Protein tyrosine phosphatase PTPN9 regulates erythroid cell development through STAT3 dephosphorylation in zebrafish

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ABSTRACT
Protein tyrosine phosphatases (PTPs) are involved in hematopoiesis, but the function of many PTPs is not well characterized in vivo. Here, we have identified Ptpn9a, an ortholog of human PTPN9, as a crucial regulator of erythroid cell development in zebrafish embryos. ptpn9a, but not ptpn9b, was expressed in the posterior lateral plate mesoderm and intermediate cell mass – two primitive hematopoietic sites during zebrafish embryogenesis. Morpholino-mediated knockdown of ptpn9a caused erythrocytes to be depleted by inhibiting erythroid cell maturation without affecting erythroid proliferation and apoptosis. Consistently, both dominant-negative PTPN9 (with mutation C515S) and siRNA against PTPN9 inhibited erythroid differentiation in human K562 cells. Mechanistically, depletion of ptpn9 in zebrafish embryos in vivo or in K562 cells in vitro increased phosphorylated STAT3, and the hyper-phosphorylated STAT3 entrapped and prevented the transcription factors GATA1 and ZBP-89 (also known as ZNF148) from regulating erythroid gene expression. These findings imply that PTPN9 plays an important role in erythropoiesis by disrupting an inhibitory complex of phosphorylated STAT3, GATA1 and ZBP-89, providing new cellular and molecular insights into the role of ptpn9a in developmental hematopoiesis.

KEY WORDS: Erythroid cell development, PTPN9, STAT3, K562 cell

INTRODUCTION
Vertebrate hematopoiesis is a sequential process that occurs in primitive and definitive waves in anatomically distinct sites (Orkin and Zon, 2008). In mammals, primitive hematopoiesis occurs in the yolk-sac blood island, and definitive hematopoiesis originates in a distinct region known as the aorta-gonad-mesonephros (Cumano et al., 1996; Müller et al., 1994). In zebrafish, the primitive wave occurs in two intraembryonic regions, known as the anterior lateral plate mesoderm (ALPM) and the posterior lateral plate mesoderm (PLPM) that subsequently form the intermediate cell mass (ICM), whereas definitive hematopoiesis begins in the ventral wall of the dorsal aorta (de Jong and Zon, 2005). The first wave produces primitive erythrocytes and macrophages, and the second wave generates hematopoietic stem cells (HSCs), which are self-renewing and pluripotent. HSCs migrate to the fetal liver and then to the bone marrow in mammals, but migrate to the caudal hematopoietic tissue and finally colonize at the kidney in zebrafish.

Primitive hematopoiesis is regulated by several transcription factors, including the stem cell leukemia gene (SCL/TAL1), the lim-only 2 gene (LMO2), PU.1 (also known as SPI1), ZBP-89 (also known as ZNF148) and GATA1. By contrast, definitive hematopoiesis is initiated by the transcription factors RUNX1 and c-MYB (Paik and Zon, 2010). The importance of these factors has been demonstrated in cell-based ex vivo assays, as well as in knockout mouse models. SCL, a transcription factor with a basic helix-loop-helix motif, is a central regulator of hematopoietic differentiation. LMO2, a LIM domain transcription factor, acts in the same manner as SCL also in embryonic hematopoiesis (Gering et al., 2003; Shivdasani et al., 1995; Warren et al., 1994). The zinc finger protein ZBP-89 regulates hematopoietic progenitor cell differentiation (Li et al., 2006; Woo et al., 2008). Another zinc finger protein, GATA1, is specifically required for the maturation of proerythroblasts (Pevny et al., 1991). PU.1, a transcription factor that contains an ETS domain, plays an indispensable role in myeloid cell development (Scott et al., 1994). RUNX1, a runt domain transcription factor, is essential for the generation of HSCs in the ventral wall of the dorsal aorta (Chen et al., 2009; Kaley-Zylinska et al., 2002; Okuda et al., 1996). The myb-family member c-MYB maintains the population of HSCs in definitive hematopoiesis (Mucenski et al., 1991). These transcription factors form a sophisticated network in order to control the processes of primitive and definitive hematopoiesis.

Protein tyrosine kinases and protein tyrosine phosphatases (PTPs) play important roles in cell proliferation, differentiation, and migration through delicate regulation of signaling pathways. Human PTPN9 is a cytoplasmic phosphatase that is hyper-activated in erythroid progenitors in the bone marrow disorder polycythemia vera (Xu et al., 2003). Previous studies have shown that PTPN9 dephosphorylates NSF, FOXO1, ERBB2, EGFR and VEGFR2 for different functions, such as promoting homotypic vesicle fusion (Huynh et al., 2004), mediating insulin signaling in hepatocytes (Cho et al., 2006), suppressing breast cancer cell growth (Yuan et al., 2010) and regulating endothelial cell function (Hao et al., 2012). Recently, we have shown that PTPN9 directly interacts with STAT3 and mediates its dephosphorylation in breast cancer cells (Su et al., 2012). Overexpression of PTPN9 decreases tyrosine phosphorylation of STAT3, whereas depletion of PTPN9 increases its phosphorylation. Here, we show that PTPN9 has undergone duplication into ptpn9a and ptpn9b in...
zebrafish, and that *ptpn9a*, but not *ptpn9b*, regulates erythroid cell development by disrupting an inhibitory protein complex of phosphorylated STAT3, GATA1 and ZBP-89 in zebrafish embryos and human K562 cells.

**RESULTS**

**ptpn9a is expressed in the primitive hematopoietic sites during zebrafish embryogenesis**

To search for essential genes encoding PTPs downstream of zebrafish *cloche*, a classical mutant with hematopoietic and endothelial cell ablation (Stainier et al., 1995; Xiong et al., 2008), we performed an RNA in situ screen and then compared the expression of candidate genes between wild-type and *cloche* mutant embryos. From this screen, *ptpn9a* was downregulated in *cloche* mutants, which was further verified by semiquantitative PCR analysis (data not shown). In the zebrafish genome, we found two homologs, *ptpn9a* and *ptpn9b*, of the human gene PTPN9. Zebrafish *ptpn9a* contains 13 exons and encodes 572 amino acids, which have 67% identity to human and mouse PTPN9. *ptpn9b* also contains 13 exons but encodes 716 amino acids, and the protein possesses a long stretch of linker – it displays 41% similarity to human and mouse PTPN9 amino acids, and the protein possesses a long stretch of linker - it displays 41% similarity to human and mouse PTPN9.

Both *ptpn9a* and *ptpn9b* are expressed in the primitive hematopoietic sites during zebrafish embryonic development by using RNA in situ hybridization. Both *ptpn9a* and *ptpn9b* were expressed maternally, and their expression was observed through early embryogenesis (supplementary material Fig. S1G–I). We found that *ptpn9a* and *ptpn9b* were diffusely expressed in the trunk after 24 hpf, without enrichment in blood vessels or the ventral wall of the dorsal aorta (the definitive hematopoietic site) at 36 hpf (Fig. 1F; supplementary material Fig. S1I). Thus, these results suggest that *ptpn9a* is strongly associated with primitive hematopoiesis.

**Knockdown of *ptpn9a* diminishes the number of primitive erythrocytes**

To investigate the role of *ptpn9a* in primitive hematopoiesis, morpholino oligonucleotides against *ptpn9a* (*ptpn9a-MOs*) were injected into zebrafish embryos at the one- to two-cell stage. Most embryos tolerated the injection of 4 ng of morpholino oligonucleotide, but higher doses were toxic. *ptpn9a* morphants developed normally in the period up to 12 hpf but showed a short body length, ventrally curved tail and small head with moderate necrosis in the anterior region at 24 hpf (Fig. 2B). The number of circulating blood cells, as detected by o-Dianisidine staining, was greatly decreased in *ptpn9a*-MO-injected embryos (Fig. 2E) compared with that of wild-type embryos (Fig. 2D) at 48 hpf. Both the abnormal appearance and lack of erythrocytes were partially rescued in *ptpn9a* morphant embryos upon co-injection of human PTPN9 mRNA (80 pg/embryo) (Fig. 2F), or zebrafish *ptpn9a* mRNA (100 pg/embryo) (supplementary material Fig. S1L–N). Neither *ptpn9b* nor SEC14-deleted *ptpn9a* mRNA was able to rescue the *ptpn9a* morphant phenotype (supplementary material Fig. S1O–T), supporting the notion of there being a specific role of *ptpn9a* in erythroid cell development. Using the transgene Tg[gal4a1:DsRed] (Traver et al., 2003) to label differentiated erythrocyths, we found that the DsRed-positive population was substantially reduced in *ptpn9a* morphants at 48 hpf (supplementary material Fig. S2S–T). However, we did not see defects in primitive hematopoiesis after morpholino-oligonucleotide-mediated knockdown of *ptpn9a* in embryos (supplementary material Fig. S1J,K). Taken together, our data suggest that *ptpn9a*, but not *ptpn9b*, is essential for primitive hematopoiesis. To gain molecular insights into the hematopoietic hypoplasia in *ptpn9a* morphants, we investigated several known essential hematopoietic transcription factors. The transcription factors SCL/tal1, LMO2 and Fli1 are required for the generation of hematopoietic progenitors within the ALPM and PLPM (Gering et al., 2003; Liu et al., 2008). The expression of these three genes appeared to be unaffected in *ptpn9a*-knockdown embryos at 14 hpf (supplementary material Fig. S2A,C,E), suggesting that *ptpn9a* is not required for the generation of hematopoietic progenitors. However, the expression of the erythroid marker *gata1* was reduced in the PLPM at 15 hpf and in the IC at 22 hpf in *ptpn9a*-MO-injected embryos (Fig. 2H,K), compared with that of wild-type embryos (Fig. 2G,J). Quantitative real-time PCR analysis further confirmed the diminished expression of the erythroid gene *gata1* (supplementary material Fig. S2W). Co-injection of human PTPN9 mRNA fully rescued *gata1* expression in *ptpn9a* morphants (Fig. 2L). These observations confirm the hypothesis that *ptpn9a* is not required for the generation of hematopoietic progenitors but is crucial for their differentiation into erythrocyths.

We then addressed whether definitive hematopoiesis is affected in *ptpn9a* morphants. Depletion of either runx1 or cmyb causes the complete absence of HSCs. We found that the populations of Runx1-positive and Cmyb-positive cells in the ventral wall of the dorsal aorta were unaltered in

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**Fig. 1. *ptpn9a* is expressed in the primitive hematopoietic sites during zebrafish embryogenesis.** RNA in situ hybridization with *ptpn9a* probe in one-cell (A), 6-hpf (B), 15-hpf (C), 18-hpf (D), 24-hpf (E) and 36-hpf (F) embryos. Note *ptpn9a* expression in the posterior lateral plate mesoderm (PLPM) (C; red arrowhead) and the intermediate cell mass (ICM) (D,E; red arrowheads) regions. Lateral views are shown with anterior to the left (D–F), and dorsal views are shown with anterior to the top (C).
ptpn9a-MO-injected embryos at 36 hpf (supplementary material Fig. S2I–L). Consistently, HSCs were detected at normal levels in ptpn9a morphants using the Tg(cd41:GFP) and Tg(cmyb:GFP) transgenic embryos (Lin et al., 2005; North et al., 2007) (supplementary material Fig. S2U–V; data not shown). Taken together, our results indicate that ptpn9a is not required for the specification of HSCs during embryogenesis.

Previous studies have shown that loss of gata1 expression results in the ectopic expression of pu.1, resulting in the conversion of primitive erythropoiesis into myelopoiesis (Galloway et al., 2005; Rhodes et al., 2005). We found comparable levels of pu.1 in the ALPM region of ptpn9a-MO-injected and wild-type embryos at 15 hpf (supplementary material Fig. S2G,H), as well as later in the ICM region at 22 hpf (data not shown). The expression of the myeloid genes l-plastin, myeloperoxidase (mpo) and lysozyme C (lyz) was mildly reduced in ptpn9a morphants (supplementary material Fig. S2N,P,R), compared with their expression in wild-type embryos (supplementary material Fig. S2M,O,Q). The mild reduction in the expression of myeloid genes in ptpn9a morphants was further verified using Tg(mpo:GFP) and Tg(lyz:dsRed) transgenic zebrfish, which have been widely used to label myeloid cells (Hall et al., 2007; Renshaw et al., 2006) (data not shown). Our observations suggest that the commitment to myeloid lineages develops normally, but that myeloid cell maturation is mildly affected, in ptpn9a morphants.

ptpn9a knockdown has no effect on erythroid cell proliferation and apoptosis in primitive hematopoiesis

To investigate the cellular mechanisms underlying ptpn9a-morphant phenotypes, we examined hematopoietic cell proliferation and apoptosis in ptpn9a-knockdown embryos. The Tg(e1205:GFP) transgene has been shown to label S, G2 and M cells with green fluorescence (Sugiyama et al., 2009). We found comparable numbers of cells that were positive for green fluorescence in the ICM region in ptpn9a-MO-injected and wild-type Tg(e1205:GFP) transgenic embryos at 24 hpf (Fig. 3A,B). Furthermore, the number of apoptotic cells in the ICM region, revealed by using a terminal transferase UTP nick-end labeling (TUNEL) assay, was also comparable in ptpn9a morphants and controls at 24 hpf (Fig. 3C,D). As expected, p53 (also known as p53) was not upregulated in the ICM region of ptpn9a-knockdown embryos (Fig. 3F). Co-injection of both ptpn9a-MOs and p53-MOs did not reverse the morphology, or the gata1 expression, in ptpn9a morphants (data not shown). To determine whether human PTPN9 also plays a similar role in mammalian developmental hematopoiesis, we used hemin in a differentiation system in order to induce K562 hematopoietic progenitors into mature erythrocytes. Consistently, inhibition of PTPN9 – by using either catalytically inactive dominant-negative

![Fig. 2. Knockdown of ptpn9a depleted embryos of primitive erythrocytes.](https://example.com/fig2.png)

**Fig. 2.** Knockdown of ptpn9a depleted embryos of primitive erythrocytes. (A–C) Brightfield images of control (A), a ptpn9a morphant (MO) (B) and a ptpn9a morphant that had been co-injected with human PTPN9 mRNA (C) at 24 hpf. (D–F) o-Dianisidine staining showed depletion of erythroid cells in ptpn9a-morphant embryos (E), compared with controls (D). Human PTPN9 mRNA partially rescued primitive erythropoiesis in ptpn9a-morphant embryos (F). In D and F, black arrowheads indicate erythrocytes. (G–L) RNA in situ hybridization was performed with gata1 probes in controls at 15 hpf (G) and 22 hpf (J), ptpn9a morphants at 15 hpf (H) and 22 hpf (K), and ptpn9a morphants that had been co-injected with human PTPN9 mRNA at 15 hpf (I) and 22 hpf (L). Note reduced gata1 expression in the PLPM (H) and ICM (K), which were partially rescued by co-injection of human PTPN9 mRNA (L). Red arrowheads indicate the ICM (J–L). Lateral views are shown with anterior to the left (A–C, D–F, J–L); dorsal views are shown with anterior to the top (G–I). The number of phenotypic embryos/total embryos is shown in the bottom left-hand corner.

![Fig. 3. ptpn9a knockdown causes no defect in hematopoietic cell proliferation and apoptosis.](https://example.com/fig3.png)

**Fig. 3.** ptpn9a knockdown causes no defect in hematopoietic cell proliferation and apoptosis. (A,B) ptpn9a-MOs were injected into Tg(e1205:GFP) transgenic embryos, in which green-fluorescence-positive cells are in phases S, G2 or M of the cell cycle. Note that there is little difference in the numbers of green-fluorescence-positive cells in the ICM between ptpn9a morphant (B) and wild-type (A) embryos at 24 hpf. (C,D) The TUNEL assay showed comparable numbers of apoptotic cells in the ICM in ptpn9a morphants (D) and controls (C) at 24 hpf. (E,F) RNA in situ hybridization revealed that expression of the p53 gene was not induced in the ICM of ptpn9a-morphant embryos (F), compared with wild-type controls (E). Red arrowheads indicate the ICM. The number of phenotypic embryos/total embryos is indicated.
PTPN9 (N9CS), which carried a mutation of cysteine residue 515 to serine, or PTPN9 siRNA (shN9) – had no effect on human K562 cell apoptosis (supplementary material Fig. S3A–F) and hemin-induced K562 cell proliferation (supplementary material Fig. S3G). These observations suggest that ptpn9a is not required for erythroid cell proliferation and apoptosis.

**PTPN9 is required for erythroid cell maturation**

We then hypothesized that ptpn9a might play a role in erythroid cell maturation. We chose to investigate the expression pattern of two markers of the terminal differentiation of erythrocytes, \( \beta^e1\)-globin and \( \beta^e2\)-globin (also known as \( hbb^e1 \) and \( hbb^e2 \), respectively), during primitive hematopoiesis. The expression of both genes gradually diminished from 26 to 48 hpf in ptpn9a morphants (Fig. 4A,C,E,G,I) compared with that in controls (Fig. 4B,D,F,H,J). Quantitative real-time PCR analysis further confirmed the diminished expression of erythroid genes, including \( \beta^e1\)-globin and \( \beta^e2\)-globin (supplementary material Fig. S2Y,Z). Co-injection of human PTPN9 mRNA (80 pg per embryo) or zebrafish ptpn9a mRNA (100 pg per embryo) rescued the expression of \( \beta^e1\)-globin in ptpn9a-MO-injected embryos (Fig. 4K,L; supplementary material Fig. S1L–N). We conclude that in ptpn9a-knockdown embryos primitive hematopoiesis is initiated, whereas the differentiation of hematopoietic progenitors into erythrocytes is blocked, resulting in the accumulation of immature erythroid cells.

We then used the differentiation system in order to induce K562 hematopoietic progenitors to mature erythrocytes in the presence of hemin. Compared with vector controls, we found that inhibition of PTPN9 function by a dominant-negative form of PTPN9 (N9CS) significantly reduced the percentage of benzidine-positive erythrocytes (Fig. 4M). Consistently, knockdown of PTPN9, by stably silencing PTPN9 in K562 cells with specific RNA interfering constructs (shN9), also led to a reduction of benzidine-positive erythrocytes (Fig. 4M). The short hairpin construct termed ShN9-3 was the most potent short hairpin among three RNAi constructs (supplementary material Fig. S4B). Furthermore, quantitative real-time PCR revealed that...
hemin-induced expression of the erythroid genes encoding γ-globin (here, specifically, HBG2). CD235a (also known as GYPE) and CD71 (also known as TFRC) was inhibited in K562 cells that expressed N9CS or shN9 (Fig. 4N–P). Taken together, these data suggest that PTPN9 is required for erythroid cell maturation.

**Increased STAT3 phosphorylation by knockdown of PTPN9 enhances formation of the STAT3–GATA1–ZBP-89 complex**

Our previous work has shown that PTPN9 directly dephosphorylates STAT3 in breast cancer cells (Su et al., 2012). Because STAT3 plays an important role in mammalian hematopoiesis, we hypothesized that Ptpn9a might also dephosphorylate Stat3 during zebrafish hematopoiesis. We found that *ptpn9a* knockdown increased, whereas overexpression of *ptpn9a* decreased, Stat3 phosphorylation in zebrafish embryos at 18 hpf (Fig. 5A). By contrast, *ptpn9b* knockdown had no effect on the phosphorylation of Stat3 in zebrafish (supplementary material Fig. S4A). In human K562 cells, STAT3 phosphorylation was also increased after overexpression of the dominant-negative PTPN9 mutant N9CS (Fig. 5B; supplementary material Fig. S4C) or transfection with *PTPN9* RNAi constructs (shN9) (supplementary material Fig. S4B). These data suggest a possible direct dephosphorylation of STAT3 by PTPN9 in zebrafish embryos and human K562 cells.

To demonstrate a physical interaction between PTPN9 and STAT3, FLAG-tagged wild-type STAT3 or FLAG-tagged dominant-negative mutant STAT3 (with the mutation Y705F) (Rong et al., 2006) was co-transfected with Myc-tagged PTPN9 (Myc–PTPN9) into 293T cells. In the presence of IL-6 and soluble IL-6 receptor after transfection, the protein complex was isolated by immunoprecipitation by using an antibody against FLAG. These experiments revealed that both endogenous PTPN9 (Fig. 5C) and overexpressed Myc–PTPN9 physically associated with STAT3 and preferentially bound to wild-type STAT3 (supplementary material Fig. S4D). Previous studies have shown that formation of the STAT3–GATA1 complex leads to the inhibition of the expression of γ-globin (Ezoe et al., 2005; Foley et al., 2002; Kiriott et al., 1998; Yao et al., 2009). Furthermore, the Krüppel-type zinc finger transcription factor ZBP-89 has been previously reported to interact with GATA1 and regulate expression of the globin genes and erythroid differentiation during hematopoiesis in zebrafish (Li et al., 2006; Ohneda et al., 2009; Woo et al., 2011; Woo et al., 2008). Based on these studies, we hypothesized that STAT3, GATA1 and ZBP-89 might form a multiprotein complex to regulate erythroid differentiation. Indeed, we found that endogenous STAT3 interacted with GATA1 and ZBP-89 (Fig. 5D; supplementary material Fig. S4H). Myc–GATA1 preferentially interacted with FLAG-tagged wild-type, but not dominant-negative, STAT3, indicating that the interaction requires activation of STAT3 phosphorylation. We also showed that ZBP-89 interacted with active STAT3 (supplementary material Fig. S4E,F) and that there is a physical interaction between GATA1 and ZBP-89 in murine erythroleukemia (MEL) cells, which is consistent with a previous report (Woo et al., 2008) (supplementary material Fig. S4G).

Owing to the inhibitory effect of phosphorylated STAT3 on erythroid cell maturation (Foley et al., 2002; Yao et al., 2009), we assumed that knockdown of *PTPN9* might increase STAT3 phosphorylation and formation of the STAT3–GATA1–ZBP-89 complex, which prevents binding of GATA1 and ZBP-89 to the promoters of their target genes. We found that increased STAT3 phosphorylation, by overexpression of dominant-negative N9CS, led to the recruitment of more GATA1 and ZBP-89 into the complex (Fig. 5E). By contrast, decreasing phosphorylated STAT3 by overexpression of wild-type PTPN9 prevented formation of the phosphorylated STAT3, GATA1 and ZBP-89 complex (Fig. 5E). Consistent with these results, we found that increasing phosphorylated STAT3, by using shN9 knockdown, caused the formation of more phosphorylated STAT3, GATA1 and ZBP-89 complexes than in controls (Fig. 5F). As predicted, we further showed that inhibition of PTPN9 by either N9CS or shN9 decreased the binding of GATA1 to its target genes γ-globin and CD235a by using chromatin immunoprecipitation assays, which was fully rescued by co-transfection of dominant-negative STAT3 (Fig. 5G,H). In addition, PTPN9 knockdown by *ptpn9a*-MO in zebrafish, or abrogation of its function, by using N9CS and shN9, in human K562 cells decreased the expression of GATA1 but not STAT3 and ZBP-89 (Fig. 2G,H; Fig. 5A; supplementary material Fig. S2W–X and Fig. S4J,M,N). GATA1 knockdown in K562 cells decreased the complex formation but had no effect on the expression of phosphorylated STAT3, total STAT3 and ZBP-89 (supplementary material Fig. S4K), and ZBP-89 knockdown in K562 cells decreased the complex formation but slightly reduced GATA1 levels and had no effect on phosphorylated STAT3 and total STAT3 (supplementary material Fig. S4L). These data strongly support the notion that STAT3 phosphorylation, which is negatively regulated by PTPN9, is crucial for the formation of an inhibitory protein complex comprising phosphorylated STAT3, GATA1 and ZBP-89.

**Dephosphorylation of STAT3 by PTPN9 contributes to erythroid cell maturation**

To further test our hypothesis on the inhibitory protein complex of phosphorylated STAT3, GATA1 and ZBP-89, we investigated erythroid cell maturation by manipulating the levels of phosphorylated STAT3, GATA1 or ZBP-89 in zebrafish embryos and human K562 cells. Remarkably, overexpression of *gata1* (50 pg per embryo), *zbp-89* (100 pg per embryo) or human dominant-negative *STAT3* (50 pg per embryo) partially rescued βε1-globin expression in *ptpn9a* morphants (Fig. 6C,L). Additionally, overexpression of *gata1* rescued erythroblasts, as identified by o-Dianisidine staining, in *ptpn9a* morphants (Fig. 6F). In K562 cells expressing N9CS or shN9, upon treatment with hemin for 48 h, we consistently found that overexpression of dominant-negative STAT3 partially rescued the number of benzidine-positive erythroid cells and the expression of erythroid genes γ-globin, CD235a and CD71 (Fig. 6M–P), whereas overexpression of dominant-negative STAT3 or constitutively active STAT3 alone had no effect on K562 cell proliferation (supplementary material Fig. S3I). Consistently, overexpression of constitutively active STAT3 decreased the benzidine-positive erythroblasts upon treatment with hemin for 48 h (supplementary material Fig. S3J). However, we found that decreasing STAT3 phosphorylation by overexpression of either wild-type PTPN9 or dominant-negative STAT3 alone had no effect on the formation of benzidine-positive erythroblasts after the induction of maturation upon treatment with hemin for 48 h (supplementary material Fig. S3H,J). Taken together, we conclude that PTPN9 plays a crucial role in erythroid cell maturation by dephosphorylating STAT3.
Fig. 5. Increasing phosphorylated STAT3 by PTPN9 knockdown stimulates the formation of an inhibitory complex comprising phosphorylated STAT3, GATA1 and ZBP-89 and reduces the GATA1 binding activity. (A) The phosphorylation (pStat3) status of endogenous Stat3 protein was elevated in \( ptn9a \)-morphant (MO) embryos but reduced in \( ptn9a \)-mRNA-injected embryos (mRNA), compared with wild-type (WT) embryos at 18 hpf, as shown by western blotting. The total amounts of Stat3 were relatively unchanged. (B) Endogenous phosphorylated STAT3, as shown by western blotting, was increased in human K562 cells that had been infected with the dominant-negative mutant PTPN9C515S (N9CS), compared with uninfected cells (WT) or cells infected with an empty vector (Vec). (C) The STAT3 protein complex, immunoprecipitated (IP) by using an antibody against STAT3, contained PTPN9, suggesting that endogenous PTPN9 physically interacts with STAT3 in K562 cells. IgG, control IP. (D) The endogenous STAT3 protein complex, immunoprecipitated by using an antibody against STAT3, contained GATA1 and ZBP-89, suggesting that endogenous STAT3 physically interacts with GATA1 and ZBP-89 in K562 cells. (E) Overexpression of the dominant-negative mutant PTPN9 (N9CS) in K562 cells increased phosphorylated STAT3 and promoted the formation of the phosphorylated-STAT3–GATA1–ZBP-89 complex, whereas overexpression of wild-type PTPN9 (N9WT) decreased phosphorylated STAT3 and the protein complex, compared with the vector control (Vec) group. (F) PTPN9 knockdown (shN9) also increased phosphorylated STAT3 and promoted the formation of the phosphorylated-STAT3–GATA1–ZBP-89 complex, compared with a scrambled construct (Scr) in K562 cells. K562 cells were induced by using IL-6 for 6 h before cell lysates were prepared for immunoprecipitation with an antibody against STAT3. (G,H) Overexpression of either dominant-negative mutant PTPN9 (N9CS) or PTPN9 knockdown (shN9) in K562 cells reduced the binding of GATA1 to its target genes \( \gamma \)-globin and CD235a, compared with vector (Vec) or scrambled (Scr) controls. Overexpression of dominant-negative STAT3 (STAT3DN) rescued the binding of GATA1 to its target genes in K562 cells that had been infected with mutant PTPN9 (N9CS) or transfected with PTPN9 shRNA (shN9). ChIP assays were performed using an antibody against GATA1 or IgG control. Quantitative PCR was used to evaluate the promoter occupancy of \( \gamma \)-globin and CD235a by GATA1 protein with or without treatment with hemin. The data are the means from three independent experiments ± s.d.; *P<0.05; ns, not significant.
DISCUSSION

In this study, we have demonstrated that PTPN9 functions in erythropoiesis in both zebrafish embryos and human K562 cells by regulating the differentiation of hematopoietic progenitors into erythroids and that PTPN9 acts through dephosphorylating STAT3, preventing the formation of an inhibitory protein complex comprising phosphorylated STAT3, GATA1 and ZBP-89. Erythrocyte hypoplasia was not caused by defects in either erythroid cell proliferation or apoptosis but was mainly due to a deficiency in erythroid cell maturation in ptpn9a-knockdown zebrafish embryos and K562 cells that lacked functional PTPN9. We note that PTPN9 plays an essential role in the formation of erythroid colonies derived from human embryonic stem cells or human endothelial colony-forming cells (Huang et al., 2011; Xu et al., 2003). Because human PTPN9 was able to partially rescue ptpn9a-morphant phenotypes in zebrafish, we suggest that the two genes have conserved functions in erythroid maturation. We also found that the number of myeloid cells was decreased in ptpn9a morphants but could not be rescued by overexpression of a moderate dose of human dominant-negative STAT3 mRNA data not shown), suggesting that other downstream target proteins of PTPN9 are involved in the regulation of myeloid cell maturation.

Mechanistically, we demonstrated that knockdown of PTPN9 increased STAT3 phosphorylation and then impaired erythroid cell development through the inhibition of erythroid gene expression by GATA1 and ZBP-89. Previous studies have elucidated that phosphorylated STAT3 inhibits GATA1 through a direct protein–protein interaction (Ezoe et al., 2005; Yao et al., 2009). The ability of phosphorylated STAT3 to suppress GATA1 transactivation has been attributed to the alteration of GATA1 binding stability. At present, it remains to be determined whether
the same mechanism is involved in the relationship between phosphorylated STAT3 and ZBP-89. Signal transducers and activators of transcription (STAT) proteins are transcription factors that are activated by phosphorylation on tyrosine residues after cytokine stimulation. During erythropoiesis, Janus kinase 2 (JAK2) transduces erythropoietin (EPO) signals to enhance erythroid cell proliferation and differentiation by phosphorylating STAT5 and STAT3. In zebrafish, knockdown of *epo* or *jak2a* decreases primitive erythropoiesis (Ma et al., 2007; Paffett-Lugassy et al., 2007). Our preliminary experiments revealed that STAT5 and STAT3 were tyrosine-phosphorylated by EPO stimulation in K562 cells (data not shown). After EPO stimulation, we noted that STAT3 phosphorylation was increased in K562 cells that expressed mutant PTPN9 (N9CS), a response similar to that seen in response to IL6 stimulation, shown in supplementary material Fig. S4C. However, STAT5 phosphorylation was unchanged in this condition, which is consistent with our previous observation that PTPN9 interacts very weakly with STAT5 (Su et al., 2012). Another study showed that PTPN9 preferentially associates with JAK1 but not TYK2 and JAK2 (Hao et al., 2012). Considering these findings together, we favor the notion that PTPN9 predominantly dephosphorylates STAT3 in erythropoiesis, which is different from the targets of other phosphatases such as SHP-1, SHP-2 and CD45 (also known as PTPN6, PTPN11 and PTPRC, respectively) (Valentino and Pierre, 2006). SHP-1 has been shown to dephosphorylate JAK2 through an interaction with GRB2 (Minoo et al., 2004) and directly dephosphorylate STAT5 in a PLC-β-dependent manner (Xiao et al., 2009). Mice deficient for Shp-1 display high proliferation of all hematopoietic lineages. Zebrafish *shp-1 (ptpn6)* is only expressed in myeloid cells during hematopoiesis (Zakrzewska et al., 2010). SHP-2, a widely distributed phosphatase, is associated with EPO receptor, c-KIT and GP130 (also known as IL6ST) in hematopoietic cells (Tauchi et al., 1996; Tauchi et al., 1994). Deletion of the *Shp-2* gene is embryonic lethal in mice, and mutations of *SHP-2* cause human Noonan syndrome. In zebrafish, *shp-2 (ptpn11)* is expressed ubiquitously during embryogenesis with the highest expression in the developing central nervous system and spinal cord (Jopling et al., 2007; Stewart et al., 2010). CD45, specifically expressed in hematopoietic cells, regulates T-cell and B-cell antigen receptor signaling. CD45-knockout mice show a loss of antigen responses in T and B lymphocytes. In zebrafish, cd45 marks a subset of aorta-gonad-mesonephros HSCs and differentiated leukocytes (Bertrand et al., 2008). Therefore, the temporal and spatial expression of phosphatases and their targets determine the specificity and roles of phosphatases in hematopoietic development.

Normal levels of STAT3 phosphorylation are required for maintaining erythroid cell proliferation during embryonic development (Jenkins et al., 2002; Jenkins et al., 2005; Kiriti et al., 1998; Mantel et al., 2012; Welte et al., 2003). However, our work showed that hyper-phosphorylated STAT3 impaired erythroid cell maturation in zebrafish and human K562 cells. In *Gp130*Y757F/Y757F mutant mice, GP130-dependent STAT3 hyperactivation results in increased numbers of hematopoietic progenitor cells (Jenkins et al., 2005). In *Gp130*Y544F mice, which contain a C-terminal truncation mutation in GP130 that eliminates GP130-mediated STAT3 activation, hematopoietic progenitor cells – including committed erythroid progenitors – are also increased (Jenkins et al., 2002). Endothelial deletion of STAT3 in mice bred from homozygous *Stat3-loxP* mice and *Tie2-Cre* mice causes defects in hematopoietic stem cell proliferation and differentiation, leading to erythroid dysplasia and increased myeloproliferation (Mantel et al., 2012; Welte et al., 2003). These studies indicate that STAT3 plays a crucial role in definitive hematopoiesis. Taken together with our data presented here and the work of others, we propose that STAT3 phosphorylation, under the control of different phosphokinases and phosphatases, plays different roles in primitive and definitive hematopoiesis and that, in zebrafish, Ptpn9 might interact with different substrates in order to regulate primitive and definitive hematopoiesis.

**MATERIALS AND METHODS**

**Fish maintenance**

Transgenic reporter zebrafish for the S, G2 and M phases of the cell cycle were generated, as described previously (Sugiyama et al., 2009). *Tg(gata1:DsRed)* were kindly provided by Shuo Lin (University of California Los Angeles, Los Angeles, CA), *Tg(mpox:GFP)*, *Tg(h2c:dsRed)*, *Tg(cdx1:GFP)* and *Tg(cmyb:GFP)* zebrafish were provided by Zilong Wen (Hong Kong University of Science and Technology, Hong Kong, China). Wild-type Tupfel long fin, AB and transgenic zebrafish were raised and handled in accordance with the guidelines of the Peking University Institutional Animal Care and Use Committee accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

**Constructs**

The zebrafish *ptpn9a* and *ptpn9b* genes were amplified and subcloned into vector pEASY Blunt and pXT7 for antisense probe and mRNA synthesis. SEC14-deleted *ptpn9a* cDNA was cloned into the pXT7 vector, which was used as a template for mRNA synthesis. Plasmid clones for the generation of probes of zebrafish *runx1, cmyb, b-def-globin, imo2* and *mpox* were kindly provided by Feng Liu (Institute of Zoology, Beijing, China). Other plasmid clones for generating probes of zebrafish *gata1, scl, pu.1, l-plastin, b-def-globin, lyc, bmp4* and *notch1b* were stocks from our laboratory. Constructs for mRNA synthesis (zebrafish *gata1* and *zbp-89*) and protein expression (human *GATA1* and *ZBP-89*) were kindly provided by Xiangen Li (Harvard University, Cambridge, MA) (Li et al., 2006). shRNA against human GATA1 (shGATA1) and human ZBP-89 (shZBP-89) were designed as described previously (Woo et al., 2011). Human PTPN9, PTPN9C515S (a dominant-negative mutant of human PTPN9; N9CS), STAT3, STAT3Y705F (a dominant-negative mutant of STAT3), STAT3CA (constitutively active STAT3 with the double mutation A661C N663C) and shPTPN9 (shRNA against human PTPN9; shN9) constructs have been described previously (Su et al., 2012).

**Morpholinos, mRNA synthesis and microinjection**

The following morpholino antiseis oligonucleotides were purchased from Gene Tools and suspended as 2 mM stocks in RNase-free water. The sequences of *ptpn9a*-MO, *ptpn9b*-MO and *p53*-MO are available in supplementary material Table S1. Capped mRNAs were synthesized from linearized pXT7 constructs using the mMessage mMachine kit (Ambion). In all the microinjection experiments, a volume of 2.3 nl was injected into one- to two-cell-stage embryos.

**Whole-mount in situ hybridization, o-Dianisidine staining and TUNEL assay**

Antisense probes were synthesized using the DIG DNA labeling kit (Roche) according to the manufacturer’s instructions, as described previously (Xiong et al., 2008). o-Dianisidine staining has been reported previously (Danilova et al., 2011). TUNEL was performed using the In Situ Cell Death Detection Kit and TMR Red (Roche) according to the manufacturer’s instructions.

**Cell culture, infection, transfection, Hoechst 33342 and benzidine staining**

K562 cells and MEL cells were cultured in 1640 medium. Hemin (50 μM. Sigma-Aldrich) was used to induce erythroid differentiation in K562 cells. Human recombinant IL-6 (100 ng/ml, R&D Systems) and human
recombinant EPO (50 U/ml, R&D Systems) were used to stimulate K562 cells. K562 cells were infected with the retroviruses pMSCV-vector, pMSCV-PTPN9WT or pMSCV-PTPN9C155S. K562 cells (1×10⁷ cells) were electroporated at 280 V and 975 μF with 20 μg of nonlinearized plasmid (STAT3Y705F, STAT3A, shGATA1, shZBP-89 and shPTPN9 constructs) using Gene-Pulser II (Bio-Rad). Stable cell-lines were selected with 0.6 mg/ml G418 and 1 μg/ml puromycin. HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium and transient transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Human recombinant IL-6 and IL-6 receptor (100 ng/ml, R&D Systems) were used to stimulate 293T cells. Benzidine staining has been reported previously (Cooper et al., 1974).

Semi-quantitative and real-time RT-PCR
Total RNA was isolated from embryos at different stages, or from harvested cells, using Trizol (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA by SuperScript II RT (Life Technologies) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad) and a SYBR Premix Ex Taq kit (Takara). Primer sequences are listed in supplementary material Table S1.

Western blot, chromatin immunoprecipitation and immunoprecipitation
Zebrafish embryos were lysed in modified radio-immunoprecipitation assay (RIPA) lysis buffer that had been supplemented with protease and phosphatase inhibitors (50 mM Tris-HCl pH 7.4, 0.25% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). K562 cells, MEL cells and 293T cells were harvested and used for immunoprecipitation reactions, as described previously (Hu et al., 2006). Chromatin immunoprecipitation (ChIP) assays were performed by using Pierce™ agarose ChIP kit according to the manufacturer’s protocol. FLAG-M2-antibody-conjugated beads (Sigma-Aldrich) were used for immunoprecipitation. The following antibodies against the indicated proteins were also used in this study: zebrafish phosphorylated Stat3 (Tyr708) (Medical and Biological Laboratories), STAT3 (C-20, Santa Cruz Biotechnology), β-actin (Cell Signaling Technology), phosphorylated Stat3 (Tyr705, D3A7) (Cell Signaling Technology), Stat3 (124H6, Cell Signaling Technology), GATA1 (D52H6, Cell Signaling Technology), ZBP-89 (H-7, Santa Cruz Biotechnology), ZBP-89 (S-15, Santa Cruz Biotechnology), phosphorylated Stat5 (Tyr694, D47E7, Cell Signaling Technology), Stat5 (Cell Signaling Technology), FLAG (M2, Sigma-Aldrich), Myc (Sigma-Aldrich), horseradish peroxidase (HRP)-conjugated light-chain-specific secondary antibodies (Abcam), and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). The rabbit polyclonal antibody against PTPN9 has been described previously (Su et al., 2012).

Statistical analysis
All experiments were repeated at least three times. A Student’s t-test was performed to evaluate the significance of differences between experimental and control groups. P<0.05 indicates a statistically significant effect.

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We thank Zhizhuang J. Zhao (University of Oklahoma, Oklahoma City, OK), Qing-Yuan Sun (Institute of Zoology, Beijing, China), Feng Liu (Institute of Zoology, Beijing, China) and lain C. Bruce (Zhejiang University, Hangzhou, China) for critical comments and reading of the manuscript; and members of Jing-Wei Xiong’s laboratory for helpful discussion and technical assistance.

Competing interests
The authors declare no competing interests.

Author contributions
Y.B. designed and performed most of the experiments, and contributed to writing the manuscript; F.S. and Z.C. provided reagents and performed some of the biochemical experiments; S.W., H.G., L.L., N.C., Q.W., K.H. and X.Z. contributed some essential reagents and performed some zebrafish experiments; K.M.

References


Supplemental Materials

Protein Tyrosine Phosphatase PTPN9 Regulates Erythroid Cell Development through STAT3 Dephosphorylation in Zebrafish

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### Supplemental Table S1. Primer and morpholino sequences.

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>Primer-R: 5’-CTCTCAATAGCCTTTATCCAGCTGAT-3’</td>
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<td>zbp-89</td>
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AAGAGAAAAAGTCAGGGCCCCCATAAGCCTAA-3’
Reverse: 5’- AGCTTTAGTGCTTA TGGGGGCCCTGACTTTTCT
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shZBP-89 Forward: 5'- GATCCCCGGGCTTTTCGATCAGGAATGAACTCT
CGAGAATTCATTTCCTGATCGAAAGCCTTTTTTA-3’
Reverse: 5’- AGCTTTAAAAAGGCTTTTCGATCAGGAATGAACT
CTCGAGAATTTCATTTCCTGATCGAAAGCCCGGG-3’

γ-globin-ChIP Forward: 5'-CGGCGGCTGGCTAGGGATGAA-3'
Reverse: 5’-CTGTGAAATGACCCATGGCG-3’

CD235a-ChIP Forward: 5’- GTGAAAATTTTACTTACCACTTTCA-3’
Reverse: 5’- TGCAAGTGTCAGTGTCTGACCTTAG-3’

ptpn9a-MO: 5’-TCGGCCATCA TCCACTCTCCGGCGC-3’

ptpn9b-MO: 5’-TTAATGTACCTTATAAGCTTG-3’

p53-MO: 5’-GCGCCATTGCTTTTGCAAGAATTG-3’
Supplemental Figure Legends

**Supplemental Fig. S1. Ptpn9a, but not ptpn9b, is crucial for early development in zebrafish.** (A) Genomic structure of ptpn9a and ptpn9b in zebrafish, showing morpholino targeting sites (ptpn9a-MO and ptpn9b-MO) and RT-PCR primers for Fig. S2H by the red lines. (B) Protein sequence alignments among human PTPN9 (hPTPN9), mouse Ptpn9 (mPtpn9), and zebrafish Ptpn9a (ptpn9a) and Ptpn9b (ptpn9b). Note hPTPN9, mPtpn9 and Ptpn9a are highly conserved while Ptpn9b has unique linker amino-acids. (C) Genomic synteny shows that Ptpn9a is a potential ortholog of hPTPN9 and mPtpn9, while Ptpn9b was derived from zebrafish genome duplication. (D-I) RNA *in situ* hybridization was performed with a ptpn9b probe in 1-cell (D), 6 hpf (E), 15 hpf (F), 18 hpf (G), 24 hpf (H), and 36 hpf (I) embryos. (J-K) No apparent blood defects were found in ptpn9b morphants (J, right panel), compared with wild-type controls (J, left panel). Ptpn9b-MO was injected at 10 ng per embryo. Semi-quantitative RT-PCR revealed that ptpn9b was not detectable in ptpn9b morphants (K), suggesting that ptpn9b-MO was effective. Gapdh was used as an internal control. (L-N) Co-injection with synthetic zebrafish ptpn9a mRNA rescued βe1-globin and embryo morphology in ptpn9a morphant embryos at 48 hpf (N), compared with controls (L). To prevent binding of synthetic zebrafish ptpn9a mRNA to ptpn9a-MO, we added the kozak sequence (GCCACC) before the coding district sequence (CDS) of ptpn9a. (O-T) Over-expression of either ptpn9b mRNA or truncated ptpn9a mRNA that the SEC14 domain of Ptpn9a was deleted had no rescue on βe1-globin and embryo morphology in ptpn9a morphants at 48 hpf (Q, T), compared with controls (O, R). Lateral views are shown with anterior to the left (G-J, L-T), dorsal views are shown with anterior to the top (F).

**Supplemental Fig. S2. ptpn9a knockdown depletes erythrocytes and has mild effects on myeloid cells but not hematopoietic stem cells.** (A-F) The hematopoietic progenitor markers scl/tal1, lmo2, and fli1 were not affected in ptpn9a morphants (B, D, F), compared with controls (A, C, E) at 14 hpf. Red arrowheads indicate the ALPM and the PLPM. (G-H) The myeloid progenitor marker pu.1 was not altered in ptpn9a morphants (H), compared with controls (G) at 15 hpf. (I-L) The hematopoietic stem cell markers runx1 and cmyb were not altered in ptpn9a morphants (J, L), compared with controls (I, K) at 36 hpf. (M-R) The myeloid genes l-plastin, myeloperoxidase (mpo) and lysozyme C (lyz) were slightly reduced in ptpn9a morphants (N, P, R), compared with controls (M, O, Q) at 60 hpf. (S-T’) Ptpn9a-MOs were injected into Tg(gata1-deRed) transgenic embryos, in which erythrocytes were labeled by DsRed. Note the erythrocytes were abolished in ptpn9a morphants at 48 hpf (T’), compared with controls (T), Bright-field images of wild-type (S) and morphant (S’) as shown in panels. (U-V’) Ptpn9a-MOs were injected into Tg(cmyb-EGFP) transgenic embryos, in which HSCs were labeled by EGFP. Note comparable numbers of EGFP-positive HSCs in the caudal hematopoietic tissue between ptpn9a morphant (V’) and wild-type (V) embryos at 48 hpf. Bright-field images of control (U) and morphant (U’). Lateral views are shown with anterior to the left (A-B; E-F; I-V’); dorsal views are shown with anterior to the top (C-D; G-H). (W-Z) Differentiated erythroid genes gata1, βe1-globin and βe2-globin reduced in ptpn9a morphants, compared with controls (W, Y and Z), but hematopoietic progenitor gene zhp-89 was normally expressed in both ptpn9a morphants and wild-type embryos (X). Erythroid gene
expression was normalized by GAPDH. Measurements are the means from 3 independent experiments ± SD. *P <0.05.

**Supplemental Fig. S3. Knockdown of PTPN9 has no effect on proliferation and apoptosis in K562 cells.** (A-F) The infected or transfected K562 cells were stained with Hoechst 33342 after hemin induction for 48 h. Knockdown PTPN9 by either N9CS or shN9 had no apoptotic mass in human K562 cells. (G, I) In the presence of Hemin, knockdown PTPN9 by either N9CS or shN9 had no effect on K562 cell proliferation (G); overexpression of either STAT3DN or STAT3CA had no effect on K562 cell proliferation (I). (H, J) Overexpression of either N9WT or STAT3DN had no effect on formation of benzidine-positive erythroids while overexpression of STAT3CA decreased formation of benzidine-positive erythroids with hemin induction (H, J).

**Supplemental Fig. S4. Increased STAT3 phosphorylation (pSTAT3) by knockdown of PTPN9 enhances pSTAT3 interactions with hematopoietic transcription factors GATA1 and ZBP-89.** (A) The phosphorylation status of endogenous Stat3 proteins was unchanged in ptpn9a morphant embryos, compared with wild-type embryos at 18 hpf. The total amounts of Stat3 were unchanged too. (B) Three PTPN9 RNAi (shN9-1, shN9-2, shN9-3) were assessed for silencing of the PTPN9 gene, noting that shN9-3 was the most potent PTPN9 RNAi. Repressing PTPN9 expression by each of the three PTPN9 RNAi led to increased pSTAT3, compared with wild-type (WT) K562 cells and cells transfected with scrambled vector (Scr). (C) The pSTAT3 was elevated in K562 cells infected with dominant-negative mutant PTPN9 (N9CS) while it was decreased in cells infected with wild-type PTPN9 (N9WT), compared with those with an empty vector (Vec). Both control and infected K562 cells were stimulated with IL-6 for 30 min after starvation. (D) PTPN9 preferentially bound to wild-type Flag-tagged STAT3, compared with Flag-tagged dominant-negative mutant STAT3. The transfected 293T cells were stimulated with IL-6 in the presence of soluble IL-6 receptor (IL-6R) 48 h after transfection. (E) GATA1 interacted with wild-type STAT3 (STAT3WT) but not dominant-negative STAT3 (STAT3DN). After 48 h post-transfection, K562 cells were stimulated with IL-6 for 1 h before making cell lysates. (F) ZBP-89 interacted with wild-type STAT3 (STAT3WT) but not dominant-negative STAT3 (STAT3DN). After 48 h post-transfection, K562 cells were stimulated with IL-6 for 1 h before making cell lysates. (G) Endogenous GATA1 interacted with ZBP-89 in mouse MEL cells. Lysates from MEL cells were immunoprecipitated with anti-GATA1 antibody. (H) Endogenous GATA1 interacted with ZBP-89 and pSTAT3 in human K562 cells. Lysates from K562 cells were immunoprecipitated with anti-GATA1 antibody. (I-J) GATA1 reduced in both N9CS and shN9 groups, compared with Vec and Scr groups after Hemin induction. (K-L) Either GATA1 RNAi (shGATA1) or ZBP-89 RNAi (shZBP-89) had high efficiency in silencing GATA1 or ZBP-89 gene. Repressing GATA1 or ZBP-89 expression by shGATA1 or shZBP-89 resulted in the disruption of GATA1, ZBP-89 and STAT3 protein interactions, compared with K562 cells transfected with scrambled vector (Scr), but had no effect on the STAT3 phosphorylation. (M-N) GATA1 but not ZBP-89 reduced in human K562 cells that PTPN9 was depleted by either N9CS or shN9 after Hemin induction for 48h. Empty vector and scramble siRNA were used as controls. Erythroid gene expression was normalized by GAPDH. Measurements are the means from 3 independent experiments ± SD. *P <0.05.
Supplemental Fig. S2

A-D: WT and MO embryo images showing the effects of PTPN9 knockdown on various markers.

E-H: L-plastin, puro1, cmmyb, and lyz images at different stages.

I, J: L-plastin, mpo, and lyz images with corresponding stage annotations.

K, L: L-plastin, mpo, and lyz images with stage annotations.

M, N: L-plastin images showing expression patterns.

O, P: mpo images showing expression patterns.

Q, R: lyz images showing expression patterns.

W: Bar graphs showing gata1 expression levels at 15hpf and 22hpf for WT and PTPN9-MO conditions.

X: Bar graphs showing zbp89 expression levels at 15hpf and 22hpf for WT and PTPN9-MO conditions.

Y: Bar graphs showing β1-globin expression levels at 26hpf, 36hpf, and 48hpf for WT and PTPN9-MO conditions.

Z: Bar graphs showing β2-globin expression levels at 26hpf, 36hpf, and 48hpf for WT and PTPN9-MO conditions.