POGZ Is Required for Silencing Mouse Embryonic β-like Hemoglobin and Human Fetal Hemoglobin Expression

Highlights

- *Pogz* is highly expressed in mouse megakaryocyte erythroid progenitors
- POGZ is required to repress murine embryonic β-like globin during erythropoiesis
- *Pogz*+/− mice develop normally and show elevated embryonic β-like globin expression
- POGZ knockdown decreases BCL11A and increases fetal globin expression in human cells

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In Brief

Gudmundsdottir et al. show that POGZ represses embryonic globin gene expression in mouse and human erythroid cells, in part by regulating Bcl11a expression in vitro and in vivo. The molecular pathways regulated by POGZ may represent potential therapeutic targets to increase fetal globin expression in patients with sickle cell disease and β-thalassemia.

Data and Software Availability

GSE113503
POGZ Is Required for Silencing Mouse Embryonic β-like Hemoglobin and Human Fetal Hemoglobin Expression

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https://doi.org/10.1016/j.celrep.2018.05.043

SUMMARY

Fetal globin genes are transcriptionally silenced during embryogenesis through hemoglobin switching. Strategies to derepress fetal globin expression in the adult could alleviate symptoms in sickle cell disease and β-thalassemia. We identified a zinc-finger protein, pogo transposable element zinc-finger domain (POGZ), expressed in hematopoietic progenitor cells. Targeted deletion of Pogz in adult hematopoietic cells in vivo results in persistence of embryonic β-like globin expression without affecting erythroid development. POGZ binds to the Bcl11a promoter and erythroid-specific intragenic regulatory regions. Pogz+/- mice show elevated embryonic β-like globin expression, suggesting that partial reduction of Pogz expression results in persistence of embryonic β-like globin expression. Knockdown of POGZ in primary human CD34+ progenitor cell-derived erythroblasts reduces BCL11A expression, a known repressor of embryonic β-like globin expression, and increases fetal hemoglobin expression. These findings are significant, since new therapeutic targets and strategies are needed to treat β-globin disorders.

INTRODUCTION

During mouse embryonic development, three distinct populations of erythroid cells are generated (Baron et al., 2013). The first are primitive erythroid cells, which arise from the yolk sac and mainly express embryonic β-like globins (Hbb-bh1 and Hbb-y) and low levels of adult-type globins (Hbb-b1 and Hbb-b2) (Kingsley et al., 2006; Palis, 2014). The second are definitive erythroid cells from the yolk sac that seed the fetal liver (FL). Initially, they express Hbb-bh1 and Hbb-y and then switch to Hbb-b1 and Hbb-b2 expression (McGrath et al., 2011). The third population is hematopoietic stem cells (HSCs) that arise from intra-embryonic sites, including the aorta-gonad mesonephros region, that initially seed the FL and then home to the bone marrow and give rise to definitive erythroid cells. These erythroid cells express the adult Hbb-b1 and Hbb-b2 globins.

Sickle cell disease (SCD) and β-thalassemia are inherited human hemoglobin disorders, which result from globin gene mutations and represent a significant global health issue. Natural variations in fetal hemoglobin expression have been linked to the severity of disease outcome, such that individuals with higher fetal hemoglobin levels have less severe symptoms in SCD and β-thalassemia (Sankaran et al., 2010). Genome-wide association studies identified three loci associated with increased fetal hemoglobin levels (Galarneau et al., 2010; Lettre et al., 2008; Uda et al., 2008), including BCL11A, which was subsequently shown to function as a transcriptional repressor of fetal hemoglobin (Sankaran et al., 2008). Conditional loss of Bcl11a...
in erythroid cells leads to increased embryonic β-like globin expression without affecting normal erythroid development, suggesting that BCL11A is a relevant therapeutic target (Xu et al., 2011). Recent experimental evidence confirmed that loss of Bcl11a expression in a preclinical model of SCD reversed sickling and end organ damage (Xu et al., 2011). Embryonic β-like globin is maintained in a repressed state by a multi-protein co-repressor complex, including BCL11A, GATA1, SOX6, and chromatin remodeling proteins, including Mi2β, HDAC1/2, LSD1/CoREST, and DMMT1 (Xu et al., 2010, 2013). These and other targets represent therapeutic opportunities to reactivate fetal globin to treat SCD and β-thalassemia (Bauer et al., 2012; Sankaran et al., 2008; Xu et al., 2013).

We identified a previously uncharacterized transcriptional regulator of hematopoiesis, POGZ (KIAA0461, ZNF280E), in a screen of a human hematopoietic progenitor cell line model (KG1) and its more differentiated progeny (Gudmundsson et al., 2007). POGZ is a zinc-finger containing protein, which binds to SP1, LEDGF, and heterochromatin proteins (Bartholomew et al., 2009; Gunther et al., 2000; Nozawa et al., 2010), suggesting POGZ may have an important role in gene regulation; however, its function in hematopoiesis is currently unknown (Gudmundsson et al., 2007; Ishikawa et al., 1997; Nomura et al., 1994; Okazaki et al., 2003). Domain structure predictions by SMART analysis (Letunic et al., 2009; Schultz et al., 1998) indicate that POGZ has at least 8 C2H2 zinc fingers, suggesting it can bind DNA (Figure S1A). We show here that Pogz is expressed in normal mouse hematopoietic stem and progenitor cells (HSPCs), with the highest levels of expression in megakaryocyte erythroid progenitors (MEPs). We discovered that POGZ is essential for normal murine embryonic development, and uncovered a function of POGZ in the regulation of embryonic β-like globin expression in vitro and in vivo. Using mouse models that conditionally delete Pogz in adult mice, we demonstrate that Pogz is intrinsically required for normal globin switching, in part, by regulating Bcl11a expression. Furthermore, we show that knockdown of POGZ expression in human erythroid cells derepresses fetal globin expression. Our data provide evidence that Pogz is a regulator of mouse embryonic β-like globin expression and human fetal hemoglobin expression.

RESULTS

POGZ Is Expressed in Normal Murine Hematopoietic Stem and Progenitor Cells

We identified POGZ, a zinc-finger-containing protein, in a screen of the human hematopoietic progenitor cell line KG1, whose potential transcription factor activity and function in hematopoiesis were unknown (Gudmundsson et al., 2007). POGZ is expressed in KG1 cells, and POGZ RNA and protein levels are decreased during differentiation, suggesting that POGZ may function in hematopoietic cells (Figure 1A). We performed a detailed analysis of Pogz expression in purified mouse hematopoietic stem and progenitor cells (HSPCs), with the highest levels of expression in megakaryocyte erythroid progenitors (MEPs). Domain structure predictions by SMART analysis (Letunic et al., 2009; Schultz et al., 1998) indicate that POGZ has at least 8 C2H2 zinc fingers, suggesting it can bind DNA (Figure S1A). We show here that Pogz is expressed in normal mouse hematopoietic stem and progenitor cells (HSPCs), with the highest levels of expression in megakaryocyte erythroid progenitors (MEPs). We discovered that POGZ is essential for normal murine embryonic development, and uncovered a function of POGZ in the regulation of embryonic β-like globin expression in vitro and in vivo. Using mouse models that conditionally delete Pogz in adult mice, we demonstrate that Pogz is intrinsically required for normal globin switching, in part, by regulating Bcl11a expression. Furthermore, we show that knockdown of POGZ expression in human erythroid cells derepresses fetal globin expression. Our data provide evidence that Pogz is a regulator of mouse embryonic β-like globin expression and human fetal hemoglobin expression.

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(CLPs) and is reduced in common myeloid progenitors (CMPs) and granulocyte macrophage progenitors (GMPs), while Pogz expression is significantly higher in MEPs (Figure 1C). Inquiry of Pogz RNA expression in the BioGPS microarray database (Su et al., 2004) confirmed that Pogz is highly expressed in murine HSPCs and increased in MEPs and that Pogz is more broadly expressed in other tissues, including neural and eye tissue (Figure S1B). Finally, we compared POGZ protein expression in a limited tissue array and found that POGZ protein is highly expressed in adult mouse thymocytes and splenocytes, with lower levels of expression in peripheral blood cells (PBCs), bone marrow cells (BMCs), and liver cells (Figure 1D). Since POZG is expressed in erythroid lineage cells, we used mouse erythroid leukemia (MEL) cells to examine the expression and subcellular localization of POGZ by immunofluorescence and determined that POGZ is mainly localized in the nucleus and is not present in the nucleolus (Figure 1E). Collectively, these results confirm that POGZ is expressed in normal mouse HSPCs, MEPs, and MEL cells, suggesting a potential role for POGZ in megakaryopoiesis and erythropoiesis.

**Reduced Output of Hematopoietic Cells and Deregulation of Genes Required for Erythropoiesis and Hemoglobin Switching in Pogz−/− FL Cells**

To uncover the physiological function of POGZ in hematopoietic development, we generated a mouse model to inactivate Pogz gene expression in vivo (Figures S1C–S1F) (Liu et al., 2003). We did not detect Pogz−/− pups at weaning in crosses of Pogz+/+ mice, suggesting that the Pogz−/− mice died during embryonic development or shortly after birth. Further analysis showed that Pogz−/− embryos rarely survived beyond embryonic day 16.5 (E16.5) when back-crossed 10 generations onto C57BL/6J background mice. Timed-pregnancy studies showed that some Pogz−/− embryos were absorbed as early as E10.5, but we observed a consistent drop in animal survival around E15.5 (Figure 2A). The Pogz−/− embryos were generally smaller and appeared anemic compared to their wild-type littermates; however, the precise cause of death is currently unknown (Figure 2B).

Since the FL is the major site of hematopoiesis in the embryo and Pogz−/− embryos survive until E15.5–E16.5, we harvested FL cells from Pogz+/+ and Pogz−/− embryos to examine lineage development by flow cytometry and performed differential gene expression analysis to identify potential pathways and target genes affected by the loss of Pogz. As expected, Pogz−/− FL was significantly smaller, with fewer cells (Figure 2C). We found that the frequency of myeloid (macrophages [Mac1+Gr1−] and neutrophils [Mac1+Gr1+]) and B cells (CD19+) in Pogz−/− FL was similar to Pogz+/+ FL; however, their total numbers were reduced due to the overall reduction in FL cellularity (Figures S2A–S2C). We observed increased frequencies of more primitive erythroid cells (S0–S2 cells) in Pogz−/− FL (Figure S2D); however, the total number of the more mature erythroid cells was decreased at all stages of development (S3–S5) in Pogz−/− FL (Figure S2E). Thus, erythroid, B, and myeloid cells are present in Pogz−/− FL, but in greatly reduced numbers.

Concomitantly, we performed microarray analysis of RNA expressed in Pogz+/+ and Pogz−/− E14.5 FL cells to identify potential target genes and pathways affected by the loss of Pogz. We found that 1,062 genes were differentially expressed in Pogz−/− versus Pogz+/+ FLs using >1.5-fold change in gene expression as a cutoff (Table S1). Ingenuity Pathway Analysis (IPA) of differentially expressed genes identified the “hematological system and development and function” as a top physiological system.
affected, and “hematological disease” as a top disease and disorder affected (Figure S2F). Differentially expressed genes were linked to erythrocytosis and hereditary persistence of fetal hemoglobin (HPFH) and included Jak2, c-kit, and c-myb (all upregulated) and Bcl11a and Tfrc (both downregulated) in Pogz−/− FL cells compared to Pogz+/+ FL cells (Figure 3A). Taken together, the data from the microarray analysis suggests that loss of Pogz may lead to deregulation of erythropoiesis and globin gene expression.

**Loss of Pogz Expression Leads to Downregulation of Bcl11a and Increased Embryonic β-like Globin Expression**

Since Pogz is highly expressed in MEPs, and loss of Pogz expression affects the expression of genes and pathways required for erythroid development and globin gene expression (Figure 3A), we determined the expression of known transcriptional regulators of erythroid and globin gene expression, including Klf1, Klf2, Nfe2, Gata1, Fog1, and Bcl11a, at E16.5 in Pogz+/+ and Pogz−/− FL cells, indicating that genes expressed during erythroid lineage development are affected by loss of Pogz expression. RNA was isolated from E14.5 Pogz+/+ and Pogz−/− FL cells (n = 3 for each genotype), and gene expression was assessed using Affymetrix Mouse 430 2.0 oligonucleotide arrays (blue indicates low expression, and red indicates high expression).

**Figure 3. Decreased Expression of Bcl11a and Increased Expression of Embryonic β-like Globins in Pogz−/− Fetal Liver Cells**

(A) Heatmap of differentially expressed genes in Pogz+/+ and Pogz−/− FL cells, indicating that genes expressed during erythroid lineage development are affected by loss of Pogz expression. RNA was isolated from E14.5 Pogz+/+ and Pogz−/− FL cells (n = 3 for each genotype), and gene expression was assessed using Affymetrix Mouse 430 2.0 oligonucleotide arrays (blue indicates low expression, and red indicates high expression).

(B) Bcl11a and Klf2 expression is downregulated in Pogz−/− FL cells. RNA was purified from E16.5 Pogz+/+ and Pogz−/− FLs, and Bcl11a, Klf2, Nfe2, Klf1, Gata1, and Fog1 expression was analyzed by real-time qPCR. Gene expression was normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

(C) Reduction in BCL11A protein levels in Pogz−/− FL cells. Western blot analysis was performed on whole-cell lysates generated from E16.5 Pogz+/+ and Pogz−/− FL cells. (D) Upregulation of embryonic β-like globins in Pogz−/− FL cells. RNA was purified from E16.5 Pogz+/+ and Pogz−/− FLs and Hbb-β1, Hbb-β1/2, Hbb-γ, Hbb-bh1, Hba-x, and Hbb-b1/2 expression analyzed by real-time qPCR. Gene expression was normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

Pogz+/+ and Pogz−/− FL by real-time qPCR. We found that the expression of Bcl11a and Klf2 was significantly reduced in RNA obtained from E16.5 Pogz−/− FL cells compared to Pogz+/+ FL cells, whereas the expression of Klf1, Gata1, and Fog1 was not significantly different (Figure 3B). We also confirmed a decrease of Bcl11a expression at the protein level in E16.5 Pogz−/− FL by western blot analysis (Figure 3C). Since BCL11A is a critical regulator of the switch between fetal and adult globin expression in definitive erythroid cells (Sankaran et al., 2008, 2009, 2010b), we analyzed the expression of the embryonic α- and β-like globins Hbb-γ, Hbb-bh1, and Hba-x in E16.5 Pogz−/− FL RNA. We found that the expression of the embryonic globins was significantly upregulated in the Pogz−/− FL compared to Pogz+/+ FL (Figure 3D). Thus, expression of Bcl11a is decreased and embryonic globin expression is increased in Pogz−/− FL cells, suggesting that Pogz may function to regulate embryonic globin gene expression.

**Persistence of Embryonic Globin Expression Is Intrinsic to Pogz−/− Hematopoietic Cells**

To determine if the increased expression of embryonic globin observed in Pogz−/− FL cells was intrinsic to hematopoietic cells and not an indirect effect due to loss of Pogz function in the microenvironment, we transplanted 1 × 10⁶ E16.5
Pogz+/+ and Pogz−/− FL cells into lethally irradiated recipient mice (Figure 4A). We found no difference in the number of differentiating erythroid cells at all stages of development (S0–S5) in the bone marrow of mice transplanted with Pogz+/+ or Pogz−/− FL cells. Gene expression was determined by real-time qPCR and normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD.

(B) Representative flow cytometry analysis of CD71 and Ter119 expression in mice transplanted with Pogz+/+; Mx1-cre and Pogzf/f; Mx1-cre BMCs 12 weeks post-pIpC treatment (n = 5 for each genotype). Gates were set around subsets of differentiating donor (CD45.2+ expression) bone marrow (BM) erythroid cells (S0–S5) as previously described (Koulnis et al., 2011). No differences in the percentages of donor-derived erythroid subsets were observed.

(C and D) Pogz mRNA is not expressed in PBCs from mice transplanted with Pogz+/+; Mx1-cre BMCs and treated with pIpC compared to mice transplanted with control Pogz+/+; Mx1-cre BMCs (C), while Hbb-y mRNA expression is significantly increased in PBCs from mice transplanted with Pogzf/f; Mx1-cre BMCs (D). Experiments were performed in triplicate. Gene expression was normalized to β-actin expression.

Figure 4. Persistence of Embryonic β-like Globin Expression Is Intrinsic to Pogz−/− Erythroid Cells

(A) Summary of FL transplantation experiments. Hbb-y RNA expression was increased and Bcl11a decreased in PBCs obtained from mice 5 weeks after transplantation of Pogz+/+ or Pogz−/− FL cells. Gene expression was determined by real-time qPCR and normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD.

Expression of Hbb-y (Figure 4A, left panel) and Bcl11a (Figure 4A, right panel) was silenced in recipient mice transplanted with Pogz+/+ FL cells (Figure 4A, left panel). The expression of Bcl11a was downregulated in mice transplanted with Pogz−/− FL cells in comparison to mice transplanted with Pogz+/+ FL cells (Figure 4A, right panel). Furthermore, analysis of Hbb-y expression 3 months post transplantation (long-term reconstitution) confirmed that Hbb-y expression remained elevated in mice transplanted with Pogz−/− FL cells, indicating that Hbb-y expression is not silenced in erythroid cells arising from long-term reconstituting HSPCs (Figure S4A). Collectively, these data suggest that loss of Pogz leads to an intrinsic derepression of embryonic β-like globin expression.

While transplantation of Pogz−/− FL cells provides evidence for an intrinsic role of Pogz in regulating embryonic globin gene expression, we sought to confirm this in a model where we could delete Pogz in hematopoietic lineage cells in adult mice and limit the potential of non-cell-autonomous effects. First, we bred Pogz conditional mice (Pogzf/f) to EpoR-cre transgenic mice (Heinrich et al., 2004) (Pogzf/f; EpoR-cre); however, Pogz was variably deleted in this model (data not shown).
Figure 5. Pogz Regulates the Expression of Bcl11a and Hbb-y in MEL Cells and Fetal Liver Cells

(A) Hbb-y expression is increased in PBC from Pogz+/- compared to Pogz+/+ mice. Gene expression was normalized to β-actin expression (n = 5 per group and data are presented as mean ± SD, *p < 0.05).

(B) Lentiviral-mediated knockdown of Pogz in MEL cells represses Bcl11a expression and induces Hbb-y expression. RNA was harvested from MEL cells 72 and 96 hr after transduction with lentiviral vector expressing Pogz shRNA or a control shRNA vector and expression of Pogz, Bcl11a, and Hbb-y analyzed by real-time qPCR. Gene expression was normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD. *p < 0.05; ***p < 0.001.

(C) Western blot analysis of POGZ, BCL11A, and ACTIN expression following lentiviral-mediated knockdown of Pogz in MEL cells. Knockdown was performed with shRNA targeting Pogz or a control shRNA vector, and cell lysates were harvested 72 hr post-transduction.

(D) Photomicrographs of cytocentrifuge preparations of MEL cells 72 hr after lentiviral-mediated knockdown indicating no effect on cell morphology.

(legend continued on next page)
number of donor-derived MEPs in BMCs of mice that lack Pogz and affects their development. We found no differences in the expression in adult mice (Figure 5A). Furthermore, since Pogz+/+;Mx1-cre and Pogzf/f;Mx1-cre into irradiated recipients to generate chimeric mice (Kühn et al., 1995). Six weeks after bone marrow transplantation (BMT), we treated mice with polyinosinic:polycytidylic acid (pIpC) to delete Pogz in hematopoietic cells. Twelve weeks after Pogz deletion we analyzed (1) BMCs to confirm that Pogz was deleted, (2) PBCs for complete blood cell (CBC) analysis, (3) BMCs for MEP and erythroid development, and (4) PBCs for expression of Pogz and Hbb-y globin. Pogz was efficiently deleted in the chimeric Pogz+/+;Mx1-cre transplanted mice (Figure S4B), and results of CBC analysis of mice 12 weeks after deletion of Pogz were normal, suggesting that Pogz is not required for normal red cell development in this model (Figure S4C). No differences in donor myeloid and B cell reconstitution were observed in mice transplanted with BMCs that lack Pogz (Figure S5A). No difference in the frequency or number of differentiating erythroid cells (S1–S5) was observed in BMCs from Pogz+/+;Mx1-cre transplanted mice compared to control transplanted mice (Figure S5B). Further, no significant differences in donor-derived erythroid cell reconstitution were observed in mice transplanted with Pogz+/+; Mx1-cre and Pogzf/f;Mx1-cre BMC when gated on donor-derived CD45.2+ cells, providing additional evidence that loss of Pogz does not affect normal adult erythroid cell development (Figure 4B). Since MEPs are restricted progenitors for erythroid cells and platelets and express significant levels of Pogz, we examined if loss of Pogz affects their development. We found no differences in the number of donor-derived MEPs in BMCs of mice that lack Pogz (Figure S5C). In addition, there were no differences in the number of megakaryocyte progenitors (MKPs) and pre-megakaryocyte/erythroid progenitors in mice transplanted with Pogz+/+ and Pogzf/f BMCs (Figure S5D), suggesting that Pogz is not required for the development of erythroid or megakaryocyte progenitors. Finally, we analyzed the expression of Pogz, Hba-x, Hbb-bh1, and Hbb-y in PBCs of transplanted mice. Our data confirmed that Pogz is not expressed (Figure 4C), while Hbb-y is expressed in all mice transplanted with Pogz+/+; Mx1-cre BMCs (Figure 4D). We also found an upregulation of Hba-x and Hbb-bh1 expression in some of the animals (Figure S6A–S6D). Collectively, these data demonstrate that Pogz is intrinsically required to repress embryonic β-like globin Hbb-y in adult red blood cells.

Interestingly, we found that the levels of Hbb-y expression were significantly increased in PBCs from adult Pogz+/− mice in comparison to Pogz+/+ mice, suggesting that Pogz-mediated repression of Hbb-y expression is dependent on the levels of Pogz expression in adult mice (Figure 5A). Furthermore, since Pogz+/− mice are viable, reproduce, and show no overt phenotype, the data suggest that reducing Pogz levels could result in persistence of embryonic globin expression without significantly altering erythroid maturation.

### Pogz Negatively Regulates Hbb-y Expression, in Part through Bcl11a

Our data suggest the possibility that POGZ represses Hbb-y expression, in part by regulating Bcl11a expression. To determine if Pogz and Bcl11a are coexpressed and developmentally regulated in a similar fashion during erythroid differentiation, we analyzed the expression profile of Pogz and Bcl11a in sorted erythroid cells from the bone marrow of normal C57BL/6J mice (Figure S6E). Our analysis shows that Pogz and Bcl11a are expressed at similar levels in all erythroid populations (Figures S6F and S6G). As a comparison, Gata1 and Klf1 are highly expressed in CD71+/+Ter119+ cells (Figures S6H and S6I). To investigate if Pogz regulates the expression levels of Bcl11a and Hbb-y, we knocked down Pogz expression in MEL cells using lentiviral-mediated delivery of Pogz-specific small hairpin RNA (shRNA) and analyzed gene expression 72–96 hr post-transduction. The knockdown resulted in significant reduction in Pogz mRNA transcripts (Figure 5B) and loss of POGZ protein expression (Figure 5C), which did not affect MEL cell differentiation (Figure 5D) compared to control-treated cells. Loss of Pogz expression in MEL cells resulted in decreased Bcl11a expression and increased Hbb-y expression levels (Figures 5B and 5C). In addition, we overexpressed Pogz in E16.5 Pogz−/− FL by retroviral transduction and examined Bcl11a and Hbb-y expression (Figure 5E). Enforced expression of Pogz resulted in upregulation of Bcl11a expression and repression of Hbb-y expression. Taken together, these data suggest that Pogz positively regulates Bcl11a and represses Hbb-y expression. Interestingly, overexpression of Bcl11a did not reduce Hbb-y expression in Pogz−/− E16.5 FL cells, which suggests that Pogz may be required for Bcl11a-mediated repression and that there are additional mechanisms by which Pogz represses mouse embryonic β-like globin (Figure S6J).

To further examine the requirement for BCL11A in Pogz-mediated regulation of Hbb-y expression, we performed double knockdown experiments in MEL cells. We found that reducing either Pogz or Bcl11a expression increases Hbb-y expression with Bcl11a knockdown showing more efficient derepression of Hbb-y expression (Figure 6A). Knocking down both Pogz and Bcl11a did not increase Hbb-y expression above knocking down Bcl11a alone (Figure 6A). To examine if Bcl11a could rescue the upregulation of Hbb-y following Pogz knockdown, we overexpressed Bcl11a in MEL cells transduced with lentiviral vectors that express shPogz. We found that enforced Bcl11a expression partially reduces the increase in Hbb-y expression mediated by Pogz knockdown in MEL cells (Figure 6B), indicating that the Hbb-y derepression upon loss of Pogz is mediated, in part through BCL11A, although additional mechanisms are likely involved in this model.

(E) Re-expression of Pogz in Pogz−/− FL cells induces Bcl11a and reduces Hbb-y RNA expression. Pogz−/− FL cells were harvested at E16.5 and transduced with a control retrovirus or retroviral vector expressing the Pogz transgene. RNA was harvested 60 hr post-transduction, and expression of Hbb-y, Bcl11a, and Pogz were analyzed by real-time qPCR. Gene expression was normalized to β-actin expression. Experiments were performed in triplicate, and data are presented as mean ± SD. *p < 0.05; **p < 0.001.
To test whether POGZ can repress Hbb-y through direct regulation of Bcl11a, we performed chromatin immunoprecipitation (ChIP) assays using MEL cells transduced with control or Pogz shRNA lentiviral vectors. POGZ binds to the Bcl11a promoter in Figure 6. POGZ Regulates Hbb-y Expression, in Part by Regulating Bcl11a Expression

(A) Western blot analysis of POGZ, BCL11A, and ACTIN protein levels, and real-time qPCR analysis of Hbb-y expression following lentiviral-mediated knockdown of Pogz, Bcl11a, or both Pogz and Bcl11a in MEL cells. MEL cells were transduced with shRNA targeting Pogz, Bcl11a, or a control shRNA vector and cell lysates harvested 72 hr post-transduction. Gene expression was normalized to β-actin expression. Experiments were performed in triplicates and data are presented as mean ± SD. PU, puromycin. BL, blasticidin.

(B) Western blot analysis of POGZ, BCL11A and ACTIN protein levels and real-time qPCR analysis of Hbb-y expression following lentiviral mediated knockdown of Pogz and overexpression of Bcl11a. Gene expression was normalized to β-actin expression. Experiments were performed in triplicates and data are presented as mean ± SD. *p < 0.05.

(C) ChIP-qPCR analysis demonstrating that POGZ binds to the Bcl11a promoter (−972) and enhancer site (+58). POGZ does not bind to a negative control region on chromosome 17. Sheared chromatin was prepared from MEL cells following lentiviral-mediated knockdown of Pogz. Knockdown was performed with shRNA targeting Pogz or a control shRNA vector, and cell lysates were harvested 72 hr post-transduction. Chromatin was immunoprecipitated with an anti-POGZ antibody and a control antibody. The Bcl11a gene indicating sites relative to the transcription start site (TSS) that were examined by ChIP is shown below. Experiments were performed in triplicate, and data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

MEL cells at −972 and is greatly reduced at −3,469, and POGZ binding to these sites is significantly reduced in cells treated with Pogz shRNA (Figure 6C). POGZ did not show significant binding to a negative control region on mouse chromosome 17. We also examined a recently described enhancer element within intron 2 of the human BCL11A gene, which contains three DNase-I-hypersensitive sites at +55, +58, and +62 kb from the transcription start site (Bauer et al., 2013; Canver et al., 2015). The mouse Bcl11a gene has orthologous sequences within intron 2 (Bauer et al., 2013). We determined that POGZ binds the orthologous +58 sequences within the enhancer region using ChIP assays (Figure 6C) and that POGZ binding to these sites is reduced when Pogz levels are lowered by shRNA. Taken together, our data suggest that POGZ may be a regulator of Hbb-y expression by directly or indirectly interacting with the Bcl11a promoter and the orthologous Bcl11a enhancer elements.
Reduction of POGZ Expression in Human Proerythroblasts Leads to Increased Expression of Fetal Hemoglobin

To investigate whether POGZ regulates fetal globin expression in human cells, we examined erythroid cells differentiated from adult human CD34+ HSPCs in a modified two-phase in vitro erythroid culture system as previously described (Migliaccio et al., 2002). We confirmed that the purified CD34+ progenitors undergo erythroid differentiation in vitro by flow cytometry using CD34, CD45, CD71, and CD235a antibodies (Figure S7A). After 2 days, the cells in these cultures are predominantly primitive CFU-E and proerythroblasts that are CD34+CD45−CD71low. Most cells undergo further differentiation to more mature CD34−CD45−CD71−CD235a+ polychromatic and orthochromatic erythroblasts after 11 days in culture (Figure S7A). In addition, compared to undifferentiated control cells (Figure S7B, left panel), the cell pellets became increasingly red, an indication of increased hemoglobinization and erythroid differentiation (Figure S7B, right panel). We found that POGZ and BCL11A proteins are highly expressed in cells from day 3 cultures (proerythroblasts) and that their expression declines during erythroid differentiation, with little expression after 12–14 days in culture (Figure 7A). To investigate whether POGZ regulates fetal hemoglobin (HBG1/2) expression in human cells, we transduced primary human CD34+ cells on day 2 of expansion culture with control shRNA or shRNA vectors targeting different regions of human POGZ coding sequence and measured fetal hemoglobin expression 10 days after transduction by real-time qPCR and western blot. Real-time qPCR analysis showed that both POGZ shRNA constructs significantly reduced POGZ transcript levels compared to control shRNA (Figure S7C). HBG1/2 expression was significantly downregulated, whereas HBE and HBA expression were marginally affected (Figure S7C).
Western blot analysis showed efficient reduction in POGZ protein expression in cultures transduced with POGZ shRNA constructs (Figure 7B), with robust increase in HBG1/2 protein levels, while POGZ or HBG1/2 protein expression was not affected in cultures transduced with control shRNA (Figure 7B). Knockdown of POGZ expression did not affect erythroid differentiation in these cultures, since there was no difference in the extent of erythroid differentiation (CD71+CD235a+ cells) in cultures treated with control shRNA and POGZ shRNA lentiviral vectors after 12 days (Figure 7C). In agreement with the mouse data above (Figure 5), BCL11A protein levels were significantly reduced upon POGZ lentiviral-vector-mediated knockdown, suggesting that POGZ may also regulate BCL11A expression in human erythroid cells (Figure 7D). In comparison, POGZ protein levels were minimally affected following lentiviral-vector-mediated BCL11A knockdown (Figure 7D). As expected, lentiviral-vector-mediated BCL11A knockdown significantly increased HBG1/2 expression (Figure 7D). HBE expression was also significantly upregulated and HBB and HBA expression significantly downregulated (Figure 7D). Finally, knockdown of POGZ results in HBG1/2 protein levels representing roughly 25% of total β-globin, as assessed by high-performance liquid chromatography (HPLC), which is therapeutically relevant (Figure 7E). Taken together, the results suggest that POGZ is a repressor of fetal hemoglobin expression in humans.

**DISCUSSION**

In this report, we identified a previously uncharacterized zinc-finger-containing protein, POGZ, which is expressed in mouse and human HSPCs and required to repress embryonic hemoglobin gene expression during normal hematopoietic development. Elevated embryonic globin expression correlated with reduced expression of Bcl11a, a known repressor of embryonic β-like globin expression, in Pogz−/− FL cells. We demonstrate, in two different animal models, that red cells develop normally in the absence of Pogz in vivo but that the red blood cells (RBCs) show increased embryonic globin expression. Thus, deregulation of embryonic globin expression is intrinsic to Pogz−/− hematopoietic cells, and embryonic globin expression can persist in adult mice after transplantation. Finally, we show that POGZ knockdown increases fetal globin expression in primary human erythroblasts, indicating that POGZ also regulates human fetal globin expression, which is the focus of our future studies. These findings are significant since improved therapeutic strategies are needed to treat hereditary globin disorders (Weatherall, 2010). Individuals affected by these diseases have moderate to severe anemia and other serious health issues; however, natural variations that result in HPFH expression are linked to lessening the severity of disease (Sankaran et al., 2010a). Therefore, POGZ may represent a potential therapeutic target to increase fetal globin expression in patients with SCD and β-thalassemia (Bauer et al., 2012).

We found that Bcl11a expression is reduced in Pogz−/− FL cells, MEL cells treated with Pogz shRNA, and human CD34+ progenitors treated with POGZ shRNA, suggesting that Pogz positively regulates Bcl11a expression in mouse and human cells. POGZ binding to the Bcl11a promoter and a recently identified intron 2 enhancer (Bauer et al., 2013; Canver et al., 2015) suggests that POGZ is directly regulating Bcl11a transcription. Future experiments, including ChIP sequencing and electrophoretic mobility shift assays (EMSA), will determine if this regulation is direct or indirect via interaction with other DNA binding proteins. We also show that BCL11A does not repress embryonic β-like globin Hbb-γ when Bcl11a is overexpressed in Pogz−/− FL erythroblasts, suggesting that POGZ may regulate embryonic globin expression by mechanisms other than regulation of Bcl11a expression. Since β-actin-Cre-mediated deletion of Pogz occurs early in development, the absence of Pogz could affect expression of genes other than Bcl11a, which could potentially affect BCL11A’s ability to properly function in these cells. Alternatively, BCL11A may require POGZ expression to repress embryonic globin gene expression. In support of this hypothesis, overexpression of Bcl11a in MEL cells transduced with shPogz RNA, where Pogz expression has been knocked down to 10%–15% of control Pogz expression levels, leads to partial repression of Hbb-γ expression. BCL11A has been the focus of numerous studies to find unique therapeutic targets in SCD and β-thalassemia (Sankaran et al., 2008, 2009, 2010b). It is hypothesized that reduction of BCL11A expression in patients with SCD and β-thalassemia could lead to derepression of fetal hemoglobin, thereby alleviating the symptoms of these disorders (Bauer et al., 2012). The decrease in Bcl11a expression and loss of repression of β-like embryonic globin expression upon loss of Pogz indicates that POGZ may have the same therapeutic potential.

Mice reconstituted with Pogz+/−; Mx1−/cre BMCs survive and show normal development of donor-derived MEP and erythroid lineage cells (S1–S5), as well as lymphoid and myeloid cells, suggesting that inhibiting POGZ function in adults would not have deleterious effects on the host hematopoietic system. However, additional studies are needed to determine whether loss of POGZ function in adults can affect HSC and multipotent progenitor development and function or affect other systems. Interestingly, we found that Pogz−/− mice, which develop normally and show no overt phenotypes, show increased embryonic globin expression levels in PBCs. Importantly, partial reduction of POGZ in human erythroblasts also derepressed fetal globin expression to levels reaching over 25% of total β-like globin. These results suggest that complete ablation of POGZ in vivo may not be required to obtain therapeutic benefits. Further in vitro and in vivo studies are needed to determine if this is feasible.

KLF1 is a master regulator of erythroid development and β-globin expression (Perkins et al., 1995). Klf1 knockout (KO) mice die in utero around E15 due to defects in the differentiation of erythroid cells at the pro-erythroblast stage (Nuez et al., 1995; Perkins et al., 1995; Pirson et al., 2008). KLF1 represses the expression of embryonic globins by upregulating the expression of Bcl11a and promotes adult β-globin expression in definitive erythroid cells (Tallack and Perkins, 2013; Zhou et al., 2010). Klf1 expression was not affected by loss of Pogz expression in FL cells. However, global gene expression analysis of Klf1−/− erythroid progenitors demonstrates that Pogz is among the significantly downregulated genes (Pilone et al., 2008). Furthermore,
analysis of submitted ChIP-sequencing data suggests that KLF1 binds the POGZ promoter in human primary erythroid cells, indicating that POGZ may be a direct KLF1 target (Su et al., 2013).

Recent evidence suggests that POGZ may have physiological functions in other systems. Specifically, studies analyzing the genetic basis of autism spectrum disorders (ASDs) and intellectual disability have detected inactivating mutations in POGZ in some of these patients (Iossifov et al., 2012; Stessman et al., 2016; Tan et al., 2016; White et al., 2016). Our preliminary studies also suggest a function for Pogz in the mammalian neural system, since loss of Pogz affects the proliferation of mouse neural progenitor cells in fetal and adult brain (K.O.G., unpublished data). Interestingly, potential disrupting mutations in the BCL11A gene have been found in ASDs, and BCL11A has been implicated in neuronal morphogenesis (Iossifov et al., 2012; John et al., 2012). In addition, it was shown in two separate studies that individuals presenting with ASD and developmental delay had common microdeletions of BCL11A rendering them haploinsufficient for the gene. Interestingly, these individuals have elevated expression of fetal hemoglobin (Basak et al., 2015; Funnell et al., 2015). These data suggest that BCL11A and POGZ could function within the same regulatory networks in the neural system.

In summary, our data show that POGZ is essential for normal embryonic development and that loss of the gene leads to deregulation of embryonic globin expression, in part through Bcl11a. Reduction of POGZ expression in erythroid cells could have therapeutic implications in SCD and β-thalassemia.

EXPERIMENTAL PROCEDURES

Mice

Conventional Pogz+/− mice and conditional Pogzfl/fl, MX1-cre mice were generated as described in Supplemental Experimental Procedures. Female mice aged 8–12 weeks were used as recipients for all transplantation experiments. Mice were housed, fed, and handled in accordance with the National Institutes of Health guidelines for animal care and use and the Guide for the Care and Use of Laboratory Animals, 8th Edition. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the National Cancer Institute at Frederick, which is accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Real-Time qPCR

Analysis of POGZ expression in KG1 cells by real-time qPCR was performed as described previously (Gudmundsson et al., 2007). Globin expression was analyzed in CD34+ HSPC-derived human erythroblasts using Taqman assays. For mouse FL cells, PBC and BMC RNA was isolated and real-time qPCR analysis performed in triplicate using Power SYBR Green PCR Master Mix (Life Technologies) and a 7500 Real-Time PCR System (Life Technologies) as previously described (Oakley et al., 2012). The ΔCt method was used to calculate relative changes in gene expression. Primer sequences are presented in Supplemental Experimental Procedures.

Flow Cytometry

Single-cell suspensions were prepared from Pogz+/+ or Pogz−/− FLs or from BMCs and PBCs from animals transplanted with Pogz+/+ or Pogz−/− FL cells or Pogz+/+: Mx1-cre or Pogz+/+: Mx1-cre BMCs. Cells were incubated with the antibodies described in Supplemental Experimental Procedures and then analyzed by FACS-Cantoll (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

FL and BMC Transplantations

FL cells were harvested from E14.5–E16.5 Pogz+/+ and Pogz−/− embryos, and BMCs were isolated from adult Pogz−/−; Mx1-cre and Pogz+/+: Mx1-cre mice and then transplanted as described in Supplemental Experimental Procedures using standard methodologies (Gudmundsson et al., 2012, 2014).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). An unpaired Student’s t test was used to calculate statistical significance. Results were considered significant if p < 0.05. Results are presented as the mean ± SD.

DATA AND SOFTWARE AVAILABILITY

The accession number for the microarray data reported in this paper is GEO: GSE113503.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.05.043.

ACKNOWLEDGMENTS

We wish to thank Mr. Steven Stull and Ms. Terri Stull for excellent animal technical support; Kathleen Noer, Roberta Matthai, and Guity Mohammad at the NCI-Frederick Flow Cytometry Core for flow cytometric analysis; Ms. Bobbi Smith for CBC analysis; and Dr. Miriam Anver at the NCI-Frederick Histotechnology/Pathology Laboratory for tissue sectioning and staining and pathology analysis. We thank Dr. Matthew Hsieh for information related to Institutional Review Board (IRB) protocols and Dr. Naoya Uchida for technical support regarding human CD34+ cell cultures. This project was funded in part by federal funds from the Frederick National Laboratory for Cancer Research, NIH (contract HHSN261200800001E) and by the intramural research program of the NHLBI and NIDDK. NIH (HL006009-09) and USUHS (R086414217). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

AUTHOR CONTRIBUTIONS

B.G., K.O.G., and J.R.K. designed and conducted experiments, analyzed results, and wrote the manuscript. J.F.T. designed and conducted experiments and analyzed results. K.D.K., S.K.S., L.S., S.S., Y.D., V.C., L.S., N.N., L. Tessarollo, L. Thorsteinsson, O.E.S., S.G., and T.R. designed and conducted experiments and analyzed results, and wrote the manuscript. J.F.T. designed and conducted experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 3, 2017
Revised: March 27, 2018
Accepted: May 14, 2018
Published: June 12, 2018

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