THE SRC HOMOLOGY 2 PROTEIN SHB PROMOTES CELL CYCLE PROGRESSION IN MURINE HEMATOPOIETIC STEM CELLS BY REGULATION OF FOCAL ADHESION KINASE ACTIVITY

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Running title: Shb and hematopoietic stem cell proliferation

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Abbreviations: 5-FU, 5-Fluorouracil; BrdU, 5-bromo-2’-deoxyuridine; FACS, fluorescence activated cell sorting; FAK, focal adhesion kinase; GMP, granulocyte-monocyte progenitor; HPC, hematopoietic progenitor cell; KLS, c-Kit+lineage+Sca-1+; LT-HSC, long-term hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor; MPP, multipotent progenitor; MAPK, mitogen activated kinase; PAK, p21-activated kinase; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol-3 kinase; PLCγ, phospholipase Cγ; SCF, stem cell factor; SH2; Src-homology-2 domain; Shb, Src-homology 2 domain containing adaptor protein B; TCR, T cell receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
Abstract

The widely expressed adaptor protein Shb has previously been reported to contribute to T cell function due to its association with the T cell receptor and furthermore, several of Shb’s known interaction partners are established regulators of blood cell development and function. In addition, Shb deficient embryonic stem cells displayed reduced blood cell colony formation upon differentiation in vitro. The aim of the current study was therefore to explore hematopoietic stem and progenitor cell function in the Shb knockout mouse. Shb deficient bone marrow contained reduced relative numbers of long-term hematopoietic stem cells (LT-HSCs) that exhibited lower proliferation rates. Despite this, Shb knockout LT-HSCs responded promptly by entering the cell cycle in response to genotoxic stress by 5-fluorouracil treatment. In competitive LT-HSC transplantations, Shb null cells initially engrafted as well as the wild-type cells but provided less myeloid expansion over time. Moreover, Shb knockout bone marrow cells exhibited elevated basal activities of focal adhesion kinase/Rac1/p21-activated kinase signaling and reduced responsiveness to Stem Cell Factor stimulation. Consequently, treatment with a focal adhesion kinase inhibitor increased Shb knockout LT-HSC proliferation. The altered signaling characteristics thus provide a plausible mechanistic explanation for the changes in LT-HSC proliferation since these signaling intermediates have all been shown to participate in LT-HSC cell cycle control. In summary, the loss of Shb dependent signaling in bone marrow cells, resulting in elevated focal adhesion kinase activity and reduced proliferative responses in LT-HSCs under steady state hematopoiesis, confers a disadvantage to the maintenance of LT-HSCs over time.

Key words: Hematopoietic stem cells, proliferation, Shb, focal adhesion kinase, stress and steady-state hematopoiesis
Introduction

The maintenance of the hematopoietic system throughout adult life requires a continuous generation of blood cells, a process dependent on a rare population of cells residing in the bone marrow, the long-term hematopoietic stem cells (LT-HSCs) [1]. Under steady state conditions the vast majority of LT-HSCs are found in the G0 stage of the cell cycle [2, 3]. Hematopoietic stress in the form of cytotoxic injury [4], blood loss [5] or stem cell stimulating cytokines [6] can however elicit an extensive proliferative response within the LT-HSC pool.

A strict regulation of the cell cycle is of utmost importance for LT-HSC and it has been extensively studied in animal knockout models. There is a fine balance between quiescence and maintaining sufficient proliferation to sustain hematopoiesis. Small shifts in the proliferative rates have been revealed to set off the system by exhaustion or reduced productivity. For instance, deletion of the cell cycle regulator p27 [7, 8] leads to increases in numbers of cycling cells and quickly depletes the system due to proliferative exhaustion of LT-HSC and hematopoietic progenitor (HPC) populations.

c-Kit and its ligand Stem Cell Factor (SCF), together with other hematopoietic cytokines, are well established regulators of LT-HSC quiescence and proliferation [9]. Nonetheless, many of the intracellular signaling pathways that govern the maintenance of the LT-HSC pool remain unknown. Recently, several reports have implicated members of the Rho GTPase family as important for the control of hematopoiesis [10, 11]. Disruption of Rac signaling results in reduced proliferation of LT-HSC and selective Rac2 deletion leads to a failure to up-hold hematopoiesis under steady state conditions [10, 11]. Since focal adhesion kinase (FAK) has been shown to regulate Rac activity [12], this signaling intermediate may also be of significance in this context.
Shb is a widely expressed Src homology-2 domain containing adaptor protein [13, 14] established as a regulator of reproduction [15], glucose homeostasis [16], angiogenesis [17] and T cell function [14, 18, 19]. Notably, Shb has also been found to regulate the cell cycle in the pancreas and blood system in a cell type dependent manner. For example, overexpression of Shb promotes proliferation in endothelial cells and in neonatal pancreatic β-cells [20]. Conversely, the loss of Shb expression results in elevated proliferative responses in T lymphoid cells to stimulation by CD3 and CD28 [19].

Shb participates in the regulation of a number of pathways also known to impact development, and does this primarily through interactions with its functional domains. The C-terminal SH2-domain of Shb [20] facilitates interactions with receptor tyrosine kinases such as vascular endothelial growth factor receptor-2 (VEGFR-2) [21], platelet derived growth factor receptor (PDGFR) [22] and the T cell receptor (TCR) [18, 23]. Moreover, Shb associates via its other domains with additional signaling elements such as SH2 domain-containing leukocyte protein of 76 kDa (Slp76) [23], Vav1 [23], c-Abl [24], FAK [17], phospholipase C-γ (PLCγ), Src, and phosphatidylinositol-3 kinase (PI3K), acting as a scaffold in signaling cascades [20]. Several of Shb’s known interaction partners, including PDGFR [25], TCR [26], Vav1 [27], and FAK [28], are established regulators of blood cell development and function. In addition, Shb deficient embryonic stem cells displayed reduced blood cell colony formation upon differentiation in vitro [29]. Thus, disruption of Shb mediated signaling could be hypothesized to result in a hematopoietic phenotype.

Utilizing the Shb knockout mouse [30], we find in the current study reduced bone marrow cell responsiveness to SCF-stimulation and elevated basal activity with respect to the FAK/Rac1/p21-activated kinase (PAK) signaling axis [11, 28, 31], proliferation defects in LT-HSCs during homeostasis, and an impairment in the long-term myeloid repopulating capacity of LT-HSCs. Unexpectedly, Shb is dispensable for stress hematopoiesis, and its loss
results in diminished myelodepression in a non-competitive hematopoietic environment after genotoxic stress.

**Materials and Methods**

*Experimental animals*

The generation of Shb knockout mice has been described previously [30]. The animals were maintained on a mixed background (129Sv/C57Bl6/FVB) as no Shb-/- offspring was generated on the C57Bl/6 strain, or on the Balb/c background in which Shb-/- pups were born. C57Bl/6 CD45.1 and Balb/c CD45.1 were from the Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the local animal ethics committees at Uppsala University.

*Flow cytometric analysis and sorting of bone marrow and peripheral blood*

Iliac bones, femurs and tibias were collected from 8 to 10 weeks old mice for all analyses. The bones were crushed and passed over a 70 µm cell strainer (BD Bioscience, Franklin Lakes, NJ) in order to obtain single-cell suspensions. The cells were stained for lineage markers with rat anti-mouse antibodies directed against CD4, B220, Mac-1, Gr-1, CD19 (Invitrogen, Carlsbad, CA), CD3 and CD8 (eBioscience, Hartfield, UK) followed by incubation with a PE-Cy5.5-conjugated goat anti-rat IgG (Invitrogen). Bone marrow cells were subsequently positively selected for c-Kit expression by magnetic separation with anti-c-Kit microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. The c-Kit purified cells were further stained for CD34-FITC, c-Kit-APC-eFluor 780, CD41-PE, CD48-PE (eBioscience), CD150-PE-Cy7, CD105-Biotin, Sca-1-APC (BioLegend, San Diego, CA), Streptavidin Alexa Fluor 680 (Invitrogen) and Flk-2-PE (BD Bioscience) in order to identify the LT-HSC, MPP (multi-potent progenitor), GMP (granulocyte-monocyte progenitor) and MEP (megakaryocyte-erythroid progenitor) populations respectively.
Peripheral blood was collected by retroorbital sampling in 0.05 mM EDTA (Merck, Whitehouse Station, NJ) and red blood cells were removed by sedimentation in 6% (w/v) Dextran T500 (GE Healthcare, Hemel Hempstead, UK) followed by lysis in Red cell lysis buffer (Sigma Aldrich, St. Louis, MO). The cells were subsequently stained with rat anti-mouse antibodies for the different lineages; CD4, CD8, CD3, B220, CD19, Gr-1 and Mac-1 and thereafter incubated with goat anti-rat PE-Cy5.5. For analysis of peripheral blood chimerism after transplantation the cells were also stained with CD45.1-FITC and CD45.2-APC (BD Bioscience).

In order to monitor FAK activity in LT-HSCs, bone marrow was isolated and promptly fixed in 4% paraformaldehyde. Cell surface staining to identify LT-HSCs was thereafter performed as described above. The cells were subsequently permeabilized with BD Cytoperm Buffer (BD Bioscience) and stained with phospho-FAK antibody (Invitrogen) followed by incubation with a PE-conjugated donkey anti-rabbit antibody (eBioscience).

Flow cytometric analysis carried out on an LSR II (BD Bioscience) and cell sortings were performed with a FACSCalibur, FACSVantage SE or FACSaria (BD Bioscience). All flow cytometric data were analyzed with FlowJo (TreeStar, Ashland, OR) or FACSDiva (BD Bioscience) software.

**Cell cycle analysis of HSC and HPCs**

DNA synthesis was determined with Hoechst 33342 (Invitrogen) staining and assessment of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Briefly, mice (8-10 weeks of age) were given intraperitoneal injections with 2mg of BrdU (BD Bioscience). Bone marrow was isolated 1 hour after the injections and cells were surface stained as described in the previous section. Cell fixation and permeabilization was thereafter carried out using the BD Cytofix/Cytoperm kit (BD Bioscience) following the manufacturer’s instruction. This was followed by DNase (300 µg/ml) treatment for 1 hour at 37°C and then by incubation with
anti-BrdU-PE (BD Bioscience). To enable identification of different cell cycle stages the cells were stained with 1 µg/ml Hoechst 33342 for 10 minutes.

Cell cycle status was also determined using the RNA binding dye Pyronin Y (Sigma Aldrich) at a concentration of 0.5 µg/ml in combination with Hoechst (10 µg/ml). In short, bone marrow was stained as previously described to enable identification of HSC- and hematopoietic progenitor cell (HPC) populations. The cells were thereafter fixed and permeabilized and subsequently stained with Hoechst for 15 minutes, Pyronin Y was added during the final 10 minutes.

Single-cell proliferation assay

CD150<sup>+</sup>–c-Kit<sup>+</sup>CD34<sup>–</sup>Flk2<sup>–</sup>CD41<sup>–</sup>CD48<sup>–</sup>Lin<sup>–</sup>LT-HSC2s from mice of 8-10 weeks of age were single-cell sorted into 96-well flat bottom plates and cultured in 5% CO<sub>2</sub> at 37° C in either 200 µl RPMI 1640 (Sigma Aldrich) with 10 % FCS (Sigma Aldrich), 2 mM L-glutamine, streptomycin (0.1 mg/ml), penicillin (100 U/ml, Gibco, Paisley, UK), or in serum-free StemSpan medium (Stem Cell Technologies, Vancouver, BC). The RPMI 1640 cultures were supplemented with the following cytokines IL-3 (10 ng/ml), SCF (10 ng/ml) and IL-6 (10 ng/ml) and the StemSpan contained IL-3 (10 ng/ml), SCF (50 ng/ml), IL-6 (10 ng/ml) and TPO (20 ng/ml) (all cytokines were purchased from PeproTech, Rocky Hill, NJ). The number of wells containing colonies was evaluated microscopically at day 10 of culture. For the determination of cell proliferation after FAK inhibition, mice were injected twice (24 hours and one hour) prior to sacrifice with 30 mg/kg body weight FAK inhibitor 14 (Tocris Bioscience, Bristol, UK) intraperitoneally. After sacrifice and staining for cell surface markers as above, LT-HSCs were single-cell sorted into 200 µl StemSpan medium supplemented with IL-3, SCF and IL-6 either in the presence or absence of 10 mM FAK inhibitor 14 and monitored for colony expansion. A fraction of the bone marrow cells was used to determine FAK activity in LT-HSC as described above.
5-Fluorouracil treatment

5-Fluorouracil (Sigma Aldrich) was administered to mice of 8-10 weeks of age via intraperitoneal injections at a dose of 150 mg/kg body weight. The mice were given 4 mg of BrdU intraperitoneally 12 hours before bone marrow isolation. LT-HSC staining was performed by incubating the cells with mouse anti-rat lineage markers and followed by staining with goat anti-rat IgG-PE-Cy5.5 as described in previous section. Sca-1 positive cells were thereafter isolated by magnetic separation with Sca-1-FITC microbeads (Miltenyi Biotec.) and subsequently surface stained for CD41-PE, CD48-PE, Flk-2-PE, and CD150-PE-Cy7. The cells were then fixed, permeabilized and DNase treated as mentioned above, followed by incubation with anti-BrdU-APC (Invitrogen) and Hoechst 33342.

Isolation and stimulation of c-Kit+ bone marrow cells

LT-HSCs are fairly rare cells, making them unsuitable for biochemical studies of signal transduction. However, most HSCs and HPCs express c-Kit and magnetic separation of c-Kit+ bone marrow cells thus results in an enrichment of stem- and progenitor cells. Single cell suspensions of bone marrow cells from mice of 8-10 weeks of age were magnetically separated based on c-Kit expression as described in the previous section. The cells were subsequently starved of serum and growth factors for 1 hour. The samples were stimulated for 3 minutes with 100 ng/ml SCF at 37°C followed by lysis in SDS sample buffer (250mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol). The Rac1 activation assay was performed according to the manufacturer’s instructions (Thermo Scientific, Waltham, MA). Briefly, stimulated cells were lysed and immunoprecipitated with a glutathione S-transferase fusion-protein corresponding to PAK1 coupled to glutathione-agarose. Whole cell lysates were subjected to separation by SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare, Uppsala, Sweden). Membranes were blocked over night in 5% BSA at 4°C and later probed with antibodies directed towards: phospho-
p38MAPK, p38MAPK, phospho-JNK, JNK, phospho-ERK, ERK, phospho-Akt, Akt, Pak, phospho-Pak, FAK (All from Cell Signaling Technology, Danvers, MA), phospho-FAK (Invitrogen) and Rac1 (Thermo Scientific).

**LT-HSC transplantation**

Competitive transplantations with LT-HSCs and 200 000 CD45.1⁺ competitor cells were carried out by retroorbital injection of sorted CD45.2⁺ donor cells into CD45.1⁺ recipients (donors, competitors and recipients were 8-10 weeks of age). LT-HSCs were defined as CD34⁻CD150⁺KLS (KLS=c-Kit⁺Lin⁻Sca1⁺) on the mixed background and as Balb/c lack Sca-1 expression CD150⁺c-Kit⁺CD34⁻Flk2⁻CD41⁻CD48⁻Lin⁻ LT-HSC2s were isolated from Balb/c mice 8-10 weeks of age. Balb/c recipients received 20, 25, 50 or 200 LT-HSC2s. The recipients were irradiated with a split dose of 9 Gy (Balb/c) or 10 Gy (mixed background) in a Nordion Gammacell 40 Exacto¹³⁷Cs irradiator (MDS Nordion, Ottawa, ON) in order to ablate endogenous bone marrow. The recipients were retroorbitally bled at 6 or 16 weeks to monitor donor contribution to peripheral blood lineages.

**Statistical analysis**

Data is presented as mean ± Standard error of the mean (SEM). For comparison of difference between two groups with normal distributed data, unpaired Students t-tests were used unless otherwise stated. For paired comparisons, one wild type and one age and sex matched knockout sample was analyzed simultaneously, under identical conditions, and the wild type and the knockout values were set as one observation each for the comparison. Comparisons between more than two groups were made by ANOVA, followed by Fischer’s LSD. For comparison of proliferation potential to in vitro stimulation with cytokines Chi-square test was used. All p-values less than 0.05 were considered statistically significant.

**Results**
Deletion of Shb results in reduced proportions of LT-HSCs

Ablation of Shb expression has been found previously to result in decreased numbers of lymphocytes and monocytes in the peripheral blood [19]. To address a cause of this impairment in hematopoiesis, we first characterized the hematopoietic progenitor and stem cell compartments of the Shb-null bone marrow. Bone marrow was isolated from Shb knockout and wild type mice and immunostained to evaluate the LT-HSC and HPC populations. As determined by flow cytometric analysis, knockout bone marrow contained significantly reduced proportions of CD34^−CD150^+KLS^+LT-HSCs (referred to as LT-HSCs, see fig 1A) [32, 33] compared to control samples (Fig. 1B, C). Since the absolute number of c-Kit^+ cells was not significantly altered in Shb null bone marrow (S. Fig 1) the decreased proportion of LT-HSCs corresponds to an absolute decrease in this cellular population. Further, there were no significant changes in the KLS (c-Kit^+ lineage^− Sca-1^− ) population [34, 35], the CD34^−CD150^−Flk2^+ KLS^+ MPPs [32, 33, 36, 37], the CD150^−CD105^−KLS^− GMPs, or the CD150^−CD105^−KLS^− MEPs (hereafter MPP, MEP, and GMP, respectively) [38] (Fig. 1B, C, D). We also examined the LT-HSC compartment of Shb null mice bred onto the Balb/c background. LT-HSCs from Balb/c mice are known to express reduced levels of Sca-1 [39] and a different immunophenotypic definition was therefore utilized to identify LT-HSCs in Balb/c bone marrow. Instead of characterizing LT-HSCs by Sca-1, Flk2, CD41, and CD48 were introduced and LT-HSCs were subsequently defined as CD150^−c-Kit^−CD34^−CD41^− CD48^−Flk2^−Lin^− LT-HSCs (henceforth referred to as LT-HSC2) [33, 40, 41]. Flow cytometric analysis revealed reduced numbers of LT-HSC2s in Shb knockout bone marrow from Balb/c mice compared to wild type samples (Fig. 2A, B), indicating that the hematopoietic defect was not strain specific. These data suggest that Shb function is dispensable for homeostasis of progenitors but point to an important role for Shb in the maintenance and expansion of LT-HSC numbers.
Shb exerts control over HSC cell cycle progression

In order to address whether the deficiency of LT-HSCs in Shb knockout bone marrow was due to aberrant regulation of proliferation, DNA synthesis was evaluated in LT-HSCs and HPCs by BrdU incorporation. Bone marrow was isolated 1 hour after BrdU delivery, and BrdU incorporation and Hoechst 33342 staining was assessed in order to ascertain proliferation rates. A reduction in total BrdU labeling suggested that Shb knockout LT-HSCs proliferate to a lesser extent than their wild type counterparts (Fig. 3A, B). In addition, Shb deficiency resulted in significantly decreased BrdU labeling in the MPP population (Fig. 3A, C). A 24-hour BrdU labeling experiment was also performed (three intraperitoneal injections of 100 mg BrdU per kg bodyweight at eight hour intervals), confirming reduced BrdU uptake in Shb knockout LT-HSCs (results not shown). Moreover, evaluation of the cell cycle by Pyronin Y and Hoechst 33342 staining provided independent confirmation that Shb knockout LT-HSC are profoundly more quiescent than their wild type counterparts (Fig. 3D and E). On the other hand, the MPP Pyronin Y+ population did not significantly differ between mutant and wild type, suggesting that any change in the number of actively cycling MPP caused by loss of Shb is minimal (Fig 3D and F). Additionally, the LT-HSC proliferative response in vitro to a defined set of hematopoietic cytokines was evaluated in a single-cell proliferation assay, revealing a reduced ability among individual Shb knockout LT-HSC2s to give rise to colonies (S. Fig. 2). The GMP population, however, showed no difference in cell expansion in vitro between single-cell sorted wild-type and Shb knockout cells (91% for wild-type and 95% for Shb knockout). The vast majority of colonies (>90%) exhibited a morphology corresponding to myeloid lineages (G, M, and GM) when the GMP-sorted cells were grown on Methocult M3434 (results not shown). These results further implicate the specificity of Shb as a positive regulator of LT-HSC cell cycle progression.

Stress hematopoiesis is unaffected by the loss of Shb
5-Fluorouracil (5-FU) is a chemoablative agent known to be toxic to cycling cells, consequently resulting in elimination of all proliferating LT-HSCs, HPCs and peripheral blood cells. This stimulates the remaining, quiescent LT-HSCs to quickly enter the cell cycle to compensate for the loss of the other cellular compartments [4]. 5-FU also transiently changes the surface phenotype of LT-HSC from c-Kit\textsuperscript{+} to c-Kit\textsuperscript{low/−} [42]. To further investigate Shb’s function in cell cycle regulation, 5-FU was administered to Shb knockout and wild type mice at a dose of 150mg/kg body weight. DNA synthesis was determined by measuring BrdU incorporation at the indicated time points. Shb null CD150\textsuperscript{+}CD41\textsuperscript{−}CD48\textsuperscript{−}Flk2\textsuperscript{−}c-Kit\textsuperscript{−}Lin\textsuperscript{−}Sca-1\textsuperscript{+} LT-HSCs promptly responded by entering the cell cycle at frequencies similar to those observed in wild type controls, both 3 and 5 days after 5-FU treatment (Fig. 4A). The proliferative response to hematopoietic stress did therefore not appear to be affected by loss of Shb function.

Additionally, as 5-FU is known to efficiently cause depletion of myeloid cells in circulation, the peripheral blood myeloid profile was therefore monitored after treatment. Wild type mice exhibited the expected, gradual myelodepression, with statistically significant reduced relative numbers of myeloid cells on day 5 when compared to untreated animals (Fig. 4B). Shb deficient myeloid cells were not equally affected by the 5-FU treatment, as the reduction in their numbers was less pronounced and failed to reach significance.

*Altered signaling characteristics in Shb knockout bone marrow cells*

The receptor tyrosine kinase c-Kit and its ligand SCF together establish a critical signaling cascade that protects LT-HSC and HPC viability [9]. Inhibition or disruption of c-Kit signaling has been demonstrated to confer altered cell cycle behavior and a failure to maintain the LT-HSC population. Stem cell factor (SCF) stimulation of wild type and Shb knockout c-Kit\textsuperscript{+} bone marrow cells revealed no major alterations in the phosphorylation at activating sites of p38MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal
kinase), ERK (extracellular-signal regulated kinase) and Akt in the absence of Shb (Fig 5A). Shb interacts with FAK and loss of Shb has been demonstrated to affect FAK signaling in response to VEGF [17]. FAK signaling was therefore estimated revealing an elevated phosphorylation at an activating site (Y397) in the absence of SCF stimulation in Shb null samples and failure of SCF to activate FAK further (Fig. 5B). The Rho family GTPase Rac1 is regulated by FAK [12]. Rac1 also displayed a significantly increased activity under basal conditions in Shb knockout cells, as indicated by the elevated levels of GTP-bound Rac1 (Fig. 5C). Found immediately downstream of Rac1 is the PAK family of serine/threonine kinases. PAK signaling also appeared aberrant in Shb knockout bone marrow with significantly increased basal phosphorylation levels as well as a lack of stimulation effect after addition of SCF in knockout samples (Fig. 5D).

To determine the activity of FAK in LT-HSCs, bone marrow was fixed and stained for pY\textsuperscript{Y397}-FAK, as well as for cell surface markers defining this cell population and subsequently analyzed by FACS. Shb deficient LT-HSCs displayed a significant increase in phospho-FAK levels indicating an aberration of FAK signaling not only in c-Kit\textsuperscript{+} bone marrow cells but also within the LT-HSC cell population (Fig. 6A-C). An attempt was also made to stain for pPAK but a robust signal could not be obtained using this mode of analysis. Overall, the data implied that loss of Shb deregulated the FAK/Rac1/PAK signaling cascade resulting in elevated basal activation and a failure to mount a ligand-stimulated response of this pathway. Signaling through FAK/Rac1/PAK has been implicated in LT-HSC and HPC cell cycle control, thus providing a mechanistic explanation for the observed differences in the LT-HSC cell cycle [10, 11, 28, 31, 43].

To directly assess the importance of elevated FAK activity for Shb knockout LT-HSC proliferation, mice were treated with a FAK inhibitor prior to sacrifice, LT-HSCs were single-cell sorted and cell proliferation determined in proliferation assays (Fig. 6D). Shb knockout
LT-HSCs exhibited a higher proliferative ability after FAK-inhibition compared with untreated cells and the number of proliferating LT-HSCs after FAK inhibition was similar to that of the corresponding wild-type cells. FAK-activity was decreased in the Shb knockout LT-HSCs (Fig. 6E) whereas no major effect was noticed in the wild-type situation (results not shown). The data confirm the notion that the altered signaling characteristics of Shb knockout LT-HSCs result in reduced proliferation.

**Reduced long-term repopulating activity of Shb knockout LT-HSCs**

The principal role of LT-HSC and HPC is their ability to reconstitute the blood system, and repopulation of lethally irradiated recipients provides the best measure of evaluating this function [1]. By transplanting purified LT-HSCs together with unfractionated bone marrow as competition, the transplanted cells are stimulated to proliferate at an accelerated rate. In the current study, two isoforms of CD45, CD45.1 and CD45.2, were used to distinguish between donors and competitors. As previously indicated, the Shb knockout was initially maintained on a mixed genetic background consisting of FVB, C57Bl/6 and Sv129. Balb/c and C57Bl/6 are the two primary strains available as CD45.1 carriers and consequently we decided to perform competitive LT-HSC transplantations using Shb null mice bred onto the Balb/c background since Shb -/- mice cannot be obtained on the C57Bl/6 background [30].

Lethally irradiated CD45.1⁺ Balb/c recipients were subsequently injected with either 20 sorted (S. Fig. 3) WT or KO LT-HSC2s each together with 200000 competitors. Engraftment appeared largely unaffected in the absence of Shb in Balb/c recipients 6 weeks after the transplantation (Fig. 7A). At 16 weeks, engraftment of all three lineages (myeloid, B cell and T cell) from donor LT-HSC2 cells was observed, confirming the notion that this cell population purified from Balb/c mice indeed contains LT-HSCs. However, when chimerism was determined at 16 weeks, there was a significant decrease in the proportions of donor-
derived cells in the myeloid lineage of Shb null recipients whereas the B and T cell lineages appeared unaffected (Fig. 7A, S. Fig. 4). Between week 6 and 16, the ratio of CD45.2+ cells would be expected to increase as the pool of transplanted LT-HSCs gradually expand their numbers to replenish the blood system; therefore, the difference in chimerism within the myeloid lineage between 6 and 16 weeks after the LT-HSC2 transplantation was determined. Wild type recipients displayed a 3-fold higher increase in the ratio of CD45.2+/CD45.1+ (a CD45.2+/CD45.1+ ratio of 3 corresponds to 75% CD45.2+ cells) compared to recipients of Shb null cells (Fig. 7B). Different doses of LT-HSC2s were also transplanted. Recipients injected with 20, 25, 50 or 200 Shb knockout LT-HSC2s displayed a statistically significant reduction of long term myeloid engraftment compared to mice receiving corresponding cell doses of wild type LT-HSC2s as visualized by plotting the calculated trend-lines (Fig. 7C). Similarly to what was observed for a dose of 20 LT-HSC2s, Shb knockout recipients showed an impaired expansion of the myeloid population between 6 and 16 weeks for the different dosages (Fig. 7D). These data highlight the role of Shb in proliferation of LT-HSC.

Discussion

A deep knowledge of the factors governing LT-HSC and HPC proliferation is imperative in understanding hematopoiesis under normal as well as neoplastic conditions. Herein we report that the adaptor protein Shb serves as a regulator of LT-HSC cell cycle control. LT-HSCs from Shb deficient mice exhibited reduced proliferation, resulting in a diminished LT-HSC pool under steady state conditions as well as in a failure to maintain contribution to peripheral blood for longer time periods following LT-HSC transplantations.

The proliferation kinetics of LT-HSCs have been extensively studied, revealing stem cells as mostly quiescent but with the ability to rapidly change their cell division frequency in response to the appropriate stimuli. Shb deficient LT-HSCs showed several proliferative
defects under homeostasis, but did not appear to be lacking in responsiveness to hematopoietic stress signals as LT-HSC engraftment was found to be unaffected 6 weeks post-transplantation. Differences in donor chimerism were instead observed 16 weeks after transplantation, at a time point when hematopoiesis has reached equilibrium again, indicating that steady state rather than stress hematopoiesis is affected. Furthermore, the Shb null LT-HSC response to cytotoxic stress in the form of 5-FU also appears normal. The milder myelodepression displayed by Shb deficient animals might be due to the fact that 5-FU is an S-phase inhibitor, thus primarily targeting proliferating cells. As the Shb knockout bone marrow generally has fewer dividing LT-HSC cells at homeostasis, it is plausible that the chemoablative effects are not as prominent in the knockout resulting in reduced myelosuppression.

After transplantation of Shb null LT-HSCs, only the myeloid lineage displays significantly reduced chimerism 16 weeks after the transplantation. This could be interpreted to suggest that the loss of Shb results in a myeloid specific differentiation defect rather than affecting cell cycle regulation. However, since the Shb knockout mice are not more severely affected by 5-FU treatment this seems unlikely. Treatment with 5-FU induces hematopoietic stress in general but also affects myelopoiesis to a larger extent due to the fast turnover of these cells. If the loss of Shb were to result in a myeloid specific differentiation defect, Shb deficient mice should display more myelosuppression than their wild type counterparts. Moreover, single-cell sorted Shb knockout GMP cells showed no difference in their proliferation and differentiation compared with wild-type controls. The effects of Shb deficiency on LT-HSC pool size and cell cycle activity were easily detected despite the absence of major hematopoietic aberrations. This discrepancy is most likely explained by the proliferative overcapacity within the HPC populations compensating for the reduced potential of Shb knockout LT-HSCs. Additionally, hematopoietic defects have already been established
in the Shb knockout mouse, with modestly decreased numbers of monocytes and lymphocytes in peripheral blood [19] at steady state conditions, thus further supporting the notion of Shb as a positive modulator of LT-HSC maintenance. In previous work with the Shb knockout, alterations in T cell function were observed that change the cytokine milieu, with increased levels of Th2 cytokines and a general increase in the proliferative rate among CD4^+ T lymphocytes [19]. Th2 cytokines are known to promote B cell expansion [44, 45] and consequently augmented peripheral proliferative responses of T and B cells seem plausible explanations for why these populations were not reduced after Shb knockout LT-HSC transplantation.

LT-HSCs can be recruited into the cell cycle given the right conditions. The signals triggering the switch from dormancy to active cycling during homeostasis remain to be identified. The receptor tyrosine kinase c-Kit and its ligand SCF have been implicated as essential factors in the maintenance of LT-HSC and HPC populations. Numerous studies have shown that complete or partial [46, 47] genetic ablation of c-Kit activity, and inhibition of c-Kit by blocking antibodies [34] result in severe hematopoietic deficiencies. Additionally, deletion of SCF in endothelial and perivascular cells has been demonstrated to severely constrain adult LT-HSC maintenance [48]. We presently note that Shb knockout bone marrow exhibits several signaling defects in FAK, Rac1 and PAK signaling with elevated basal activity and reduced responsiveness to SCF stimulation.

FAK is highly expressed in LT-HSCs but due to the embryonic lethality of FAK knockouts little is known about its effects on the hematopoietic system [28, 43]. Conditional deletion of FAK in bone marrow has revealed extensive but opposing effects on proliferation in hematopoietic progenitor populations [28, 49]. Erythroid progenitors from FAK null mice displayed decreased proliferative responses to SCF stimulation [28]. In a population of FAK deficient KLS cells that primarily corresponded to multi-potent progenitors, a larger fraction
of cells was found to be actively cycling [49], suggesting that the effect of FAK signaling is highly dependent on which hematopoietic cell type is observed. *Shb* knockout c-Kit+ bone marrow cells as well as LT-HSCs exhibited significant hyperphosphorylation of FAK in the absence of stimulation suggesting that the proliferative defect could be effected by elevated FAK signaling. This notion was confirmed by assessing LT-HSC proliferation after FAK inhibition using a single-cell proliferation assay. An important downstream effector of FAK activity is Rac1 [12]. Western blot analysis of c-Kit+ cells also revealed elevated levels of active Rac1 and its immediate downstream target PAK in *Shb* deficient cells under basal conditions. Rac1 has been implicated as an important regulator of LT-HSC and HPC cell cycle progression [11]. In addition, PAK has been demonstrated to regulate proliferation in several different hematopoietic lineages including mast cells and T cells [50, 51] and PAK hyperactivation was recently linked to elevated LT-HSC proliferation in CD48 null mice [31]. The corroboration of elevated FAK-activity in the *Shb* knockout LT-HSCs lends support to the notion that the signaling characteristics of the c-Kit+ bone marrow cells are representative for the LT-HSC population. These findings are further reinforced by the signaling signature of *Shb* knockout endothelial cells in which elevated FAK and Rac1 activities and reduced ligand (VEGF) responsiveness were reported [17, 52]. Whether the presently observed alterations of Rac1 and PAK activities are the relevant effectors of the FAK-dependent suppression of LT-HSC proliferation is not directly addressed by our present data.

The common denominator for the signaling components that are deregulated in the absence of *Shb* is that they not only exhibited increased baseline activation but also a poor stimulation response. We have extensively searched for a direct association between Shb and c-Kit but have failed to detect such an interaction (results not shown). It therefore seems most plausible that the reduced SCF-responsiveness is an indirect consequence of the *Shb* knockout LT-HSC signaling signature. However, the fact that FAK inhibition promoted *Shb* knockout
LT-HSC proliferation argues against a major role of SCF-responsiveness in this context. At present, the reason why elevated FAK activity would reduce steady-state LT-HSC proliferation remains unclear but this could reflect FAK-induced alterations of cellular responses in relation to their natural niche. Experiments using a conditional Shb knockout would address this issue further.

Conclusions

Collectively, our data define a role for the adaptor protein Shb as a positive regulator of the LT-HSC cell cycle and that absence of Shb confers a disadvantage to maintaining this population in the long term by acting as a modulator of the FAK/Rac1/PAK signaling axis. Particularly elevated basal FAK activity appears to be an important component maintaining LT-HSC in a quiescent state. Shb might therefore provide useful insights into the factors that determine LT-HSC cell cycle dormancy, thus increasing our understanding of stem and progenitor cell driven leukemia as well as further development of stem cell therapies.

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collection and final approval of manuscript; SLMF: Conception and design, data
collection, data analysis and interpretation, final approval of manuscript; GQD:
Conception and design, financial support, data interpretation, manuscript writing, final
approval of manuscript; MW; Conception and design, financial support, data collection,
data analysis and interpretation, manuscript writing and final approval of manuscript.
References


Titles and legends to figures

**Figure 1. Immunophenotypic estimation of relative numbers of LT-HSCs and HPCs.** (A) Schematic diagram of the hematopoietic stem and progenitor cells hierarchy including the markers defining each subset. (B) The plots are representative of flow cytometric identification of LT-HSC and HPC in Shb knockout and wild type mice on a mixed genetic background. The different populations are identified by six-color fluorescence activated cell sorting (FACS) analysis of the differential expression of lineage defining markers, c-Kit, Sca-1, CD34, CD150 and CD105. (C and D) The relative numbers of KLS, LT-HSC, MPP, MEP and GMP populations as defined according to plots in B. KLS is given as percentage of Lin-cells, LT-HSC and MPP are given as percentages of KLS. MEP and GMP are percentages of c-Kit+/Lin-/Sca1- cells. Data are presented as average ± SEM from 9-14 mice and ** denotes p< 0.01 as determined by paired Student’s t-test.

**Figure 2. Flow cytometric analysis of relative numbers of LT-HSC2 on Balb/c background.** (A) Plots representative of LT-HSC2s in Shb knockout and wild type mice. The different populations are identified by the differential expression of lineage defining markers, c-Kit, CD41, CD48, Flk2, CD34, and CD150. (B) The relative numbers of LT-HSC2s (percent of total c-Kit+ cells) as defined in the previous plots. Data are presented as average ± SEM from 6 mice and * denotes p< 0.05 as determined by paired Student’s t-test.

**Figure 3. Cell cycle analysis in LT-HSC and HPC by BrdU incorporation and Pyronin Y staining.** (A) Representative histograms of BrdU fluorescence 1 hour after BrdU administration gated on the LT-HSC, MPP, MEP and GMP populations. (B and C). The percentage of BrdU positive cells, used to determine the relative numbers of cycling cells within the different subsets. (D) Plots representative of Hoechst 33342 and Pyronin Y fluorescence gated on LT-HSC, MPP, MEP and GMP populations. (E and F) The relative number of all cycling cells within each population as determined by Pyronin Y and Hoechst.
staining. Data are mean values ± SEM from 8 mice where *denotes p< 0.05 and
**denotes p< 0.001 as determined by paired Student’s t-test.

**Figure 4. Effects of 5-FU induced hematological injury on LT-HSCs proliferation and**
**peripheral blood myelosuppression.** (A) Percentage of actively cycling CD150−CD41−
CD48−Flk2−c-Kit−Lin−Sca-1+ LT-HSCs assessed by BrdU incorporation and Hoechst 33342
uptake 3 and 5 days after treatment commencement. Data are presented as mean ±SEM for 4
mice each. (B) Relative numbers of myeloid cells in peripheral blood, identified by Gr-1 and
Mac-1 cell surface expression in 2-color FACS analysis. Data are presented as mean ±SEM
from mice at day 0 (n=4 mice each genotype), 3 (n= 5 mice each genotype) and 7 (n=7 mice
each genotype), where **denotes p< 0.001 as determined by Student’s t-test.

**Figure 5. Effects of SCF stimulation on c-Kit enriched bone marrow cells.** (A) MAPK
and Akt signaling was examined by SCF stimulation of bone marrow cells for 3 minutes
followed by Western blot analysis with phoshospecific antibodies. The blot presented is
representative of 3 independent experiments. (B) FAK activity was evaluated by
immunoblotting for phospho- and total FAK, respectively. Proteinphosphorylation was
related to total protein content on the same blot and signal strength was estimated by
densitometric analysis. Values represented are arbitrary units and based on 5 independently
performed experiments. (C) Rac1 activity under basal and c-Kit stimulating conditions was
estimated by Pak1-GST pulldown. The upper panel represents active GTP-binding Rac1 and
total Rac1 in lysates from 1 of the 3-4 independently performed experiments. Quantification
of Rac1 activity was performed by densitometry and values were normalized against
unstimulated Shb knockout samples. (D) Level of PAK activation was determined by
densitometric analysis of Western blots, comparing phosphoprotein to total protein and values
are presented in arbitrary units from 4 different experiments. All results are represented as
mean values ±SEM, where *denotes p< 0.05 as determined by paired Student’s t-test unless
otherwise stated and ** denotes p< 0.01 by two-way ANOVA followed by Fischer’s LSD.
Figure 6. LT-HSC FAK activation and its importance for proliferation. (A) LT-HSC FAK activity in the absence of stimulating ligands was estimated by flow cytometric analysis of phospho-FAK levels. Plots are representative of gating strategies for identification of LT-HSCs. (B) Histogram plot representative of FAK activity within the LT-HSC population. (C) The relative mean fluorescence intensity (MFI) for phosphorylated FAK. Data in C is presented as mean values from 3 mice where ** denotes p<0.01 as determined by paired Student’s t-test. (D) Effects of FAK inhibition on LT-HSC cell proliferation using a single-cell proliferation assay. Wild-type (WT) or Shb knockout (KO) mice were either injected with FAK inhibitor 14 or left untreated. LT-HSCs were subsequently sorted and cultured either in the presence or absence of FAK inhibitor 14 together with cytokines. Untreated mice and cells or mice and cells treated with the FAK inhibitor were analyzed in parallel. *** indicates p<0.001 with a chi-square test. The figure is representative of three separate experiments with the FAK inhibitor. (E) Histogram plot of FAK activation after treatment with FAK inhibitor 14 in vivo of Shb knockout (KO) LT-HSC cells as described in D.

Figure 7. LT-HSC2 repopulating ability estimated by transplantation into lethally irradiated CD45.1+ recipients Balb/c background. (A) The ratio of CD45.2+ over CD45.1+ cells following transplantation of 20 LT-HSC2 were determined by FACS analysis. Peripheral blood cell populations were identified as Gr-1+Mac-1+ myeloid cells, B220+CD19+ B cells and CD3+CD4+CD8+ T cells and subsequently gated for CD45.2+ and CD45.1+ cells to determine chimerism. (B) The relative increase between 6 and 16 weeks after transplantation in numbers of CD45.2+ cells within the myeloid lineage was calculated. (C) LT-HSC2s were transplanted at doses of 20, 25, 50 and 200 respectively and the ratio of CD45.2+ to CD45.1+ cells within the peripheral blood myeloid compartment was analyzed by flow cytometry 16 weeks post-transplantion. The different doses are presented on a logarithmic scale and trend-lines were calculated based on the mean CD45.2+/CD45.1+ ratios at the different cell doses. Means ±SEM are shown for 2-6 mice at each point. The experiments are from three separate cell sortings transplanted at three different occasions and the total numbers of mice were 13-17. * indicates p<0.05 when the difference in mean value at each cell dose for the WT and
Shb knockout were compared using a paired Students’ t-test. (D) The relative increase in myeloid chimerism between 6 and 16 weeks was estimated for the four different LT-HSC2 doses (20, 25, 50 and 200). Data are represented as mean values ±SEM increase in myeloid engraftment between week 6 and 16 for the different cell doses where *denotes p< 0.05 as determined by a paired Student’s t-test. Individual engraftment (CD45.2+/CD45.1+ ratio) of myeloid cells in each mouse is shown in S. Table 1.
Figure 2, Gustafsson et al, top

A

WT

KO

44.6

44.1

Lineage

c-Kit

c-Kit

3.71

3.28

Flk2/CD41/CD48

CD150

CD34

LT-HSC2

28.9

21.1


B

Relative numbers of LT-HSC2

Percent

WT

KO

0.5

0.4

0.3

0.2

0.1

0

*
Figure 4, Gustafsson et al, top

A
LT-HSC BrdU labeling after 5-FU treatment

B
Myeloid cell numbers after 5-FU treatment
Figure 7, Gustafsson et al, top

A

Peripheral blood chimerism

CD45.2/CD45.1 ratio

Weeks

0 0,5 1 1,5 2 2,5 3 3,5

6 16 6 16 16

Myeloid B cell T cell

WT KO

**

B

Myeloid chimerism increase 6 to 16 weeks

CD45.2/CD45.1 ratio

WT KO

*

C

LT-HSC myeloid transplantation efficiency

CD45.2/CD45.1 ratio

y = 0,1087x + 4,5071
R² = 0,94009

Number of transplanted LT-HSCs (log scale)

y = 0,1085x + 1,247
R² = 0,97198

D

Myeloid chimerism increase 6 to 16 weeks

CD45.2/CD45.1 ratio

WT KO

*
Supplementary figure 1.

**Absolute numbers of c-Kit enriched cells.** The numbers of cells from wild type and *Shb* knockout mice on both mixed and Balb/c background were determined after magnetic separation for c-Kit⁺ cells by counting in a Bürker chamber. Results are given as mean values ±SEM from 12 mice on the mixed background and 6 Balb/c mice, respectively.
Supplementary figure 2.

**Effect of cytokine stimulation on LT-HSC2 proliferation.** CD150<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>−</sup> Flk2<sup>+</sup>CD41<sup>−</sup>CD48<sup>−</sup>Lin<sup>−</sup> LT-HSC2s were single cell sorted into 96-well plates containing either serum-free StemSpan (IL-3 10 ng/ml, SCF 50 ng/ml, IL-6 10 ng/ml and TPO 20 ng/ml) or RPMI 1640 (IL-3 10 ng/ml, SCF 10 ng/ml and IL-6 10 ng/ml). The number of wells containing more than one cell was determined at day 10. The results are from three mice each genotype sorted at three different occasions.

***denotes p< 0.001 as determined by Chi-square test.
Supplementary figure 3.

Post-sort analysis of sorted LT-HSC2. The sorted LT-HSC2 cells were analyzed by the same FACS that performed the sorting in order to ensure the purity of the samples prior to transplantation. LT-HSC2s were defined as Lin^−^c-Kit^+^Flk2^−^CD41^−^CD48^−^CD34^+^CD150^+^ by labeling with fluorescent antibodies.
Supplementary figure 4.

Analysis of peripheral blood chimerism 6 weeks post-transplantation in the myeloid and B cell lineages. The FACS plots presented are representative of the ratio of CD45.2$^+$ to CD45.1$^+$ cells among myeloid cells and B cells in peripheral blood from transplanted recipients. The myeloid lineage is defined as Gr-1$^+$/Mac-1$^+$ and the B cells are identified as B220$^+$/CD19$^+$. 
Supplemental Table 1: Relative engraftment of donor LT-HSC cells (CD45.2+) over competitor cells (CD45.1+) after transplantation to bone marrow-ablated recipient mice at 16 weeks after transplantation in each individual animal.

<table>
<thead>
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<th>WT CD45.2+/CD45.1+</th>
<th>KO CD45.2+/CD45.1+</th>
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</thead>
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<tr>
<td>0.7 (25)</td>
<td>0.1 (25)</td>
</tr>
<tr>
<td>1.3 (50)</td>
<td>0.5 (25)</td>
</tr>
<tr>
<td>2.1 (20)</td>
<td>1.0 (20)</td>
</tr>
<tr>
<td>2.3 (20)</td>
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<td>3.7 (200)</td>
<td>1.4 (50)</td>
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<td>67.5 (200)</td>
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</table>

CD45.2+/CD45.1+ ratio of myeloid cell engraftment for each individual mouse is shown. Number in parenthesis is the number of transplanted LT-HSC cells.