Profilin 1 is essential for retention and metabolism of mouse hematopoietic stem cells in bone marrow

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Running Title: Pfn1 is essential for HSC retention and metabolism

Scientific Section Heading: Hematopoiesis and Stem Cells
**Key points**

1. The deletion of pfn1 led to bone marrow failure, loss of quiescence, increased apoptosis and mobilization, and a metabolic switch of HSCs.

2. pfn1 partially acts through the axis of pfn1/Gα13/EGR1 to regulate the stem cell retention and metabolism in the bone marrow.

**Abstract**

How stem cells interact with the microenvironment to regulate their cell fates and metabolism is largely unknown. Here we demonstrated that the deletion of the cytoskeleton-modulating protein profilin 1 (pfn1) in hematopoietic stem cell (HSCs) led to bone marrow failure, loss of quiescence, and mobilization and apoptosis of HSCs in vivo. A switch from glycolysis to mitochondrial respiration with increased ROS level was also observed in HSCs upon pfn1 deletion. Importantly, treatment of pfn1-deficient mice with the antioxidant N-acetyl-L-cysteine reversed the ROS level and loss of quiescence of HSCs, suggesting that the metabolism is mechanistically linked to the cell cycle quiescence of stem cells. The actin-binding and proline-binding activities of pfn1 are required for its function in HSCs. Our study provided evidence that pfn1 at least partially acts through the axis of pfn1/Gα13/EGR1 to regulate the stem cell retention and metabolism in the bone marrow.
Introduction

In the bone marrow (BM) microenvironment, the fates of hematopoietic stem cells (HSCs) are balanced among quiescence, self-renewal, differentiation, apoptosis, and motility. This balance is achieved through the dynamic interaction of factors including transcription factors, epigenetic regulators, cytoskeleton molecules, and extrinsic cues from the environment. Recently, we demonstrated that HSCs possess a distinct metabolic profile with a preference for glycolysis rather than mitochondrial respiration. However, the relationship among cell fates, metabolism, and the microenvironment of HSCs is not well understood.

Pfn1 is a ubiquitously expressed member of the profilin family. Pfn1 enhances ADP-to-ATP exchange on G-actin and is capable of adding ATP-bound G-actin to the barbed ends of growing filaments. Pfn1 was therefore thought to play a central role in the regulation of de novo actin polymerization. Pfn1 also binds to phosphoinositides, phosphatidylinositol-3,4,5-triphosphate and a number of proline-rich proteins ranging from those participating in cytoskeletal organization to transcriptional control in cells. Binding of profilins to signaling molecules, such as Arp2/3 complex, Mena, VASP, N-WASP, dynamin I, and others, further implicates them as regulators of diverse activities including proliferation, migration, endocytosis, mRNA splicing, and transcription.

The pfn1 gene was first identified as a survival essential gene in Drosophila. Homozygous pfn1 knockout mouse embryos die as early as the two-cell stage, indicating
its essential role in survival and cell division of embryonic development. The roles of pfn1 in motility are not well consistent among different cell types. A number of studies indicated that pfn1 stimulates migration of endothelial cells, chondrocytes, human mesenchymal stem cells, and granule neurons. By contrast, pfn1 decreases motility and invasiveness of breast cancer cells in a mouse model and is downregulated in invasive bladder cancer cells compared to non-invasive counterparts.

The in vivo role of pfn1 in tissue-specific stem cells has not been reported. The availability of the pfn1 $^{\text{flox/flox}}$ mice provided us an opportunity to clarify the function of pfn1 in different tissues and stem cells in the whole animal. We bred HSC-specific Cre-ER mice that express inducible Cre in HSCs and pfn1 $^{\text{flox/flox}}$ mice to inducibly delete pfn1 in HSCs. We used this model to study the roles of pfn1 in hematopoietic development and to investigate the relationships of BM environment and metabolism and cell fates of HSCs. We showed that, different from its roles in many other types of cells, pfn1 is essential for the retention and quiescence of HSCs in the BM. Pfn1 also maintains glycolysis to directly control HSC quiescence, indicating that the unique metabolic property of HSCs is a determinant of quiescence of these stem cells.
Methods

Mice

C57 BL/6 CD45.2 and CD45.1 mice were purchased from the National Cancer Institute and from the University of Texas Southwestern Medical Center animal breeding core facility. To obtain an HSC-specific deletion of *pfn1*, C57BL/6 mice carrying the loxP-flanked *pfn1* gene were crossed with transgenic C57BL/6 mice expressing the tamoxifen-inducible Cre recombinase under the control of stem cell leukemia (Scl) HSC enhancer to produce Scl*pfn1* mice (sTable 1). For induction of Cre-ER recombinase, mice received intraperitoneal tamoxifen (Sigma, 1 mg/0.1 ml of corn oil) injections as described. Mice were maintained at the University of Texas Southwestern Medical Center animal facility. All animal experiments were performed with the approval of UT Southwestern Committee on Animal Care.

Flow cytometry, mouse HSC culture, competitive reconstitution analysis, and homing assay

The isolation of Lin*Sca-1+Kit*Flk2*CD34* cells (LT-HSCs), analysis of repopulation of mouse HSCs, and the Hoechst 33342/pyronin Y staining and BrdU incorporation were performed as described. Indicated numbers of BM Lin*Sca-1+Kit*Flk2*CD34* cells were cultured in serum-free medium supplemented with SCF, TPO, and FGF-1 as described. The competitive reconstitution analysis was conducted as we described before. Homing assays were performed as described. Details are included in the Supplementary Materials.
Measurement of $^{13}$C lactate production, ATP assay, and oxygen consumption analysis

The metabolic assays were performed essentially as we described $^{1,15}$. Details are described in the Supplementary Materials.

Measurement of reactive oxygen species

The measure of reactive oxygen species was performed essentially as we described $^{15}$. Briefly, control and Selpfn1 Lin$^-$ cells were incubated with 1 µM 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) (C-369, Invitrogen) for 30 min at 37°C in the dark. Then, cells were stained for HSCs markers Sca-1-PE/Cy5.5, C-Kit-APC, CD34-PE and Flk2-PE and assayed by flow cytometer. Antibodies were all purchased from BD Biosciences.

Treatment with N-acetyl-L-cysteine

Control and Selpfn1 mice were treated with tamoxifen for 7 days followed by treatment with N-acetyl-L-cysteine (NAC, Sigma) at a dose of 100 mg/kg body weight for another 8 days. NAC was injected into mice intraperitoneally.

Retrovirus infection and quantitative RT-PCR

The retrovirus infection and real-time RT-PCR were performed essentially as described $^{12}$ and details are included in the Supplementary Materials. Real-time RT-PCR primers were described in sTable 2.
Colony assays
The CFU-E, BFU-E, CFU-GM, and CFU-Pre-B assays were performed as described previously. Details are described in the Supplementary Materials.

Transwell experiments
20,000 of FACS-collected WT and Sclpfm1 Lin−Sca-1−Kit−Flk2−CD34− cells were suspended in 300 μl of HBSS/0.1%BSA in the upper chamber and allowed to migrate across the 5-μm pore inserts (Corning-Costar, MA) toward the lower chamber containing HBSS/0.1%BSA and 160 ng/mL CXCL12 (Peprotech). Transmigrated cells were harvested after 4 hours. The transmigrated cells and the cells remained at the upper chamber were analyzed by flow cytometry.

Statistical analyses
Data are expressed as mean ± SEM. Data were analyzed by Student’s t-test and were considered statistically significant if $p < 0.05$. 
Results

The maintenance of BM HSCs requires Pfn1 function

To obtain an inducible loss-of-function model for pfn1 in HSCs, we crossed pfn1\textsuperscript{fl/fl} mice with transgenic mice expressing the tamoxifen-inducible Cre recombinase under the control of stem cell leukemia (Scl) HSC enhancer, which deletes floxed genes in HSCs and hematopoietic progenitors upon tamoxifen treatment \cite{10} (sTable 1). The resultant Scl-Cre-ER/pfn1\textsuperscript{fl/fl} mice (Sclpfn1; Fig. 1A) and the control mice (Scl-Cre-ER/ pfn1\textsuperscript{+/+}) were injected with tamoxifen to induce Cre expression in HSCs. Specific primers are designed to distinguish the WT, pfn1\textsuperscript{fl/fl} and pfn1\textsuperscript{-/-} mice (upon tamoxifen treatment, sFig. 1A).

Genotyping of BM long-term HSCs (LT-HSCs) as Lin\textsuperscript{-}Sca-1\textsuperscript{+}Kit\textsuperscript{+}CD34\textsuperscript{-}Flk2\textsuperscript{-} (LSKFC) cells and differentiated hematopoietic Lin\textsuperscript{+} cells of the Sclpfn1 mice revealed that loss of pfn1 had occurred in HSCs, but not in Lin\textsuperscript{+} cells at 24 h after treatment of tamoxifen (Fig. 1B). This pfn1 loss was dramatically increased in all hematopoietic cells at day 6 after the differentiation of HSCs (Fig. 1B).

To evaluate the role of pfn1 in HSC maintenance and function, we analyzed the frequency and number of LSK cells and LSKFC cells (as LT-HSCs) in control and Sclpfn1 mice at different time points after tamoxifen treatment (Fig. 2A-B). LSKFC cells in control mice were 0.011±0.0038\% and 0.012±0.0036\% of the total bone marrow cells at day 8 and day 14, respectively. In contrast, LSKFC cells accounted for only 0.0058±0.0017\% of BM cells at day 8 and 0.0008±0.00021\% at day 14 in Sclpfn1 mice (Fig. 2B). Total numbers of BM cells in Sclpfn1 mice dramatically decreased from day 8
to day 14 consistent with exhaustion of HSCs (sFig. 1B). Consistently, the total HSC number in BM decreased from $5.3 \pm 0.067 \times 10^3$ at day 0 to $0.067 \pm 0.019 \times 10^3$ at day 14 (Fig. 2C). Meanwhile, lineage distribution in BM was changed at day 14 in Sclpf1 mice compared to the wild-type distribution with increases in percentages of CD3$^+$ and B220$^+$ lymphoid cells and CD71$^+$Ter119$^+$ erythroid cells, but decreases in Mac-1$^+$ and Gr-1$^+$ myeloid cells (sFig. 1C). A colony assay was performed to assess the dynamics of progenitor cells after $pfn1$ deletion. As shown in sFig. 1D-E, numbers of myeloid progenitor cells (CFU-GM), but not erythroid progenitors (CFU-E) or B cell progenitors (CFU-pre-B), were dramatically decreased to $40.70\pm8.01\%$ of the control at 4 days after tamoxifen treatment. The flow cytometry analyses also showed that the common myeloid progenitors (CMP) decreased dramatically by more than 60% than the control (sFig. 1H&I). The loss of cellularity of BM and dislocation of HSCs in bone marrow niches in pf1-null mice were also evident (sFig. 1F-G). Overall, our data in Fig. 2 and sFig. 1 indicate that $pfn1$ deletion results in the exhaustion of HSCs and the fast cycling myeloid progenitors in the mouse BM.

To further determine whether the reduction in phenotypic HSCs in Sclpf1 mice reflected an intrinsic defect, we performed two types of competitive transplantation experiments. In the first experiment, we transplanted $2 \times 10^5$ BM cells from either tamoxifen-treated Sclpf1 mice or control animals with $2 \times 10^5$ CD45.1 competitors into lethally irradiated CD45.1 recipients. Significant numbers of donor-derived cells were observed in the peripheral blood of mice injected with control HSCs ($23.21\pm1.53\%$, $47.97\pm3.47\%$, and $57.28\pm4.61\%$ repopulation at 3, 8, and 16 weeks, respectively); however, donor Sclpf1
cells only contributed to minor chimerism (4.15±0.52%, 8.04±7.04%, and 11.69±11.15% repopulation at 3, 8, and 16 weeks, respectively, Fig. 2D). Nevertheless, Sclpfn1 donor cells did repopulate the lymphoid and myeloid lineages (Fig. 2E), indicating differentiation was not impaired in \( pfnl^{-/-} \) HSCs. Sclpfn1 donor cells treated with tamoxifen repopulated lethally irradiated CD45.1 recipients at barely detectable levels (sFig. 2), which further confirmed that pfnl plays a crucial role in maintenance of HSC activity.

In the other experiment, we transplanted \( 2 \times 10^5 \) Sclpfn1 cells (without tamoxifen treatment) or control BM cells with \( 2 \times 10^5 \) CD45.1 competitors into lethally irradiated CD45.1 recipients. Both groups were repopulated at similar efficiencies (42.61±2.74% and 35.77±5.64%, respectively, Fig. 2F) at 8 weeks post-transplantation. When \( pfnl \) deletion was induced by tamoxifen treatment, levels of the \( pfnl \)-deficient HSCs significantly decreased over time from 35.77±5.64% at 4 weeks to 4.21±0.60% after 17 weeks after treatment began (Fig. 2F). The repopulation of the control group remained relatively constant (46.54±5.78%, 50.71±6.36%, 54.03±8.07%, and 53.21±9.88% at weeks 4, 7, 10, and 17, respectively). The \( pfnl^{-/-} \) donor cells repopulated all lymphoid and myeloid lineages (Fig. 2G). Collectively, these results indicate that pfnl is essential for the repopulating activity of HSCs.

**Spontaneous and constitutive mobilization of HSCs in Sclpfn1 mice**

A number of possible mechanisms may account for the decreased HSC frequency and activity observed in Sclpfn1 mice including 1) decreased proliferation of HSCs, 2)
increased mobilization, and 3) increased apoptosis. We first determined the impact of
pfn1 deficiency on HSC quiescence/proliferation by analyzing the cell-cycle profile of
pfn1−/− BM HSCs. Hoechst 33342 and pyronin Y staining 16 of sorted HSCs (as LSKFC
cells) revealed no significant difference between Sclpfn1 and control mice at day 4 after
tamoxifen treatment. However, there was an approximately 50% decrease in G0 phase of
pfn1−/− HSCs compared with wild-type controls at day 8 after tamoxifen treatment (Fig.
3A). This decreased quiescence of pfn1−/− HSCs in BM was further confirmed by
bromodeoxyuridine (BrdU) incorporation assays, which showed 2-fold greater (24.3 ±
4.2% versus 11.8 ± 3.8% in HSCs, and 36.7 ± 4.3% versus 24.2 ± 3.6% in LSK cells)
incorporation of BrdU into pfn1−/− HSCs or LSK cells as compared to control HSCs or
LSK cells (Fig. 3B). Therefore pfn1−/− HSCs exhibited decreased quiescence in BM.

Next, we tested whether pfn1 plays a role in retaining HSCs in the BM. To this end, we
analyzed LT-HSC frequency and number in the peripheral blood of Sclpfn1 and control
mice. Beginning at day 4 after tamoxifen treatment, the frequency of circulating HSCs in
Sclpfn1 mice increased about 3-fold compared to control (Fig. 3C). At days 8 and 14
post-treatment with tamoxifen, the frequencies of circulating Sclpfn1 HSCs were
dramatically increased by up to 10-fold in Sclpfn1 mice relative to controls. Because the
spontaneous mobilization of LT-HSCs started at day 4 after tamoxifen treatment (Fig.
3C), concordantly with the decrease of the frequency of HSCs in BM (starting after day
4; Fig. 2B), HSC mobilization is likely a cause of the decrease of HSCs in BM in Sclpfn1
mice.
To investigate the mechanistic role of \( \text{PFN1} \) in retention of HSCs in the BM, we performed a transwell experiment with control and Sclpfn1 HSCs treated for 8 days with tamoxifen. As shown in Fig. 3D, Sclpfn1 HSCs had significantly decreased chemoattraction for SDF-1 compared to control cells, indicating that \( \text{PFN1} \) regulates the BM retention of HSCs, possibly through SDF-1-mediated chemotaxis.

Both mobilization and homing are measures of HSC motility. Although Sclpfn1 HSCs had drastically increased mobilization, and \( \text{PFN1} \) deletion up-regulates CXCR4 expression in LT-HSCs (sFig. 1H&J), the Sclpfn1 HSCs did not show significant difference in homing to BM compared to controls (Fig. 3E). This result suggests that \( \text{PFN1} \) is essential for the retention but not for the immediate homing of HSCs in the BM niche. Therefore \( \text{PFN1} \) deficiency induces an uncommon uncoupling of homing and mobilization of HSCs in BM.

It was reported that EGR1 regulates HSC migration and retention in the BM niche, and their knockout showed similar phenotypes as \( \text{PFN1}^{-/-} \) mice \(^{17,18} \). To test the possible role of \( \text{PFN1} \) in regulation of EGR1, we examined EGR1 expression in control and Sclpfn1 HSCs treated with tamoxifen for 3 days by using quantitative RT-PCR. Interestingly, we found that the EGR1 level in Sclpfn1 HSCs decreased to 38.99±4.76\% of that in control (sFig. 3A). Another protein, MLL, which was shown to support HSC BM retention \(^{18} \), was also decreased in Sclpfn1 HSCs (sFig. 3B). To determine how \( \text{PFN1} \) regulates HSC BM retention, we demonstrated that Pfn1 was capable of interacting with a guanine nucleotide-binding protein \( \text{G}^{\alpha}13 \) (Fig. 3F & sFig. 3C), which was known to transactivate...
the expression of EGR1. Importantly, a retroviral expression of EGR1 rescued the defective retention of Sclpfn1 HSCs in the BM but not in the spleen, and the decreased quiescence of Sclpfn1 HSCs (Fig. 3G-H). These results indicate that Pfn1 interacts with Gα13, which upregulates EGR1 to maintain HSCs in the BM.

Finally, to determine the role of pfn1 in apoptosis of HSCs, we assessed Annexin V and 7-AAD staining of LSKFC cells as a function of time after pfn1 deletion. As shown in Figure 3I, apoptosis of HSCs was dramatically increased in Sclpfn1 mice compared to controls at 16 days, but not at 4 days after tamoxifen treatment. Furthermore, the increased apoptosis of Sclpfn1 HSCs can also be rescued by EGR1 (Fig. 3J). Overall Pfn1 plays a significant role in the survival of HSCs.

To understand the relationship between apoptosis and BM exhaustion of Sclpfn1 HSCs, we analyzed the growth of pfn1−/− and control HSCs in vitro. Similar to the observed loss of quiescence in vivo, null HSCs had faster cycling in culture than wild-type HSCs (Fig. 4A). This suggests that pfn1 negatively regulates HSC proliferation. The null HSCs expanded much more slowly than their WT counterparts in vitro (Fig. 4B-C). To explain these seemingly contradictory observations, we analyzed apoptosis and differentiation of control and pfn1−/− HSCs in culture (Fig. 4D, sFig. 4A). We found that null HSCs had significantly increased levels of apoptosis relative to controls (Fig. 4D). Similar to results obtained in vivo, pfn1−/− HSCs differentiated normally in culture (sFig. 4A). Overall, these in vivo and in vitro results suggest that pfn1 supports the survival and quiescence of
HSCs. The deletion of *pfn1* in HSCs led to increased apoptosis, cycling, and mobilization of HSCs, all of which account for the BM failure of the mutant mice.

**Pfn1 supports glycolysis of HSCs**

Previous studies suggested that some cytoskeleton molecules, including moesin, γ-actin and cofillin-1, may be involved in metabolic regulation. We recently demonstrated that HSCs have a distinct metabolic profile and prefer to utilize glycolysis rather than mitochondrial oxidation as their main energy source. We thus sought to test whether *pfn1*, as an important cytoskeleton molecule, plays a role in HSC metabolism. To this end, we first examined whether metabolic properties of HSCs changed over the course of the cell cycle. We collected LT-HSCs at G0 and G1 by FACS and measured their ATP content, oxygen consumption, and glycolytic flux relative to ATP level. We found that the G0 HSCs, which are quiescent, had 41.10±4.30% ATP content and 3.59±0.16 folds higher glycolytic flux per ATP than did the G1 population (Fig. 5A). G0 HSCs also showed a lower level of oxygen consumption on average (Fig. 5A). These results, for the first time, demonstrate that HSCs in different stages of the cell cycle have different metabolic preferences.

Because *pfn1* deletion in HSCs leads to decrease of G0 that possesses greater glycotic flux, we sought to test whether *pfn1* plays a role in regulation of HSC metabolism. We started to measure the expression of Hif-1α, an important regulator of hypoxia and metabolism, by real-time RT-PCR in control and *pfn1*−/− HSCs. As shown in Fig. 5B, Hif-1α expression was dramatically decreased to 35.91±2.40% or 27.02±0.09% at day 1.
or 3 respectively after tamoxifen treatment in null HSCs. This result is accordant with our finding that pfn1 upregulates EGR1 (sFig. 3A, and see below Fig. 6E), and the fact that EGR1 directly transactivates Hif-1α\(^22\) (also see below Fig. 6D, F). Our results thus suggest that pfn1 may play a role in regulation of glycolysis and mitochondrial oxidation. Indeed, the HSC-specific deletion of pfn1 resulted in decreased glycolytic flux per ATP (40.5±0.94% compared to that of control) at only 2 days after tamoxifen treatment (Fig. 5C). At 8 days after tamoxifen treatment, the Sclpfn1 HSCs had 6.56±1.42 -folds increased levels of ATP, 2.5-fold (2.50±0.12%) increase of oxygen consumption, and 97.05±0.21% decrease of glycolytic flux per ATP compared to controls (Fig. 5D). Consistently, the glycolysis related enzymes were downregulated and Kreb cycle related enzymes PDHA1 and PDHB were upregulated in Sclpfn1 HSCs (sFig. 5A, B). Interestingly, this metabolic switch upon pfn1 deletion is specific to HSCs, because it did not occur in various hematopoietic progenitors (sFig. 5C-E). These results clearly demonstrate that deletion of pfn1 results in a switch from anaerobic glycolytic metabolism to oxidative phosphorylation in HSCs.

To identify the possible cause for this metabolic switch in HSCs upon pfn1 deletion, we examined the ATP production and oxygen consumption of control and Sclpfn1 HSCs during \textit{in vitro} culture (sFig. 4B-C). While the \textit{in vitro} cultured Sclpfn1 HSCs have increased apoptosis (Fig. 4D), unlike the \textit{in vivo} situation, they did not show metabolic switch (sFig. 4B-C). In addition, the \textit{in vivo} metabolic switch happens in pfn1 deleted HSCs much earlier than the apoptosis changes. These results indicate that the altered metabolic profiling of Sclpfn1 HSCs does not result from the apoptosis of Sclpfn1 HSCs.
Given that pfn1 upregulates EGR1 (sFig. 3A) that suppresses mobilization\textsuperscript{17}, and EGR1 transactivates Hif-1α\textsuperscript{22} (also see Fig. 6D-F) and partially rescues pfn1-null metabolic phenotype (sFig. 3D), we reasoned that the change of metabolic profiling upon pfn1 deletion intrinsically links to the mobilization of HSCs.

Mitochondria are considered as the major source of reactive oxygen species (ROS) in cells and play important roles in aging, degenerative diseases, and HSC dysfunction\textsuperscript{23-25}. We found that the level of mitochondrial super-oxide (MitoSOX) is much lower in G0 cells than G1 cells in WT LT-HSCs, and MitoSOX levels in either G0 or G1 cells of Sclpfn1 LT-HSCs are higher than that of controls respectively (Fig. 5E). Accordantly, the mitochondrial potential in these cells showed a similar trend (sFig. 5F). Our results are consistent with the view that ROS can induce loss of quiescence and apoptosis of HSCs\textsuperscript{26}. Our previous results indicate that ROS production in HSCs plays an important role in regulation of the metabolic phenotype and cell fates\textsuperscript{15}. To investigate whether ROS is responsible for the phenotypes of Sclpfn1 HSCs, we treated Sclpfn1 and control mice with the antioxidant \textit{N}-acetyl-L-cysteine (NAC) for 7-8 days and then examined the cell fates of HSCs. As shown in Fig. 5F, the level of ROS in Sclpfn1 HSCs was significantly higher than in control cells and was statistically decreased after a 8-day treatment with NAC. Interestingly, NAC treatment reversed the loss of quiescence of the null HSCs (Fig. 5G, sFig. 5G), but had no effect on HSC frequency or apoptosis in Sclpfn1 mice (Fig. 5H-I, sFig. 5H-I). Taken together, these data indicate that metabolic changes are one of the earliest events in \textit{pfn1}-deficient HSCs in BM, which accounts for their loss of quiescence.
**Actin and poly-proline binding are critical to pf11’s function in HSCs**

Previous studies showed that the actin binding and the poly-proline binding ability of pf11 are critical for the fission yeast viability and chondrocyte function. To investigate the relative contributions of actin and non-actin ligands to pf11 activity in HSCs, we infected Sclpf1 HSCs with retroviruses encoding wild-type pf11, an actin binding-deficient point mutant of pf11 (R74E), or pf11 point mutants (Y6D and H133S) with reduced affinities for poly-proline. Five weeks after transplantation of the infected HSCs, tamoxifen was used to induce deletion of pf11 to produce pf11-/- donor HSCs. As expected, retroviral infection with the wild-type pf11, but not the actin binding-deficient mutant or the poly-proline binding-deficient mutants, was able to rescue the repopulation activity of null HSCs (Fig. 6A-C). Retroviral expression of EGR1 was able to increase Hif-1α level in Sclpf1 cells (Fig. 6D, F), and wild-type pf11 expression also upregulated EGR1 and Hif-1α expression (Fig. 6E-F). By contrast, the actin binding-deficient mutant or the poly-proline binding-deficient mutant pf11 had a lower ability to elevate EGR1 and Hif-1α expression (Fig. 6E-F). It is of note that the proline-binding mutants skewed myeloid/B-lymphoid lineage repopulation (Fig. 6C). Taken together, these data suggest that the actin binding and poly-proline binding activities of pf11 are important for its regulation of HSC activity.

In sum, we provide strong evidence that pf11 plays critical roles in the maintenance of multiple cell fates and metabolism of HSCs. As summarized in Fig. 6, the loss of pf11 in HSCs leads to increased mobilization and the metabolism switch, and meanwhile
apoptosis of HSCs over time in vivo, all of which contribute to bone marrow failure.
Discussion

It is well established that pfnn1 plays critical roles in cell survival, proliferation, and motility in various cell types \(^2\text{--}^5,^8,^27\). Its *in vivo* role in stem cells, however, has not been studied. Here we showed that Pfn1 plays multiple essential roles in HSCs and is required for the steady-state hematopoiesis. Different from its migration/proliferation roles in many cell types \(^3\text{--}^5,^7\), pfnn1 is key to maintain the retention and quiescence of HSCs in the BM. It is also critical for the survival but plays no significant role in differentiation or homing of HSCs. Therefore, although pfnn1 is ubiquitously expressed, it has specific function in HSCs. This function is partially contributed by its upregulation of the expression of EGR1, which has been shown to be required for the quiescence and retention of HSCs in the BM \(^17,^18\). While it is known that the forced expression of EGR1 causes changes in differentiation \(^28\), we demonstrated that it can partially rescue the Sclpfnn1 HSC phenotype. Importantly, EGR1 is known to be able to bind to and activate the promoter of Hif-1\(\alpha\) \(^22\), as confirmed in our system. Therefore, at least partially through the axis of pfnn1/G\(\alpha\)13/EGR1, pfnn1 regulates BM retention of HSCs that contributes to maintaining their glycolytic metabolism. Treatment of *pfnn1*-deficient mice with NAC reversed the ROS level and loss of quiescence of HSCs, suggesting that pfnn1 maintained metabolism is required for the quiescence of HSCs. We further demonstrated that pfnn1’s function in HSCs depends on its actin-binding and poly-proline-binding activities.

Recently, we demonstrate that HSCs have a distinct metabolic profile, and Meis1 and Hif-1a are involved in the regulation of this unique metabolism \(^1,^15\). However, the
relationship between metabolism and cell fates (quiescence, self-renewal, differentiation, apoptosis, and motility) of HSCs is not known. Here, our results suggest that a metabolic quiescence is mechanistically linked to the cell cycle quiescence of stem cells. We showed that pfn1 plays an important role in regulation of the metabolism of HSCs. Deletion of pfn1 in HSCs leads to downregulation of the metabolism master regulator Hif-1α and a switch from glycolysis to mitochondrial respiration. Importantly, this metabolic change happened earlier than the loss of quiescence of HSCs. It is well known that mitochondrial oxidation is accompanied by significantly increased ROS production \(^{29-33}\), and the free radical scavenger NAC restored quiescence of pfn1-null HSCs. Therefore the metabolic preference of HSCs regulates HSC cell cycle status. We also found that quiescent HSCs (at G0) have higher levels of glycolysis than proliferating HSCs (at G1). Therefore the altered metabolic profiling of the Sclpfn1 HSCs may also be partially contributed from the cell cycle change. We speculate that a feedback loop controls the metabolism and cell cycle state of stem cells. Further investigation will be needed to uncover the underlying mechanism for this regulation.

The relationships among different cell fates of HSCs are complicated. For example, whether homing and mobilization are two sides of the same coin is not clear, although in many cases homing and mobilization are clearly coupled. Expression of integrin α4/β1 and SDF1/CXCR4 are needed for both homing enhancement and retention of HSCs in BM \(^{34,35}\). However, Gαs is critical for the homing but not the mobilization of HSCs \(^{36}\). That there are meaningful molecular distinctions between homing, engraftment, and retention processes is also demonstrated by our finding that pfn1 deficiency uncouples
these events. Unlike Gαs, pfn1 is essential for the mobilization but not homing of HSCs. Our results also suggest that quiescence and mobilization/survival of HSCs can be independent events. More detailed studies, including those utilizing the high-resolution, real-time imaging tools to elucidate the roles of pfn1 in regulation of the activities of HSCs in and outside of BM will provide important new insights into the relationships among HSCs, niche, and metabolism.

Here we demonstrated that both the actin-binding and the poly-proline binding abilities of pfn1 are essential to HSC activity. In the future studies, it will be important to dissect which of these two activities of pfn1 is responsible for BM retention, survival, differentiation, and metabolic preference of HSCs. The role of the proline-binding function will be particularly interesting. It is known that profilins interact with more than a dozen proline-rich-peptide-containing proteins, including those involved in cytoskeleton polymerization (Arp2/3), focal adhesion (EVL, Palladin, Mena, and VASP), synaptic scaffold synthesis (Delphilin, Aczonlin, Drebrin and Gephyrin), membrane trafficking (VCP, Clathrin, and Annexin I), Rho GTPase signaling (WIP, WAVE, WASP, VF6, FRL, and mDiaphanous), and nuclear export (Exportin 6 and SMN) ³⁷,³⁸. Here we added one more molecule, Gα13, to this list, and our study suggested that EGR1, as a target of Gα13, is a mediator of pfn1’s function in HSC BM retention and links pfn1 to Hif-1α upregulation and glycolytic metabolism. It is noteworthy that Pfn null HSC phenotype is much more severe than that of the EGR1 null mice. Given the large number of pfn1-interacting proteins, it is likely that the pfn1/Gα13/EGR1 axis represents a branch of pfn1’s downstream effectors’ network. The clarification of the
contribution of these and other molecules to pfn1’s function in HSCs will lead to a significantly improved understanding of the interplay between stem cells and their niches and the pathology of HSC-related diseases.
Acknowledgments

We thank Dr. Joachim R. Goethert from Universitaetsklinikum Essen and Dr. Reinhard Fassler from Max Planck Institute of Biochemistry for kindly providing SCL-CRE/ER mice and pfn1\textsuperscript{fl/fl} mice respectively. We thank the support from NIH grant 1R01CA172268, DOD W81XWH-10-1-0429, CPRIT RP100402, National Natural Science Foundation of China 21328503, Robert A. Welch Foundation grant I-1834, and the Gabrielle’s Angel Foundation.

Authorship


Conflict-of-interest disclosure

The authors declare no competing financial interests.
References

Figure Legends

Figure 1. Conditional Deletion of \( pfn1 \) in HSCs. A) Schematic of \( pfn1 \) floxed allele showing deletion of floxed exon 1 following Cre recombinase activity. Use of Scl-Cre-ER\(^T\) results in specific deletion of the \( pfn1 \) gene in HSCs following tamoxifen treatment. B) Genotype analyses of LT-HSCs (Lin\(^-\)Sca-1\(^+\)Kit\(^+\)Flk2\(^-\)CD34\(^-\), LSKFC) and Lin\(^+\), Lin\(^-\), Lin\(^-\)Kit\(^+\) (LK) cells and whole bone marrow (WBM) cells at 1 day and 6 days after tamoxifen injections. Deletion of \( pfn1 \) is indicated by the arrow (700bp).

Figure 2. The maintenance of BM HSCs requires \( pfn1 \) function. A) Representative flow cytometry profile of Lin\(^-\)Sca-1\(^+\)Kit\(^+\) cells from control and Sclpfn1 mice. B) The frequency of LSKFC in the Sclpfn1 mouse was dramatically decreased compared to the control mouse (1.4% vs. 0.01%) at day 14 after tamoxifen treatment. Deletion of \( pfn1 \) over time is indicated by the arrow. B-C) Quantification of HSC (Lin\(^-\)Sca-1\(^+\)Kit\(^+\)Flk2\(^-\)CD34\(^-\)) frequency and number in BM of control and Sclpfn1 mice (n = 5-8) at different time points (*, p<0.05). D) Competitive reconstitution analysis of control and Sclpfn1 HSCs treated with tamoxifen for 4 days. BM CD45.2 cells (2 x 10\(^5\) cells) from donor mice along with 2 x 10\(^5\) freshly isolated CD45.1 competitor cells were transplanted into lethally irradiated CD45.1 recipient mice. The mice (n = 5 per group) were analyzed for engraftment through 16 weeks post-transplant (*, p < 0.05). E) Multilineage contribution of donor cells in the recipients at 14 weeks post-transplant (n = 5). F) Control and Sclpfn1 donor cells (2 x 10\(^5\) cells) along with 2 x 10\(^5\) competitor cells were transplanted into lethally irradiated CD45.1 recipient mice. These primary transplanted mice (n = 5) were treated with tamoxifen at 8 weeks post-transplant and were then analyzed for the engraftment from 2 to 17 weeks after tamoxifen treatment. (*, p < 0.05). Deletion of \( pfn1 \) at 2 weeks after tamoxifen treatment is indicated by the arrow. G) Multilineage contribution of donor cells in the recipients at 16 weeks post-transplant (n = 5).

Figure 3. Spontaneous and constitutive mobilization of HSCs in Sclpfn1 mice. A) Sclpfn1 BM HSCs are less quiescent than control HSCs. In the left panel, LT-HSCs (as Lin\(^-\)Sca-1\(^+\)Kit\(^+\)Flk2\(^-\)CD34\(^-\) cells) from a wild-type mouse, stained with Hoechst 33342 and pyronin Y, were analyzed for cell cycle stage. In the right panel, the percentages of
G0 cells in control and Sclpf1 mice (n = 3-4) at day 4 and 8 after tamoxifen treatment are shown (*, p < 0.05). B) BrdU incorporation indicates a decreased cycling in HSCs isolated from control mice compared to Sclpf1 mice (n = 3; *, p < 0.05). C) Relative frequency of LT-HSCs in peripheral blood (PB) was analyzed over time in control and Sclpf1 mice after tamoxifen treatment (n = 3-7; *, p < 0.05). D) The migrations of LT-HSCs (as Lin°Sca-1°Kit°Flk2°CD34° cells) isolated from control and Sclpf1 mice 8 days after tamoxifen treatment were compared in a transwell experiment (n = 3; *, p < 0.05). E) Control and Sclpf1 HSCs home equivalently to recipient BM. BM from control or Sclpf1 mice (n = 5) was labeled with 5- and 6-carboxyfluorescein succinimidyl ester (CFSE), and 1 x 10⁷ cells were transplanted into lethally irradiated recipients. After 16 hours, the total percentage of CFSE⁺ cells in the BM, spleen, and liver and LT-HSCs (CFSE⁺Lin°Sca-1°Kit⁺Flk2°CD34° cells) in BM were determined by flow cytometry. F) Lysates of wild-type mouse BM cells were co-immunoprecipitated with anti-Gs o antibody or control rabbit IgG, and precipitation was then determined by western blotting using anti-Gld-type mouse BM cells were c G-H) Rescue of Sclpf1 LT-HSC by EGR1. Control or EGR1 overexpressed Sclpf1 BM cells were transplanted into CD45.1 recipients with competitors. Mice were treated with tamoxifen after 5 weeks. Panel G, percentages of donor derived CD45.2⁺ LT-HSC cells were determined in BM and spleen (n = 3-4, * p < 0.05). Panel H, LT-HSCs were sorted from the BM of control or EGR1 rescued mice, and cell cycle was then measured by Hoechst 33342 and pyronin Y staining (n = 3-4, * p < 0.05). I) Control and Sclpf1 Lin°Sca-1°Kit⁺Flk2°CD34° cells were analyzed for apoptosis by using Annexin V/7-AAD staining (n = 3-5; *, p < 0.05). J) LT-HSCs from the BM of control or EGR1 rescued mice were cultured for 8 days, and apoptosis were measured (n = 3-4; *, p < 0.05).

**Figure 4. Pfn1 supports the survival of HSCs.** A) Cultured HSCs were stained with Hoechst 33342 and pyronin Y and analyzed for cell cycle stage. The percentages of G0 cells in cultured control and Sclpf1 HSCs are shown (n = 3; *, p < 0.05). B) LT-HSCs (as Lin°Sca-1°Kit°Flk2°CD34° cells) were isolated from control and Sclpf1 mice 4 days after tamoxifen treatment and cultured in STF medium for 8-10 days. Figures are representative for samples after a 10-day culture. C) Quantification of control and
Sclpfn1 HSCs after 10 days in culture (n = 3; *, p < 0.05). D) Cultured HSCs from control and Sclpfn1 mice were analyzed for apoptosis using Annexin V/7-AAD staining (n = 3; *, p < 0.05).

Figure 5. Pfn1 regulates metabolism of HSCs. A) Measurement of ATP content, oxygen consumption, and labeled glycolytic flux per ATP in G0 and G1 fractions of LT-HSCs (as Lin−Sca-1+Kit+Flk2−CD34− cells) (n = 3-6; *, p < 0.05). B) Hif-1a expression in control and Sclpfn1 HSCs at days 1 and 3 after tamoxifen treatment (n = 3; *, p < 0.05). C-D) Metabolic profiles (ATP content, oxygen consumption and glycolytic flux per ATP) in control and Sclpfn1 HSCs at (C) day 2 or (D) day 8 after tamoxifen treatment. Sclpfn1 HSCs are much less glycolytic (n = 3-6; *, p < 0.05). E) Mitochondrial super-oxide (MitoSOX) were analyzed in G0 and G1 cells from control and Sclpfn1 LT-HSCs (n=3). F-I) Control and Sclpfn1 mice were treated with tamoxifen for 7 days followed by NAC treatment for another 8 days. (F) Levels of reactive oxygen species (ROS) were determined by analysis for carboxy-DCFDA, and percentage of DCFDA+ LT-HSCs are shown (n = 4-6; */**/***; p < 0.05). (J) Cell cycle status, cell frequency, and levels of apoptosis of HSCs were analyzed (n = 3; *, p < 0.05).

Figure 6. Actin and poly-proline binding is critical to pfni’s function in HSCs. A-B) E16 fetal liver Sclpfn1 Lin− cells were infected with retrovirus encoding wild-type (WT), actin-binding-deficient pfni or poly-proline-binding-deficient pfni and were then transplanted into lethally irradiated CD45.1 recipients. Five to ten weeks later, the mice were treated with tamoxifen for 2 weeks. Donor engraftment at indicated time post-transplant is shown (n = 5). C) Multilineage contribution of donor cells in recipients at 10 or 15 weeks post-transplant (n = 5). D) Control or EGR1 retrovirally-expressed Sclpfn1 BM cells were transplanted into CD45.1 recipients with competitors. Mice were treated with tamoxifen after 5 weeks. Donor derived CD45.2+ LSK cells were sorted and expression of Hif-1α was measured (n = 3). E) Pfni or Pfni-mutants were retrovially-expressed in wild-type LSK cells, and expression of EGR1 was measured (n = 3). F) Pfni, Pfni-mutants, or EGR1 was retrovially-expressed in wild-type LSK cells, and expression of Hif-1α was measured (n = 3). *, p < 0.05, different from control values; **, different from mutant pfni values.
Fig. 1

A

[Diagram showing the deletion of an exonic fragment using Cre-ER<sup>T</sup> and Scl recombination system]

B

-Apellido1 LSKFC
-WT control
-Apellido1 Lin<sup>+</sup>
-Apellido1 WBM
-Apellido1 Lin<sup>-</sup>
-Apellido1 Lin<sup>+</sup>
-Apellido1 LK
-Apellido1 LSKFC

Deletion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 day</th>
<th>6 days</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>700bp</td>
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Fig. 3

A

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B

PFN-WT

PFN-KO

C

% LT-HSCs in PB

D

Cell No migrated cells

E

% Homing Cells

F

GNA13

Pfn1

IgG-Light Chain

G

% LT-HSC Cells

H

% in LT-HSC Cells

I

% Apoptotic HSCs

J

% Apoptotic LT-HSCs

Control

Sclpnf1

Control

EGR1

Control

Sclpnf1

Control

Sclpnf1

Control

Sclpnf1

Control

Sclpnf1

Control

Sclpnf1
Fig. 4

A

% Cultured cells in G0

Control   Sclpfn1

B

Control   Sclpfn1

C

% Cell No (Control)

Control   Sclpfn1

D

% Apoptotic cells

Control   Sclpfn1
Fig. 5

A. ATP (RLU)

B. Relative mRNA expression of HIF-1α (control)

C. ATP (RLU)

D. ATP (RLU)

E. Flow cytometry analysis of MitoSOX and colorimetric analysis of ATP, oxygen consumption, and lactate production

F. % of DCFDA+ cells

G. % LT-HSCs in G0

H. % LT-HSCs in BM

I. % Apoptotic HSCs
Profilin 1 is essential for retention and metabolism of mouse hematopoietic stem cells in bone marrow

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