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Requirement for Ssbp2 in Hematopoietic Stem Cell Maintenance and Stress Response

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Transcriptional mechanisms governing hematopoietic stem cell (HSC) quiescence, self-renewal, and differentiation are not fully understood. Sequence-specific ssDNA–binding protein 2 (SSBP2) is a candidate acute myelogenous leukemia (AML) suppressor gene located at chromosome 5q14. SSBP2 binds the transcriptional adaptor protein Lim domain–binding protein 1 (LDB1) and enhances LDB1 stability to regulate gene expression. Notably, Ldb1 is essential for HSC specification during early development and maintenance in adults. We previously reported shortened lifespan and greater susceptibility to B cell lymphomas and carcinomas in Ssbp2−/− mice. However, whether Ssbp2 plays a regulatory role in normal HSC function and leukemogenesis is unknown. In this study, we provide several lines of evidence to demonstrate a requirement for Ssbp2 in the function and transcriptional program of hematopoietic stem and progenitor cells (HSPCs) in vivo. We found that hematopoietic tissues were hypoplastic in Ssbp2−/− mice, and the frequency of lymphoid-primed multipotent progenitor cells in bone marrow was reduced. Other significant features of these mice were delayed recovery from 5-fluorouracil treatment and diminished multilineage reconstitution in lethally irradiated bone marrow recipients. Dramatic reduction of Notch1 transcripts and increased expression of transcripts encoding the transcription factor E2a and its downstream target Cdkn1a also distinguished Ssbp2−/− HSPCs from wild-type HSPCs. Finally, a tendency toward coordinated expression of SSBP2 and the AML suppressor NOTCH1 in a subset of the Cancer Genome Atlas AML cases suggested a role for SSBP2 in AML pathogenesis. Collectively, our results uncovered a critical regulatory function for SSBP2 in HSPC gene expression and function. The Journal of Immunology, 2014, 193: 000–000.

A small pool of pluripotent stem cells capable of self-renewal maintains normal cellular hematopoiesis and recovery from cytotoxic stress (1, 2). The delicate balance between hematopoietic stem cell (HSC) self-renewal and differentiation during these processes requires precise coordination of gene expression with the abundance of their protein products. The core multiprotein transcriptional complex that regulates hematopoietic and progenitor cell stem (HSPC) activity is composed of the b-HLH protein TAL1/SCL, the Lim-only protein 2 (LMO2), LIM domain–binding protein 1 (LDB1), and the zinc finger protein GATA2. Initial gene-targeting studies demonstrated an absolute requirement for the HSC-expressed and lineage-restricted factor, TAL1/SCL1, in embryonic HSC specification (3). In contrast, TAL1/SCL appears to be dispensable for hematopoietic maintenance in adults because of functional redundancy with LYL1 (4). In erythroid and megakaryocytic progenitor cells, the non–DNA-binding transcriptional adaptor LMO2 is thought to bridge TAL1/SCL1 and the self-dimerizing cofactor LDB1 to nucleate the assembly of b-HLH-GATA factor– and LDB1-containing multisubunit complexes (5, 6). This pathway raises the question of whether a similar mechanism occurs in HSCs. Furthermore, unlike TAL1/SCL1, LMO2 and LDB1 are continuously required for postnatal HSC maintenance (7–9). Elegant global chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-Seq) studies have localized LDB1, TAL1/SCL1, and GATA2 to conserved promoter elements on the TAL1/SCL1,
Gata2, Runx1, Lmo2, E2a, and Myb genes. Furthermore, decreased expression of Tal1/Scl1, Gata2, Runx1, Lmo2, and Myb in Ldb1−/− mouse HSPCs has unequivocally demonstrated these encoded proteins to be positive regulators of HSPC-specific gene expression (9).

Human sequence specific ssDNA-binding protein 2 (SSBP2) was positionally cloned as a myeloid leukemia suppressor from a chromosomal disruption within a critical region of loss in 5q13-14 in an acute myelogenous leukemia (AML) cell line (10). SSBP2 and the related SSBP3 and SSBP4 were so designated because of the in vitro ssDNA-binding activity of the founding member CSDP, the chicken ortholog of SSBP3 (11); however, the significance of this putative ssDNA-binding activity in vivo is unknown. All three proteins can bind LDB1 through a highly conserved N-terminal domain; in turn, LDB1 binds the LIM domains of LMO or LIM homeodomain proteins (LHX) through an evolutionarily conserved COOH-terminal LIM-interacting domain.

We and others have established that SSBPs enhance promoter occupancy and transcriptional activity of LMO2- and LHX-containing complexes by preventing the ubiquitilation and ultimately the proteasomal degradation of LDB1 by the E3 ubiquitin ligase RNF12/RLIM (12–15). Clearly, SSBPs have been selected for and evolutionarily maintained as key modulators of LDB1 activity. In Caenorhabditis elegans, for example, the sole SSB ortholog SAM-10 coordinates with the LDB ortholog in a cell-autonomous fashion to regulate synaptic differentiation (16).

Similarly, the expression of the Drosophila ortholog, SSDP, is a rate-limiting cofactor that regulates combinatorial transcriptional signals from CHIP (LDB1)-APTEROUS (LHX) or CHIP (LDB1)-PANNIER (GATA) complexes (17, 18). Finally, in zebrafish, SSDPs regulate neural patterning and sensory neuronal growth in part through LDB1 stabilization (19). Although SSBPs from all of these species bind and stabilizes LDB1, consistent with extensive evolutionary conservation of these members of this family (>99% identity), there also may be distinct function for each member. Consistent with this notion, Ssbp2−/− mice are viable (15), whereas Ssbp3−/− mice die in utero (20, 21). In addition, a genome-wide in vivo screen by genetic cross of two strains of mice that differ in hippocampal neurogenesis suggested Ssbp2 to be a quantitative trait locus regulating neuronal survival and regeneration (22). Finally, the highly penetrant autoimmune defects and enhanced predisposition to B cell lymphomas and tissue harvests was shown in Ssbp2−/− mice (23).

The pivotal role of Ldb1 in HSPC maintenance (9, 23) suggests that Ssbp2 plays a similarly important role. In this study, we report that Ssbp2 exerts a nonoverlapping regulatory function to maintain murine HSPCs. In addition, reestablishment of homeostasis after elimination of cycling HSPCs by myeloablative treatment is impaired upon Ssbp2 ablation. Moreover, Ssbp2−/− bone marrow (BM) competes poorly in multilineage reconstitution of lethally irradiated recipients. Furthermore, decreased expression of Notch1 and elevated expression of E2a and its transcriptional target Cdkn1a in Ssbp2−/− HSPCs identify a role for Ssbp2 in the HSPC-specific gene expression program. Finally, because accumulating evidence suggests a myeloid suppressor role for Notch1 (24, 25), we evaluated the expression of Notch1 and Ssbp2 from data obtained from the Cancer Genome Atlas (TCGA). Interestingly, Notch1 expression was modestly correlated with Ssbp2 expression in primary AML samples. Taken together, these initial biological findings lay the foundation for future mechanistic investigations on SSBP2-regulated transcriptional networks in HSPCs.

Materials and Methods

Mice

Ssbp2−/− mice were described previously (15). For the competitive transplantation experiments, the mice were backcrossed to a C57BL/6 (CD45.2) background for 10 generations. Mouse bleeding and tissue harvests were according to Institutional Animal Care and User Committee-approved protocols following standard operating procedures.

Immunofluorescence analysis

BM mononuclear cells enriched by centrifugation or plated from lineage-committed cells through magnetic-activated cell separation were stained for (Ssbp2−/−). Lin Scilc−/− or LSK) cells were purified by staining the Lin cells with anti-Sca-1–PE and anti-c-Kit–FITC Abs (CD45.2+ BM Pharmsing), followed by cell sorting using a BD FACS Aria IIu sorter. Cells were centrifuged onto slides and fixed in 4% paraformaldehyde in PBS for 20 min, which was followed by permeabilization and then staining with rabbit polyclonal anti-SSBP2 Ab or mouse anti-LDB1 Ab (Molecular Probes) as detailed previously (26).

Phenotypic analysis of HSPCs by flow cytometry

Seven-color FACS analysis (Pacific Blue, Alexa 488, allospecificy-cyanocyn-Cy7, Alexa 647, PE, and PerCP-Cy5.5) was performed using a Fortessa cell analyzer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star). The Abs used against mouse Ags, including Pacific Blue lineage mixture (Pacific Blue anti-mCD3, -mLy-6G/Ly-6C), -mCD11b, -mCD45R(B220), and -mTer-119, Calix-FITC, c-Kit–allospecificy-cyanocyn-Cy7, CD34–Alexa 647, Flt3–PE, and CD48–PerCP-Cy5.5 were from BD Pharmingen, eBioscience, or BioLegend. BM mononuclear cells were stained with Pacific Blue–conjugated lineage mixture along with Sca1–Alexa 488, c-Kit–allospecificy-cyanocyn-Cy7, and Sca1–c-Kit–cells were defined as LSK cells. Similar procedures were used to analyze Flt3–CD34+ LSK cells (LT-HSCs), Flt3+CD34+ LSK cells (ST-HSCs), Flt3+CD34+ LSK cells (multipotent progenitor cells [MPPs]), Flt3+CD34+ LSK cells (lymphoid–primed multipotent progenitor cells [LMPPs]), and CD48+ or CD48– LSK cells.

Hematopoietic recovery from myelotoxic agents

Mice were administered a single i.p. dose of 5-fluorouracil (5-FU) at 150 mg/kg body weight. Fifty-microliter aliquots of peripheral blood samples collected from 5-FU–treated and control (untreated) mice were analyzed at 6, 12, and 18 d after 5-FU injection in a Siemens Advia 120 analyzer for total and differential counts. Two complementary approaches were taken to examine the role of Ssbp2 in HSPC recovery from 5-FU. In the first set, wild-type (WT) BM was transplanted into lethally irradiated Ssbp2 null mice, and the mice were challenged with 5-FU 4 mo after transplantation. In the second set, donor BM cells (CD45.2) were prepared from Ssbp2−/− or Ssbp2−/− control mice and mixed with congenic competitor BM cells (CD45.1) at a 1:1 ratio. The mixed cells (5 × 105) were transplanted into the irradiated recipients (CD45.1−) by tail vein injection, and the mice were challenged with 5-FU as detailed above.

Competitive BM transplantation

Age-matched female congenic B6.SJL-PtcaPep3b/BoyJ (B6.BoyJ;CD45.1+) mice (The Jackson Laboratory) were used as transplant recipients. These mice were lethally irradiated (9.5 Gy). Donor BM cells (CD45.2) were prepared from Ssbp2−/− or Ssbp2−/− control mice and were mixed with congenic competitor BM cells (CD45.1) at 1:1 or 10:1 ratio. The mixed cells (5 × 105) were transplanted into the irradiated recipients (CD45.1−) by tail vein injection. Donor-derived engraftment and multilineage reconstitution in BM and peripheral blood from recipients were assessed by flow cytometry using CD45.1–allospecificy-cyanocyn or CD45.2–PE-Cy7 at 4 and 16 wk posttransplantation to evaluate for short- and long-term engraftment, respectively.

Real-time quantitative PCR

RNA was isolated using the RNeasy mini kit (Qiagen). cDNA was generated using SuperScript II reverse transcriptase (Invitrogen). Samples were normalized using 18S RNA, and gene expression levels were determined using the relevant primers and probes on a TaqMan AB7900HT instrument (Applied Biosystems). Each experiment was performed five times in triplicate. Data are expressed as mean ± SD.
Analysis and data mining

TCGA AML database (27) was accessed, and the pertinent data were analyzed with cBioPortal for AML cases with mutations, copy number alterations, or an at least 2-fold change in expression level by RNA-sequence analysis (28, 29).

Statistical analysis

Data were analyzed for significance using two-tailed Student t test. A p value < 0.05 was considered statistically significant.

Results

Expression of Ssbp2 in HSPCs and hypoplasia of hematopoietic organs in Ssbp2−/− mice

To determine whether Ssbp2 expression was regulated in a differentiation-specific manner, we examined BM cells enriched for HSPC subpopulations for transcript levels. Overall, Ssbp2 expression was higher in the long-term [Flt3−Lin−Sca+c-Kit+ (Flt3−LSK)] and short-term (Flt3−LSK) HSPC populations than in committed progenitor cells (LKs), and expression was lower in the lineage-positive (Lin+) cells than in the committed progenitor cells (Fig. 1A). These findings suggested that Ssbp2 expression was repressed as the cells differentiated. Immunofluorescence staining of whole BM confirmed restricted expression, with expression in ∼5% of all cells (Fig. 1B). Consistent with our previous characterization of the high specificity of anti-SSBP2 Abs (30), no specific signal was detected in Ssbp2−/− BM. Flow cytometry–sorted populations revealed abundant levels of nuclear expression and colocalization with LDB1 in both Flt3−LSKs and Flt3−LSKs (Fig. 1C). These images reflected the relative change in expression shown in Fig. 1A. The localization of SSBP2 to nuclear speckles in LK and Lin− cells was reminiscent of our previous findings with epithelial cells and fibroblasts in which SSBP2 localized to nuclear structures (26, 31). Although the nature of these structures is unknown, our work in progress suggests that non-LDB1-interacting elements also may be present.

To evaluate whether hematopoiesis was affected in the absence of Ssbp2, we first assessed mice 6–8 wk of age for the absolute number of total nucleated cells in the BM, spleen, and thymus. In all three tissues, the mononuclear population was reduced by 30–40% (p < 0.05 for each tissue type), suggesting an overall reduction in hematopoietic activity (Fig. 2A). The absolute number of PBLs was twice as high in WT than in null mice (Fig. 2B, left panel). Although there was a tendency for an increase in granulocytes, the values did not reach statistical significance (Fig. 2B, right panel). Likewise, the absolute number of RBCs and platelets in WT and Ssbp2-null mice were not significantly different (data not shown).

HSPC homeostasis is impaired in Ssbp2−/− mice

The abundant expression of SSBP2 in HSPCs prompted us to determine whether normal HSPC homeostasis was perturbed in the absence of Ssbp2. Accordingly, flow cytometric analyses for stem and early progenitor cell subpopulations were performed with null mice and their WT littermates. Characterization of the LSK population, which is composed of stem and early progenitor cells, revealed no significant difference in frequencies (Fig. 3B). Within the LSK compartment, the frequency of CD34− cells was significantly lower in WT than null mice, suggesting an increase in the ratio of early immature HSPCs (Supplemental Fig. 1B). CD34 and FLT3 expression were used to further subdivide the LSK subpopulation (Fig. 3C). The CD34−FLT3− fraction, which corresponded to LT-HSCs, was twice as high in null than in control mice, whereas there was no significant difference in the ST-HSC or CD34+FLT3− population (Fig. 3E). FLT3 expression is associated with lymphoid priming and increased lymphoid gene expression program. The frequency of CD34−FLT3hi LMPPs was reduced by one-third. Although there was a trend toward fewer LMPPs, the differences were not statistically significant in this small group of mice (WT, n = 4; null, n = 5). Furthermore, there was no compensatory increase in the frequency of CD34−FLT3lo population, suggesting the decrease in FLT3hi expression in flow cytometry was not due to absence of Flt3 upregulation by SSBP2 in the pre-LMPP cells. Likewise, the signaling lymphocyte activation molecule marker CD150 distinguishes LT-HSCs in the larger the LSK population, whereas CD48 expression detects an uncommitted, early progenitor population (32). Once again, the frequency of CD48− cells representing LT-HSCs was increased in the absence of Ssbp2 in contrast to the decreased frequency of CD48+ uncommitted progenitor cells with restricted amplifying potential (Fig. 3D, 3F).

In summary, the frequency of both CD34− cells and FLT3− cells within the LSK compartment increased and that of LSK cells expressing CD34 or FLT3 decreased (Supplemental Figs. 1, 2). That these results would reflect a change in absolute numbers if a larger cohort of mice were used cannot be ruled out. Collectively, these data suggested a seminal role for Ssbp2 in maintaining early HSPC homeostasis.

Delayed hematopoietic recovery of Ssbp2−/− mice after myeloablative treatment

To assess whether loss of Ssbp2 affected the hematopoietic response to the cytotoxic stress we observed, we challenged mice with a single dose of the myeloblative drug 5-FU, which eliminates cycling HSPCs. Ssbp2−/− mice displayed a markedly delayed recovery as reflected in serial peripheral blood leukocyte counts postdrug injection. On day 12, the total percentage of peripheral blood leukocytes was 75 ± 10% in WT mice but only 20 ± 5% in null mice (p < 0.05) (Fig. 4A). The recovery of both myeloid cells and lymphoid cells was impaired; likewise, platelet and RBC recovery were delayed (data not shown). The normal BM cellularity of a WT mouse on day 12 was noticeably altered, with decreased BM cellularity observed in null mice (Fig. 4B). Hypoplasia of both myeloid and erythroid lineages was evident in Ssbp2−/− BM (data not shown).

Weissman et al. (33) demonstrated that early stages of recovery from 5-FU treatment are characterized by a transient expansion of Lin−Sca− HSPCs. Therefore, we examined the BM of null and WT mice by flow cytometry at 48 and 96 h after 5-FU treatment. Unlike the 50% expansion of Lin−Sca− cells seen in the WT mice (from 30 to 46%) between days 2 and 4, Ssbp2−/− mice showed a more modest 12% expansion (from 33 to 36%) (Fig. 4C). These findings suggested a diminished potential for stress-mediated HSPC expansion in the absence of Ssbp2.

These mice were homozygously deleted for Ssbp2 in all tissues, so it was important to examine cell intrinsic versus microenvironmental mechanisms producing these results. To determine whether the defects were intrinsic or extrinsic to HSCs, we transplanted isogenic (CD45.2) WT BM cells into lethally irradiated congenic Ssbp2−/− mice, which had been lethally irradiated to ablate autologous hematopoiesis. The recipients also were challenged with 5-FU after they had been fully engrafted (4 mo after the transplantation), and we found no significant difference in the recovery rate between transplanted null mice and the control mice at that point. Regardless, the poor reconstituting potential of Ssbp2−/− BM prevented us from conducting the reverse experiments (i.e., testing the 5-FU response in WT mice reconstituted with null mouse BM). In a complementary approach, Ssbp2−/− or WT BM cells (CD45.2) were mixed with WT CD45.1 donor BM.
cells at a ratio of 10:1 and transplanted into lethally irradiated WT CD45.1 mice (Fig. 4E) and the recipients challenged with 5-FU after they had been fully engrafted (4 mo after the transplantation). Surprisingly, we found no significant difference in the recovery rate between mice transplanted with Ssbp2<sup>−/−</sup> or WT-enriched BM (Fig. 4E). However, because of technical limitations in monitoring CD45.1 or CD45.2 alleles during recovery, these findings should be interpreted with caution. Overall, these findings are consistent with a contribution from nonhematopoietic cells of the BM niche to the recovery Ssbp2<sup>−/−</sup> HSPCs from cytotoxic stress.

Multilineage repopulating activity of Ssbp2<sup>−/−</sup> BM is severely impaired

Next, we tested the ability of Ssbp2<sup>−/−</sup> BM cells to competitively reconstitute hematopoiesis in lethally irradiated mice. We assessed their relative short- and long-term repopulating potential at 4 and 16 wk by transplanting BM cell mixtures containing different ratios of Ssbp2<sup>−/−</sup> donor to WT competitor cells into lethally irradiated recipients. Initial transplantations at a ratio of 1:1 donor to competitor revealed that WT cells predominated in both BM (7 versus 1%; p < 0.05) and peripheral blood (13 versus 2%; p < 0.05) at 4 wk.
posttransplantation (Fig. 5A). However, when the ratio was increased to 10:1, Ssbp2/2 cells could be detected in ∼50–70% of the recipients (Fig. 5A). Despite the transplantation of a much higher number of Ssbp2/2 than WT cells, the frequency of donor cells in the Ssbp2/2 recipients was still significantly lower than that of WT recipients at 4 wk (72.2 ± 6.7 versus 89.6 ± 6.1%; p < 0.05) and at 16 wk (55 ± 7.5 versus 96 ± 12.2%; p < 0.05), suggesting a diminished potential for short- or long-term reconstruction (Fig. 5B).

We next examined the peripheral blood contribution of Ssbp2/2 cells to specific hematopoietic lineages. Consistent with the decreased bone contribution to BM homeostasis, the peripheral blood cell types, including granulocytes, macrophages, and B and T lymphocytes, was severely compromised (Fig. 5C).

To rule out the possibility that the reduced repopulating capacity of Ssbp2/2 HSCs was due to impaired homing of these cells to BM, we compared the ability of Ssbp2/2 cells (CD45.2) and their WT counterparts to move into the BM of irradiated recipients (CD45.1). At 24 and 48 h posttransplantation, there was no significant difference between the percentage of CD45.2−expressing WT and Ssbp2−/− cells in homing to the BM (24 h: 1.2 versus 1.0%; 48 h: 2.7 versus 2.8%) (Supplemental Fig. 2A, 2B). In addition, we sorted the transplanted cells for Flt3 expression 18 h after transplantation and found no significant difference between WT and null donors (32 versus 29%, Supplemental Fig. 2C).

Taken together, these data demonstrated reduced multilineage repopulating activity in Ssbp2−/− HSPCs.

Abundant expression of b-HLH factor E2a and loss of Notch1 expression in the absence of Ssbp2

To assess whether the impaired HSC activity in Ssbp2−/− mice correlates with an altered gene expression pattern, we evaluated transcript levels of 16 genes encoding known regulators of quiescence and self-renewal in purified HSPCs: Trp53, Myc, p27Kip1, Cdkn1a (p21), Gfi1, Ldb1, Runx1, E2a, HoxB4, Bmi1, Klf10, Ski, Sox4, Trim27, p18Ink4c, and Notch1. Of these, E2a
and Cdkn1A (p21) were expressed at a significantly higher level and Gfi1 at a significantly lower level in null HSPCs than in WT HSPCs (p < 0.05 for each gene). Although high expression levels of E2a- and Cdkn1a-positive regulators of quiescence and a modest decrease in Gfi1 expression a negative regulator of HSC quiescence appear contradictory, the net outcome reflected impaired stress recovery and engraftment defects and suggested perturbation in the transcriptional network overall.

Accumulating evidence has suggested a myeloid leukemia suppressor role for NOTCH1 (24, 25). Therefore, we queried TCGA AML dataset for SSBP2 and NOTCH1 expression as determined by RNA-Seq studies. As shown in Fig. 6C, 21 of 166 identified cases (12.6%) had loss of SSBP2 expression and 27 cases (16.2%) had increased expression. Twenty-four percent of the patients with low SSBP2 expression also had decreased NOTCH1 expression, whereas 9 of the 27 cases (37% of total) with higher SSBP2 expression also had elevated NOTCH1 expression. The coordinated expression of both genes was moderately significant (p = 0.046). Considering the genetic and phenotypic heterogeneity in AML (27), these results, taken together with the absence of Notch1 expression in Ssbp2−/− HSPCs, suggest that this axis is preserved in a subset of AMLs.

**Discussion**

Our studies revealed Sshb2 as a novel positive regulator of HSC homeostasis and recovery from cytotoxic stress. These findings address the consequences of disrupting a critical transcriptional pathway in HSPC homeostasis because SSBPs directly interacting with LDB1 and are present in all of the same transcriptional complexes examined to date. Earlier ChIP-Seq studies established the colocalization of LDB1, SCL1, and GATA2 on the Tal1/SCL1, Gata2, Runx1, Lmo2, E2a, and Myb gene promoters in HSPCs. More important, a feed-forward autoregulatory loop may exist because the expression all these genes except E2A was decreased in the absence of LDB1 (9).

**Abundant SSBP2 expression and colocalization with LDB1 in HSPCs**

High expression of Ssbp2 in undifferentiated stem and progenitor cells, mirroring Ldb1 expression (9), is indicative of a role for Ssbp2 in normal hematopoiesis (Fig. 1A). These findings are in agreement with global expression profiling studies. SSBP2 is frequently on the list of genes preferentially expressed in HSPCs (34–37). Because nuclear localization of Drosophila orthologs (SSDPs) depends on the nuclear localization signal of LDB1 (38), the colocalization with LDB1 raises the possibility of an LDB1-dependent role for SSBP2 in HSPCs (Fig. 1C). Such an interaction suggests that some of the phenotypic consequences of Ssbp2 loss may be Ldb1 dependent. Moreover, the unexpected localization of SSBP2 to nuclear speckles in differentiated cells suggests that SSBP2–LDB1 interactions may be less crucial in undifferentiated HSPCs, mirroring SSBP2 expression in normal hematopoiesis (Fig. 1A). These findings are in agreement with global expression profiling studies. SSBP2 is frequently on the list of genes preferentially expressed in HSPCs (34–37). Because nuclear localization of Drosophila orthologs (SSDPs) depends on the nuclear localization signal of LDB1 (38), the colocalization with LDB1 raises the possibility of an LDB1-dependent role for SSBP2 in HSPCs (Fig. 1C). Such an interaction suggests that some of the phenotypic consequences of Ssbp2 loss may be Ldb1 dependent. Moreover, the unexpected localization of SSBP2 to nuclear speckles in differentiated cells suggests that SSBP2–LDB1 interactions may be less crucial in undifferentiated HSPCs.
(MCF7, HeLa, and A549) and in fibroblasts (IMR90), SSBP2 localizes to punctate structures in both the nucleus and nucleolus (26, 31). In some of these cells, SSBP2 colocalized with the DNA/RNA-binding protein families of ILF2 and EWS (H. Liang et al., manuscript in preparation). Future studies should shed light on the role of LDB1-dependent and -independent SSBP2 pathways in hematopoiesis.

Impaired HSPC homeostasis and stress response in Ssbp2−/− mice

The biological consequences of Ssbp2 ablation, reduced cellularity in multiple hematopoietic tissues (Fig. 2), increased frequency of LT-HSCs, and reduced frequency of LMPPs, underscore the significance of high Ssbp2 expression in HSPCs (Fig. 3). Although the absolute number of HSPC subpopulations was not significantly

![FIGURE 5](image_url)

**FIGURE 5.** Long-term and short-term repopulating ability is decreased in the absence of Ssbp2. (A) Ssbp2−/− BM cells do not reconstitute at a 1:1 donor-to-competitor ratio. Short-term engraftment (4 wk) results as determined with CD45.2-expressing cells in BM and peripheral blood are depicted. Data represent mean ± SD from five recipient mice. (B) Reduced frequency of Ssbp2−/− BM donor cells in peripheral blood of recipients transplanted at a donor-to-competitor ratio of 10:1. Engraftment analyses were performed at 4 wk (short term) and 16 wk (long term) after transplantation. Data represent mean ± SD from one of two experiments each with five mice per genotype. *p < 0.05 as determined by unpaired two-tailed t test between WT and Ssbp2−/− mice. (C) Contribution to all the peripheral blood lineages is compromised in the absence of Ssbp2. Representative analysis showing percent CD45.1 and CD45.2 populations within granulocyte (Gr1+), macrophage (Mac1+), B lymphocyte (B220+), and T lymphocyte (CD3+) in the peripheral blood from Ssbp2−/− BM recipients 16 wk after transplantation.

![FIGURE 6](image_url)

**FIGURE 6.** Gene expression alterations in Ssbp2−/− HSPCs. (A and B) E2a and Cdkn1a transcript levels are increased and Notch1 transcript levels are decreased in the absence of Ssbp2. Quantitative RT-PCR results illustrate differences between Ssbp2−/− and WT counterparts in the expression of HSC quiescence (A) and self-renewal regulators (B) normalized to 18sRNA control. Representative results of triplicates from one of two separate cDNA pools from a group of five mice. *p < 0.05 between paired samples. (C) Tendency toward coordinated SSBP2 and NOTCH1 expression in primary AMLs. The cBIOPortal database was queried for at least 2-fold alteration in expression as determined by RNA-Seq; 166 AML cases were identified. Each column represents a patient. p value at 95% confidence interval is 0.047.

![gene_expression alterations](image_url)
altered, the increased frequency of LT-HSCs and decreased frequency of LMPPs pointed to impediments to normal HSPC differentiation and stress response in the absence of Ssbp2. A larger cohort of mice may better detect a small but biologically significant change in the absolute number of cells.

When cycling HSPCs are eliminated by 5-FU treatment or other hematopoietic stress, quiescent HSCs capable of self-renewal enter the cell cycle rapidly and re-establish the stem cell pool before returning to dormancy (33, 39, 40). The initial proliferative burst of HSCs, which expands the Lin−Sca+ population, was severely compromised in the absence of Ssbp2 (Fig. 4). Whether the HSPC defects are HSC intrinsic or due to a niche contribution or some combination of the two cannot be readily answered by the present studies. These findings, regardless, raise the possibility of a role for Ssbp2 in an interplay between HSC and BM niche because reconstitution with either WT marrow or the microenvironment rescued the delayed recovery from cytotoxic stress. Finally, the possibility that Ssbp2 may be a strain-specific quantitative trait locus in hematopoiesis analogous to neuronal survival (22) cannot be ruled out.

The overall decrease in the short- and long-term reconstituting potential of BM from null mice likewise implicates Ssbp2 in HSPC expansion (Fig. 5). The subtle but significant differences in the LT-HSC and LMPP frequency and pronounced defects in myeloproliferative response, and competitive repopulating activity of knockout BM cells underscore a requirement for Ssbp2 in maintaining normal HSPC integrity.

Aberrant expression of regulatory genes and potential role in AML

Our characterization of aberrant E2a and Notch1 expression in the absence of Ssbp2 raises a number of questions. Both E2a and Notch1 have critical regulatory roles and potentially important roles in HSPC maintenance, and our findings implicated SSBP2 directly or indirectly in modulating E2a and Notch1 transcription. E2a expression, which is first detectable in LT-HSCs, increases steadily as those cells develop into MPPs and LMPPs, then rises dramatically in common lymphoid progenitor cells (41). The enhanced LT-HSC cycling and stem cell exhaustion seen in E2a−/− mice has uncovered a role for E2A in HSC cycling through transcriptional upregulation of CDK inhibitors (42, 43). Elegant serial adoptive transfer studies with compound heterozygotes for E2a and Cdkn1a suggest an absolute requirement for this pathway in maintaining LT-HSCs (44). Furthermore, a global genetic screen identified E2a promoted Cdkn1a expression as a regulator of cell cycle arrest in the absence of cell death in several tumor cell lines (45). Although high expression levels of E2a and Cdkn1a potential positive regulators of quiescence and the modest decrease in G61 expression a negative regulator of HSC quiescence, appear contradictory, the net outcome of Ssbp2 loss was impaired stress recovery and engraftment defects.

In normal BM, Notch1 expression increases modestly from LT-HSC to ST-HSCs. Similar to E2a expression, Notch1 expression increases progressively from LT-HSC to MPPs to LMPPs before rising dramatically in common lymphoid progenitor cells (46). The reduced frequency of LMPPs in Ssbp2−/− BM could potentially be a direct consequence of increased E2a expression because HSPCs and downstream progeny are exquisitely sensitive to E2a dosage (42) and to loss of Notch1 expression, which is critical for LMPP expansion.

Clearly, the resolution of HSPC analyses and gene expression changes in subpopulations presented in this report can be enhanced considerably with the use of larger cohorts of mice and more sensitive signaling lymphocyte activation molecule markers (47). ChIP studies will further elucidate the mechanistic underpinnings of SSBP2-regulated transcriptional network. Regardless, the preliminary findings shown in Fig. 6 identify Ssbp2−/− mice as a novel tool with which to examine the complex E2A-NOTCH1 axis in regulating HSPC integrity.

Human SSBP2 was positionally cloned as a candidate myelodysplasia/AML suppressor gene from chromosome 5q14 (10). Although most patients harbor large deletions, the smallest region of overlap is thought to map to 5q31. However, a recent study with high-density single nucleotide polymorphism arrays demonstrated that larger deletions are associated with poorer outcome and shortened disease-free survival (48). In addition, SSBP2 expression is decreased in primary AML stem cells (49) and cell lines (30) in contrast to normal HSCs. Unlike murine HSCs, which acquire self-renewal abilities without transformation, with HoxB4 overexpression in two primates induced AML in both (50). One of these monkeys harbored retroviral integration in the SSBP2 gene and an associated reduction in expression by 70%. Moreover, inducible expression of SSBP2 in the myelomonocytic cell line U937 resulted in growth arrest and differentiation, although the mechanisms are unknown (30). Although these findings suggest a role for AML suppressor, direct evidence for SSBP2-mediated myeloid leukemia suppression is lacking (50).

AML is genetically diverse (27, 51, 52). The role of NOTCH1 in myeloid differentiation and transformation is controversial and may reflect loss and gain of function roles. Overexpression of HES1, a NOTCH1 downstream target, exacerbates chronic myelogenous leukemia blast crisis in BCR-ABL–induced murine models (53). Furthermore, inactivating NOTCH1 mutations are found in chronic myelomonocytic leukemia. Taken together, loss of SSBP2 and altered NOTCH1 expression may identify a distinct subset of leukemia initiating cells originating HSCs or MPPs, whereas enhanced SSBP2 and NOTCH1 expression may define LMPPs as a target of transformation. The present report is an early step in connecting these intricate networks with HSPC activity and, potentially, AML pathogenesis.

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Disclosures

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References


