fetal liver cells at all developmental stages analyzed and Gata1 was not induced to wt expression levels from stage E 13.5 onwards (Figure 8A). Expression of Glycophorin A (GYP A), an erythroid differentiation marker gene was only induced at a very late stage (E 15.5) in Gfi1b deficient cells and never reached wt expression levels (Figure 8A).

To test whether the deregulation of globin genes is also maintained in adult Gfi1b deficient mice, we used FACS sorted CD71+, TER119+ (proerythroblast) or CD71+, TER119− (late erythroblast) bone marrow cells from two surviving adult EpoR-Cre Gfi1bfl/fl mice for Q-PCR analysis (Figure 8B). Only a partial deletion of the floxed Gfi1b allele was detected in TER119+ cells (Figure 8C). However, despite this partial deletion, expression of beta-like embryonic globin genes (Hbb-y and Hbb-bh1) was still strongly up-regulated in these cells over 60 to over 100 fold, respectively, compared to wild type controls (Figure 8B). Hba-x or the adult hemoglobin genes alpha (Hba) and beta (Hbb) or Gata1 were only mildly affected by Gfi1b deficiency in these cells (Figure 8B). When we compared the effect of Gfi1b deficiency on a number of known and suspected regulators of globin gene expression (Figure S8) between fetal liver and adult TER119+ bone marrow cells from our array data, the only significant overlap turned out to be a strong overexpression of Gata2 and of the fetal globin genes themselves. This suggests an important role for Gata2 in the effect of Gfi1b deficiency on fetal globin gene expression and a more direct involvement of Gfi1b in the regulation of fetal globin gene expression.

Discussion

In this study we present evidence that the transcriptional repressor Gfi1b is an important factor for murine embryonic and adult definitive erythropoiesis. It has been described previously that Gfi1b is highly expressed in megakaryocyte and erythrocyte progenitors (MEPs) and to a lower extent throughout erythrocyte maturation [1]. However a complete study of the role of Gfi1b in erythroid differentiation throughout development and in adult stages was hampered by the early embryonic lethality of germline Gfi1b knockout mice. We have analyzed three different mouse models, which enabled the deletion of conditional Gfi1b alleles either specifically in erythropoiesis at early developmental stages (EpoR-Cre mediated) or upon treatment with either pIpC (Mx-Cre) or tamoxifen (Rosa-Cre-ERT). The results from analyses of all three models indicate that Gfi1b is an essential factor required for erythroid maturation during embryonic development in the fetal liver and in adult stages for the production of mature erythrocyte cells in the bone marrow. This is supported by the reduced frequencies of TER119+ erythroblasts that were observed in all Gfi1b deficient mice regardless how the ablation was achieved. Analysis of mice 2 and 9 months after a deletion of the conditional Gfi1b allele even suggested that Gfi1b is absolutely essential to maintain erythropoiesis at long term.

We also found that MEPs are present and even increased in percentage in adult Gfi1b deficient mice, which excludes a lack of precursor cells as a the underlying cause for the low frequencies of erythroblasts in the absence of Gfi1b and supports a regulatory role.
of Gfi1b during erythroid commitment and development. Probably as a result of the erythroid maturation defect, adult Gfi1b deficient mice suffer from anemia as indicated by the low RBC counts, the low hematocrit and hemoglobin levels. In addition, adult Gfi1b deficient mice also show extramedullary erythropoiesis, which may be a consequence of the anemia.

Mice in which Gfi1b ablation was mediated by the EpoR-Cre transgene did not die at midgestation but mainly at birth. This finding points to the possibility that a delayed or inhibited erythroid development is not entirely responsible for the embryonic lethality observed in germline Gfi1b knockout mice. However, it cannot be ruled out that incomplete deletion of the floxed Gfi1b
Figure 8. Insufficient activation of Sox6, Gata1 and Gpa in Gfi1b deficient cells. A: Q-PCR analysis of the relative expression levels of regulators of globin gene expression (Gata1), globin gene switch (Bcl11a, Sox6) and glycophorin A (Gypa) normalized to Gapdh. Sample sizes were as described in Figure 7B. B: Q-PCR analysis on RNA from CD71+, TER119- (pro-erythroblasts) and TER119+ (late erythroblasts) live bone marrow cells from a surviving EpoR-Cre induced Gfi1b-KO mouse compared to a wild type littermate. All measurements were done in triplicates. C: RT-PCR detection of EpoR-Cre and Gfi1b wt, flox and KO (excised) alleles on total RNA from bone marrow of a surviving mouse with erythroid specific inactivation of Gfi1b by EpoR-Cre.
doi:10.1371/journal.pone.0096636.g008
alleles has allowed enough erythrocytes to mature to allow full development past E13.5–14.5. It remains unclear however, why most EpoR-Cre, Gfi1b<sup>fl/fl</sup> mice die shortly after birth. Additional studies are necessary to clarify this, but a recently described role of the EpoR in vascular cells and hypoxic stress [43] may have contributed to this lethality. Different from what was observed in fetal liver, ablation of Gfi1b in adult mice, whether erythroid specific or not, did not lead to noticeable accumulation of proerythroblasts (CD71<sup>+</sup>, TER119<sup>+</sup>) cells in the bone marrow, but rather to a loss of erythroblast populations (CD71<sup>+</sup>, TER119<sup>+</sup>) cells. It is thus possible that a role of Gfi1b in proerythroblast maturation is different in embryonic and adult development.

Our data also demonstrate that Gfi1b plays an important role in the regulation of the expression of embryonic globin genes. Regardless how Gfi1b ablation was achieved, all animals that lack or are deficient of Gfi1b showed a significant increase in embryonic globin gene expression both in fetal liver cells and in bone marrow derived adult erythroid cells. The expression of embryonic globin genes is dependent on fine-tuning by many transcription factors, co-activators and co-repressors. An important role in this regulation has previously been assigned to two complexes, the NF-Y/Gata2 activator hub and the BCL11a/COPiTII/GATA1 repressor hub that are both present in embryonic and adult erythroid cells on repressed and active y-globin regulatory sequences [44]. The embryonic globin gene repressor function of BCL11a is dependent on the presence of another factor, SOX6, to form long-range interactions [45]. Our experiments showed a strong down-regulation of the expression of both Sox6 and Gata1 in Gfi1b deficient fetal liver cells and largely unaffected levels of Bcl11a expression. Since SOX6 and GATA1 are both required for a functional repressor complex that occupies the embryonic beta globin locus at regulatory sequences, these findings provide compelling evidence that the deregulation of these two genes is responsible for impaired repression of the embryonic globin genes in Gfi1b deficient mice. In contrast, TER119<sup>+</sup> cells from adult Gfi1b knockout mice showed up-regulation of Gata2 mRNA levels, but almost no change in other known regulators of embryonic globin gene expression. Gata2 overexpression is known to stimulate fetal globin gene expression [46] and moreover, downregulation of Gata1 induces Gata2 expression and results in impaired differentiation of erythroblasts [47]. This phenotype is similar to what we observe in our Gfi1b knockouts. The analysis of published ChIP-seq data of Gfi1b [48] did not reveal a direct occupation of the embryonic globin genes suggesting that Gfi1b affects the expression of embryonic globin genes likely via a different mechanism in erythroid cells from fetal liver or adult bone marrow. Although our data clearly establish a role of Gfi1b in embryonic globin expression, future studies will have to show whether this occurs through a direct repression of the globin locus by the GFI1B/LSD1/CoREST repressor complex, or whether Gfi1b acts indirectly on globin expression possibly in a complex with other regulatory factors.

Supporting Information

Figure S1 Analysis of mice from crossings between Gfi1b<sup>fl/fl</sup> and EpoR-Cre transgenic animals. A: Newborn mice from a Gfi1b<sup>fl/fl</sup> x Gfi1b<sup>Wt/Ntf</sup>/EpoR-Cre crossing (upper panel). PCR from tail tip DNA identifies floxed or wt Gfi1b alleles and the presence of the EpoR-Cre transgene (lower panel). The genotype of each pup is given for both Gfi1b and the EpoR-Cre transgene. B: PCR analysis of recombination of the Gfi1b allele in fetal liver cells from two littersmates from a Gfi1b<sup>fl/fl</sup> x Gfi1b<sup>Wt/Ntf</sup>/EpoR-Cre crossing at 14.5 dpc (upper panel). The different alleles detected are indicated; only in the presence of EpoR-Cre the recombinated knockout Gfi1b allele is detected. Recombination of the floxed Gfi1b allele is incomplete, which is possibly due to the presence of non-erythroid cells in fetal liver, but more likely is a consequence of a specific selection for non Gfi1b deleted cells during erythropoiesis. C: Although the Gfi1b-KO embryos look pale, the fetal livers of these embryos can barely be discriminated from wt fetal livers. (TIF)

Figure S2 RT-PCR analysis of tissues and cells from Mx-Cre, Gfi1b<sup>Wt/Ntf</sup> transgenic mice. An efficient, but not complete recombination of the floxed Gfi1b alleles was detected by RT-PCR using FACS sorted TER119<sup>+</sup> cells or total bone marrow (BM), spleen (Sp) or thymus (Thy). (TIF)

Figure S3 Analysis of spleens from Mx-Cre, Gfi1b<sup>Wt/Ntf</sup> animals after pIpC induced deletion. A: Spleens and normalized spleen weight from animals with the indicated genotype. B: Flow cytometric analysis of splenocytes from the indicated animals for the markers CD71 and TER119. (TIF)

Figure S4 RT-PCR analysis of bone marrow cells from Rosa-Cre-ERT, Gfi1b<sup>Wt/Ff</sup> and Rosa-Cre-ERT, Gfi1b<sup>Ff</sup>/Ff transgenic mice. Complete recombination of the floxed Gfi1b alleles was detected by RT-PCR in Rosa-Cre-ERT Gfi1b<sup>Ff</sup>/Ff mice upon tamoxifen treatment. (TIF)

Figure S5 Effect of tamoxifen mediated ablation of Gfi1b in adult Rosa-Cre-ERT, Gfi1b<sup>Wt</sup> mice. A: Flow cytometric analysis of cells from the indicated mice to detect different erythroblast cell populations according to CD71 and TER119 marker expression. B: Schema of Tamoxifen treatment. Mice were analyzed 2–8 days after receiving two IP injections of tamoxifen in two days (100 mg/kg the first day and 50 mg/kg the second day). (C) 4 to 7 mice were analyzed for both genotypes at all time points and plotted as mean ± SD for the four erythroblast cell populations. (TIF)

Figure S6 Long term effect of tamoxifen mediated ablation of Gfi1b in adult Rosa-Cre-ERT, Gfi1b<sup>Wt</sup> mice. A: Flow cytometric analysis of cells from the indicated mice to detect different erythroblast cell populations according to CD71 and TER119 marker expression. Mice were analyzed 2 months after tamoxifen treatment as described in Figure S5. B: Quantification of the frequency of the indicated cell subsets from the mice characterized in (A). Proerythroblast: CD71<sup>+</sup>, TER119<sup>+</sup>, Basophilic erythroblasts: CD71<sup>+</sup>, TER119<sup>+</sup>, Polychromatophilic erythroblasts: CD71<sup>med</sup>, TER119<sup>+</sup>, Orthochromatophilic erythroblasts: CD71<sup>lo</sup>, TER119<sup>+</sup>, C: PCR analysis of DNA from total bone marrow from Rosa-Cre-ERT, Gfi1b<sup>Wt/Wt</sup> or Rosa-Cre-ERT, Gfi1b<sup>Ff/Ff</sup> mice to detect the wt, floxed or excised (KO) alleles. (TIF)

Figure S7 Box-and-Whisker plot of gene level normalized intensity for Gfi1b in wt and Gfi1b-KO fetal liver cells. The upper plot shows smallest value, first quantile, median, third quantile and largest value of the Gfi1b-gene level normalized intensities of wild type (red) and Gfi1b knockout (blue) TER119<sup>+</sup> fetal liver cells analyzed in duplicates on Affymetrix gene-1.0 ST arrays that allow for exon-level analysis. Exons 1048130, 1048130 and 10481310 (including first ATG) are bordered with loxP sites in the conditional Gfi1b-KO and should be deleted by
CRE recombination. This is a proof for the deletion of Gfi1b by EpoR-Cre in TER119 fetal liver cells. The lower plot does show the exon-intron structure and gene-1OSF array probesets covering the Gfi1b-gene and analyzed here. Both plots were generated using the web-tool “Gene array analyzer” (http://gaa.mpib-berlin.mpg.de/) [49].

Figure S8 Change of expression of globin genes and their regulators induced by Gfi1b deficiency. A: Scatter plot demonstrating the relation of the magnitude of gene expression changes induced by Gfi1b deficiency in fetal liver cells at day 14.5 relative to the probability of a significant change of expression (rawp). Values were taken from array data sets described in Figure S7. Genes visualized are either globin genes or known or suspected regulators of globin gene expression. Labels represent the official gene symbols and dots represent the data for gene level analysis of array data. B: Bar graph representing the magnitude of gene expression changes induced by Gfi1b deficiency in TER119 bone marrow cells from adult mice as measured on 16-fold log-scale. Dotted lines indicate the levels of 1.5-fold or 2-fold changes in gene expression level as indicated. (TIF)

Acknowledgments
We are indebted to Mathieu Lapointe for technical assistance and Marie-Claude Lavallée, Mélanie St-Germain and Jade Dussureault for excellent animal care, Eric Massicotte, Julie Lord for FACS and cell sorting.

Author Contributions
Conceived and designed the experiments: LV HB WM JT. Performed the experiments: LV HB WI JK. Analyzed the data: LV HB. Contributed reagents/materials/analysis tools: TM MT. Wrote the paper: LV HB TM.

References
1. Vassen L, Okayama T, Moroy T (2007) Gfi1b green fluorescent protein knock-in mice reveal a dynamic expression pattern of Gfi1b during hematopoiesis that is largely complementary to Gfi1. Blood 109: 2356–2364.
2. Feldk A, Herranzo R, Vassen L, Zeng H, Hemesh O, et al. (2006) Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development. EMBO Rep 7: 326–333.
3. Yucel R, Kossan C, Heyd F, Moroy T (2004) Gfi1 green fluorescent protein knock-in mutant reveals differential expression and autoregulation of the growth factor independence 1 (Gfi1) gene during lymphocyte development. J Biol Chem 279: 49066–49071.
4. Hock H, Hamblen MJ, Roake HM, Tavzer D, Bronson RT, et al. (2003) Intramolecular requirement for zinc finger transcription factor Gfi1 in neutrophil differentiation. Immunity 18: 109–120.
5. Karsunky H, Zeng H, Schmidt T, Zevnik B, Kluge R, et al. (2002) Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nat Genet 30: 295–300.
6. Saleqs S, Cameron S, Orkin SH (2002) The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. Genes Dev 16: 301–306.
7. Yucel R, Karsunky H, Klein-Hitpass L, Moroy T (2003) The transcriptional repressor Gfi1 controls development of early, uncommitted c-kit+ T cell progenitors and CD4+CD8+ lineage decision in the thymus. J Exp Med 197: 631–644.
8. Zeng H, Yucel R, Kossan C, Klein-Hitpass L, Moroy T (2004) Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. EMBO J 23: 4116–4125.
9. Khandanpour C, Sharif-Akari E, Vassen L, Gaudreau MC, Zhu J, et al. (2010) Evidence that growth factor independence 1b regulates dormancy and peripheral mobilization of hematopoietic stem cells. Blood 116: 5149–5161.
10. Vassen L, Duhren U, Kossan C, Zeng H, Moroy T (2012) Growth factor independence 1 (Gfi1) regulates cell-fate decision of a bipotential granulocytic-monocytic precursor defined by expression of Gfi1 and CD48. Am J Blood Res 2: 228–242.
11. Schulz D, Vassen L, Chow KT, McWhiter SM, Amin RH, et al. (2012) Gfi1b negatively regulates Rag expression directly and via the repression of FoxO1. J Exp Med 209: 187–199.
12. Koldhoff M, Zakrzewski JL, Beelen DW, Elmaagacli AH (2013) Additive pathway in lymphoblastic leukemia. Cancer Cell 23: 200–214.
13. Zornig M, Schmidt T, Karsunky H, Grzeschick A, Moroy T (1996) zinc finger protein GFI-1 cooperates with myc and pim-1 in T-cell lymphomagenesis by reducing the requirements for IL-2. Oncogene 12: 1789–1801.
14. Khandanpour C, Phelan JD, Vassen L, Schutte J, Chen R, et al. (2013) Growth factor independence 1 protects hematopoietic stem cells against apoptosis but also prevents the development of a myeloproliferative-like disease. Stem Cells 29: 376–385.
15. Khandanpour C, Theide C, Valk PJ, Sharif-Akari E, Nuckel H, et al. (2010) A variant allelle of Growth Factor Independence 1 (GFI1) is associated with acute myeloid leukemia. Blood 115: 2462–2472.
16. D’Souza S, del Prete D, Jin S, Sun Q, Huston AJ, et al. (2011) Gfi1 expressed in bone marrow cells is a novel osteoblast suppressor in patients with multiple myeloma bone disease. Blood 118: 6871–6880.
17. Khandanpour C, Kossan C, Gaudreau MC, Duhren U, Hebert J, et al. (2011) Growth factor independence 1 protects hematopoietic stem cells against affymetrix MOE430-2 expression arrays. Data are from single array experiments, not allowing for p-value determination. The same genes as in (A) were analyzed. Multiple probesets for single genes were averaged. Gene expression changes are indicated in log-scale. Dotted lines indicate the levels of 1.5-fold or 2-fold changes in gene expression level as indicated. (TIF)
35. Jegalian AG, Wu H (2002) Regulation of Socs gene expression by the proto-oncoprotein GFI-1B: two routes for STAT5 target gene induction by erythropoietin. J Biol Chem 277: 2345–2352.
36. Sprussel A, Schulte JH, Weber S, Necke M, Handschke K, et al. (2012) Lysine-specific demethylase 1 restricts hematopoietic progenitor proliferation and is essential for terminal differentiation. Leukemia 26: 2039–2051.
37. Laurent B, Randrianarison-Huetz V, Frisan E, Andrieu-Soler C, Soler E, et al. (2012) A short Gfi-1B isoform controls erythroid differentiation by recruiting the LSD1-CoREST complex through the dimethylation of its SNAG domain. J Cell Sci 125: 993–1002.
38. Chowdhury AH, Ramroop JR, Upadhyay G, Sengupta A, Andrzejczyk A, et al. (2013) Differential transcriptional regulation of meis1 by Gfi1b and its co-factors LSD1 and CoREST. PLoS One 8: e53666.
39. Emig D, Salomonis N, Baumbach J, Lengauer T, Conklin BR, et al. (2010) AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. Nucleic Acids Res 38: W755–762.
40. Joshi A, Hannah R, Diamanti E, Gottgens B (2013) Gene set control analysis predicts hematopoietic control mechanisms from genome-wide transcription factor binding data. Exp Hematol 41: 354–366 e314.
41. Heinrich AC, Pelanda R, Klingmüller U (2004) A mouse model for visualization and conditional mutations in the erythroid lineage. Blood 104: 659–666.
42. Ikonomi P, Noguchi CT, Miller W, Kasahara H, Hardison R, et al. (2000) Levels of GATA-1/GATA-2 transcription factors modulate expression of embryonic and fetal hemoglobin. Gene 261: 277–287.
43. Suzuki M, Kobayashi-Ouaki M, Tsutsumi S, Pan X, Ohmori S, et al. (2013) GATA factor switching from GATA2 to GATA1 involves long-range interactions and cooperation with SOX6. Genes Dev 27: 783–790.
44. Kuhn R, Schwenk F, Aguet M, Rajewsky K (1995) Inducible gene targeting in mice. Science 269: 1427–1429.
45. Ogunshola OO, Bogdanova AY (2013) Epo and non-hematopoietic cells: what do we know? Methods Mol Biol 982: 13–41.
46. Xu J, Sankaran VG, Ni M, Mermel TF, Furum RV, et al. (2010) Transcriptional silencing of (γ-globin)-globin by BCL11A involves long-range interactions and cooperation with SOX6. Genes Dev 24: 783–790.
47. Wilson NK, Foster SD, Wang X, Knezevic K, Schutte J, et al. (2010) Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. Cell Stem Cell 7: 532–544.
48. Gelbert P, Teranishi M, Jesnickes K, De Gaspari P, John D, et al. (2012) Gene Array Analyzer: alternative usage of gene arrays to study alternative splicing events. Nucleic Acids Res 40: 2414–2423.