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REVIEW

# BLOS2 maintains hematopoietic stem cells in the fetal liver via repressing Notch signaling

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**During development, hematopoietic stem cells (HSCs) undergo a rapid expansion in the fetal liver (FL) after their emergence in the aorta–gonad–mesonephros (AGM) region. We recently reported that the endolysosomal trafficking factor BLOS2, encoded by the *Bloc1s2* gene, regulates HSC/hematopoietic progenitor cell emergence in the AGM region; however, whether it plays a role in the FL remains unknown. Here, we show that BLOS2 plays an essential role in the regulation of HSC proliferation and differentiation in the FL. *Bloc1s2* depletion leads to elevated Notch signaling, with an increased frequency but weakened self-renewal ability of FL HSCs. Functional assays show that *Bloc1s2*$^{-/-}$ FL HSCs harbor impaired lymphoid and myeloid differentiation abilities. These findings reveal that balanced control of Notch signaling by BLOS2 is required for HSC homeostasis during FL hematopoiesis.** 

Hematopoietic stem cells (HSCs) in the mouse fetus first appear at embryonic day 10.5 (E10.5) in the aorta–gonad–mesonephros (AGM) region [1,2]. Then, they migrate to the fetal liver (FL) [3] until bone marrow (BM) is formed; HSCs reside in the BM during adulthood [4]. In contrast to adult hematopoiesis in the BM, embryonic HSCs actively expand in the FL [5]. Although a recent study suggested that rapid maturation of pre-HSCs occurs in the AGM between E9.5 and E11.5 [6], the FL is an ideal niche for a higher frequency of cycling HSCs undergoing expansion and differentiation [5]. However, the mechanisms involved in the balance of expansion and lineage commitment of fetal HSCs are incompletely understood.

Cell cycle status is essential for the regulation of HSC self-renewal and fate commitment [7,8]. Emerging evidence suggests that actively cycling HSCs cause the phenotype of aging [9]. An increase in HSC numbers, combined with blockage of committed and differentiated cell generation, severely impairs hematopoiesis and embryonic lethality [10].

BLOS2 is a subunit of biogenesis of lysosome-related organelles complex-1 (BLOC-1), which has been reported to function in endolysosomal trafficking and biogenesis of lysosome-related organelles [11]. Our previous study described for the first time BLOS2 as a novel negative regulator of Notch signaling through lysosomal trafficking [12]. In the AGM, loss of BLOS2 can enhance HSC emergence by elevating Notch signaling at the expense of myeloid and lymphoid differentiation [12], suggesting that BLOS2 could be an important regulator of HSC development. However, whether it is involved in HSC maintenance in other hematopoietic organs remains unclear. In this article, we investigate the role of BLOS2 in the regulation of HSC proliferation and differentiation in the FL.

## Methods

### *Mice*

The *Bloc1s2* knockout mice (*Bloc1s2*$^{-/-}$) were described previously [12]. The mice were bred at the animal facility of the Institute of Zoology, Chinese Academy of Sciences. This study was approved

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by the Institutional Animal Care and Use Committees at the Institute of Zoology, Chinese Academy of Sciences.

*Flow cytometry*

Single cells were obtained from E13.5 FL. Antibody staining was performed for 30 min at 4°C using antibodies specific to c-Kit-APC, Sca-1-PE, CD45-APC, CD34-PE, CD11b-FITC, CD127-PE-CY7, and TER119-PE-CY7 (eBioscience) using the Lineage-FITC kit (BD Biosciences). Cell sorting was performed on a MoFlo XDP (Beckman Coulter) or Cytoflex (Beckman Coulter) sorter.

*Cell cycle analysis*

Fresh FL cells were stained with antibodies against Sca-1-PE (eBioscience) and c-Kit-APC (eBioscience) using the Lineage-eFluo 450 kit (eBioscience). Cells were subsequently stained with anti-Ki67-FITC or an isotype control FITC conjugate along with 7-amino-actinomycin D (7-AAD) according to the manufacturer's instructions (eBioscience).

*Cell culture*

Fresh FL cells ($6 \times 10^4$) were isolated from E13.5 *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ embryos. The separated cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin, and 1% L-glutamine. In addition, cytokines, including stem cell factor at 50 ng/mL, interleukin-3 at 50 ng/mL, and FLT3 ligands at 50 ng/mL, were added to the medium. The cells were cultured at 37°C for 7 days and used for fluorescence-activated cell sorting (FACS) analysis.

*Transplantation assay*

For the transplantation assay, 0.125 embryo equivalents (ee) of *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL suspension cells (CD45.2) were mixed with $1 \times 10^4$ BM cells (CD45.1) and injected into lethally irradiated recipient mice (CD45.1). For secondary transplantation, $1 \times 10^6$ chimeric BM cells from the primary recipients were transplanted into the secondary recipient mice 16 weeks after primary transplantation.

*Treatment with dibenzazepine*

For dibenzazepine (DBZ) treatment, $6 \times 10^4$ fresh cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL were treated with the Notch pathway inhibitor DBZ (8 μmol/L, SML0649, Sigma-Aldrich) for 4 days. Semi-adherent cells were then carefully harvested, and antibody staining was performed as previously described [13] with antibodies specific to Sca-1-PE (eBioscience) and c-Kit-APC (eBioscience) using the Lineage-eFluo 450 kit (eBioscience).

*Carboxyfluorescein diacetate succinimidyl ester dilution assays*

Equal amounts of Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells (5,000) in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 μmol/L, BioLegend) as previously described [14]. On day 3, cells were harvested, and the CFSE fluorescent staining was analyzed by flow cytometry.

*Quantitative real-time polymerase chain reaction analysis*

Cells were isolated from *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL by FACS, and total RNA was extracted with RNeasy (Qiagen). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using the SYBR qRT-PCR Master Mix (Promega) and the CFX96 Real-Time PCR system (Bio-Rad). GAPDH expression was used for normalization. The PCR primers are listed in Supplementary Table E1 (online only, available at www.exphem.org).

*Colony-forming unit culture assay*

Equal amounts of cells (1,000) in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL were cultured in 24-well dishes filled with methylcellulose semi-solid medium M3434 (STEMCELL Technologies) for 7–10 days. The numbers of myeloid (colony-forming unit granulocytes/macrophage [CFU-GM]), erythroid (burst-forming unit-erythroid [BFU-E]), and mixed (colony-forming unit granulocyte-erythrocyte-monocyte-megakaryocyte [CFU-GEMM]) colonies were counted under a microscope (Nikon, Japan).

*Statistical analysis*

All experiments were performed at least three times. Data are shown as the mean ± standard error of the mean (SEM). Student *t* test was used for statistical comparisons.

## Results and discussion

In mouse E13.5 FL, *Bloc1s2* expression was enriched in LSK cells and especially in common myeloid progenitor cells, but very low in certain mature cells (Lin$^+$), including granulocytes and monocytes (Supplemental Figure E1A, online only, available at www.exphem.org). Based on the expression pattern, we investigated whether *Bloc1s2* deficiency had an impact on definitive hematopoiesis at this stage. E13.5 *Bloc1s2*$^{-/-}$ fetuses had smaller livers compared with *Bloc1s2*$^{+/+}$ fetuses (Supplemental Figure E1B, online only, available at www.exphem.org). However, FACS analysis detected an approximately twofold increase in the frequency of LSK cells in *Bloc1s2*$^{-/-}$ E13.5 FL (Fig. 1A). Moreover, loss of *Bloc1s2* also resulted in a higher frequency of hematopoietic stem/progenitor cells (HSPCs; Lin$^-$c-Kit$^+$CD34$^+$; Fig. 1A). Therefore, BLOS2 loss of function causes an increase in the frequency of HSCs and HSPCs in the FL at E13.5.

To obtain further insight into the increase of HSCs upon loss of *Bloc1s2* in the FL, we tested the cell-cycle status of LSK cells. Ki67/7–AAD staining revealed a significantly higher frequency of cycling LSK cells (S/G2/M) in *Bloc1s2*$^{-/-}$ embryos compared with that in *Bloc1s2*$^{+/+}$ embryos (47.2% in *Bloc1s2*$^{-/-}$ vs. 24% in *Bloc1s2*$^{+/+}$; Supplemental Figures E2A and E2B, online only, available at www.exphem.org). The aberrant proliferation of LSK cells in *Bloc1s2*$^{-/-}$ FL might result from faster division rates as measured by CFSE dilution assay after 72 hours of culture *in vitro* [14] (Supplemental Figure E2C, online only, available at www.exphem.org). qRT-PCR analysis confirmed that LSK cells in *Bloc1s2*$^{-/-}$ FL exhibited increased expression of cell cycle-related genes, which are required for HSC proliferation (Supplemental Figure E2D, online only, available at www.exphem.org). In addition, there was no obvious alteration in the apoptosis status of LSK cells in either *Bloc1s2*$^{+/+}$ or *Bloc1s2*$^{-/-}$ E13.5 FL (Supplemental Figure E2E, online only, available at www.exphem.org). Therefore, loss of *Bloc1s2* drives aberrant proliferation of HSCs. Collectively, these data suggest that BLOS2 plays an important role in maintaining HSC homeostasis in the FL.

These observations prompted us to further examine the repopulation ability and self-renewal potential of *Bloc1s2*$^{-/-}$

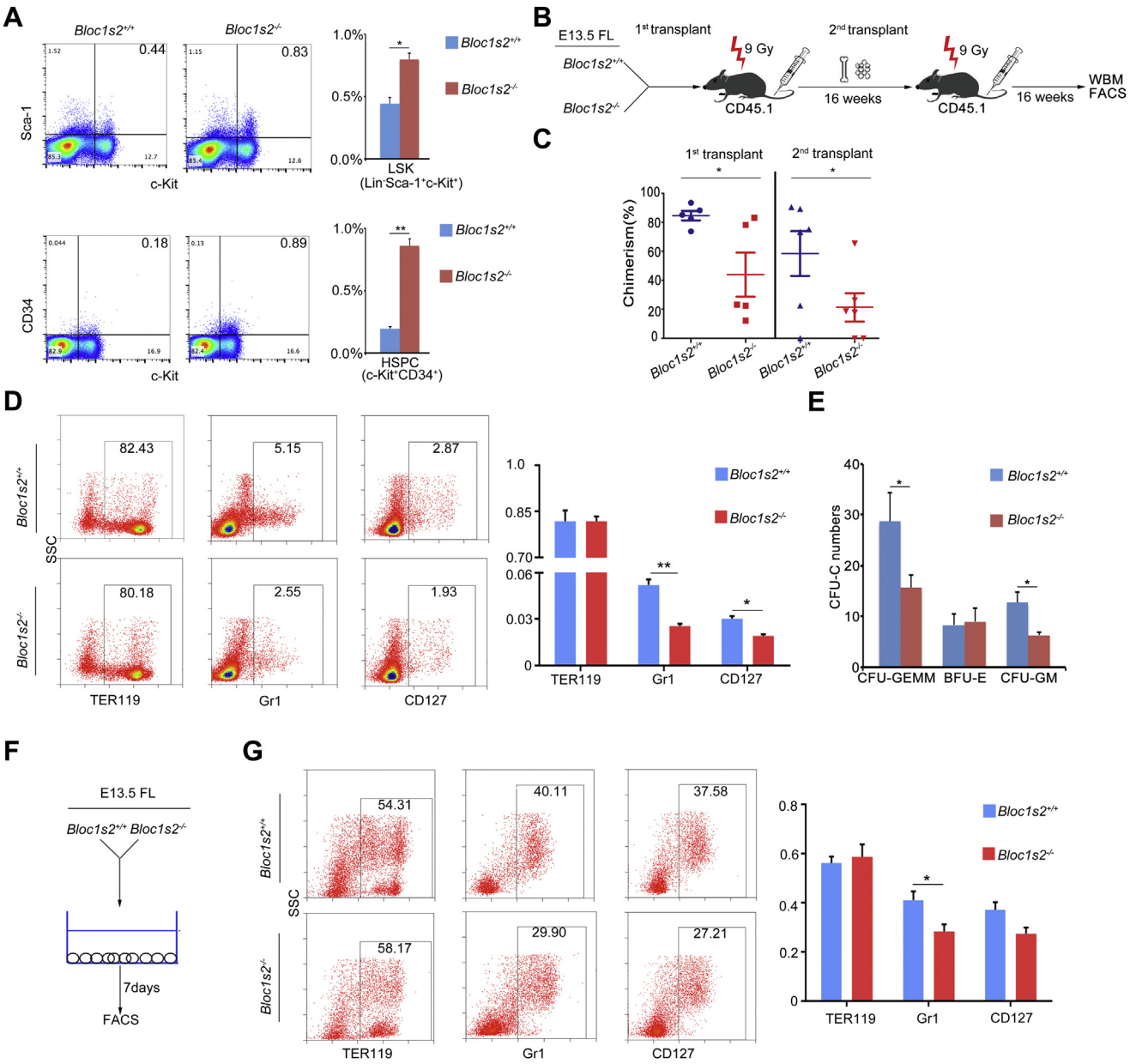


**Figure 1.** BLOS2 deficiency impaired the self-renewal and differentiation of HSCs in the FL. (**A**) FACS analysis of LSK cells and c-Kit$^+$CD34$^+$ cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL. Representative FACS profiles are shown on the left and quantification data on the right ($n = 2$ embryos for each group, 3 independent experiments). (**B**) Experimental design for transplantation with cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL. (**C**) Chimerism of donor-derived cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL after the first and second transplantation assay (for the first transplantation, $n = 5$ mice per group; for the second transplantation, $n = 6$ mice per group). (**D**) FACS analysis of TER119$^+$, Gr1$^+$, and CD127$^+$ cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL. Representative FACS profiles are shown on the left and quantification data on the right ($n = 2$ embryos for each group, three independent experiments). (**E**) CFU assay using equal amounts of LSK cells from *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL ($n = 2$ embryos for each group, three independent experiments). (**F**) Experimental design for the *in vitro* culture with equal amounts of cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL. (**G**) FACS analysis of TER119$^+$, Gr1$^+$, and CD127$^+$ cells using $6 \times 10^4$ cells from *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL after 7 days. Representative FACS profiles are shown on the left and quantification data on the right ($n = 2$ embryos for each group, three independent experiments). All graphs are mean ± SEM. *$p < 0.05$. **$p < 0.01$.

LSK cells by BM transplantation and serial transplantation assays (Fig. 1B). We transplanted 0.125 ee of E13.5 *Bloc1s2*$^{+/+}$ or *Bloc1s2*$^{-/-}$ FL suspension cells (CD45.2) into lethally irradiated CD45.1 recipient mice. This assay revealed that the long-term reconstitution ability of LSK cells in *Bloc1s2*$^{-/-}$ FL was compromised (Fig. 1C). Furthermore, after 16 weeks, secondary transplantation was conducted using $1 \times 10^6$ donor BM nucleated cells (BMNCs; CD45.2) from repopulated primary recipients with $1 \times 10^4$ BMNCs (CD45.1) to promote short-term survival. Sixteen weeks after secondary transplantation, the reconstitution ability was further decreased in the *Bloc1s2*$^{-/-}$ group, indicating a

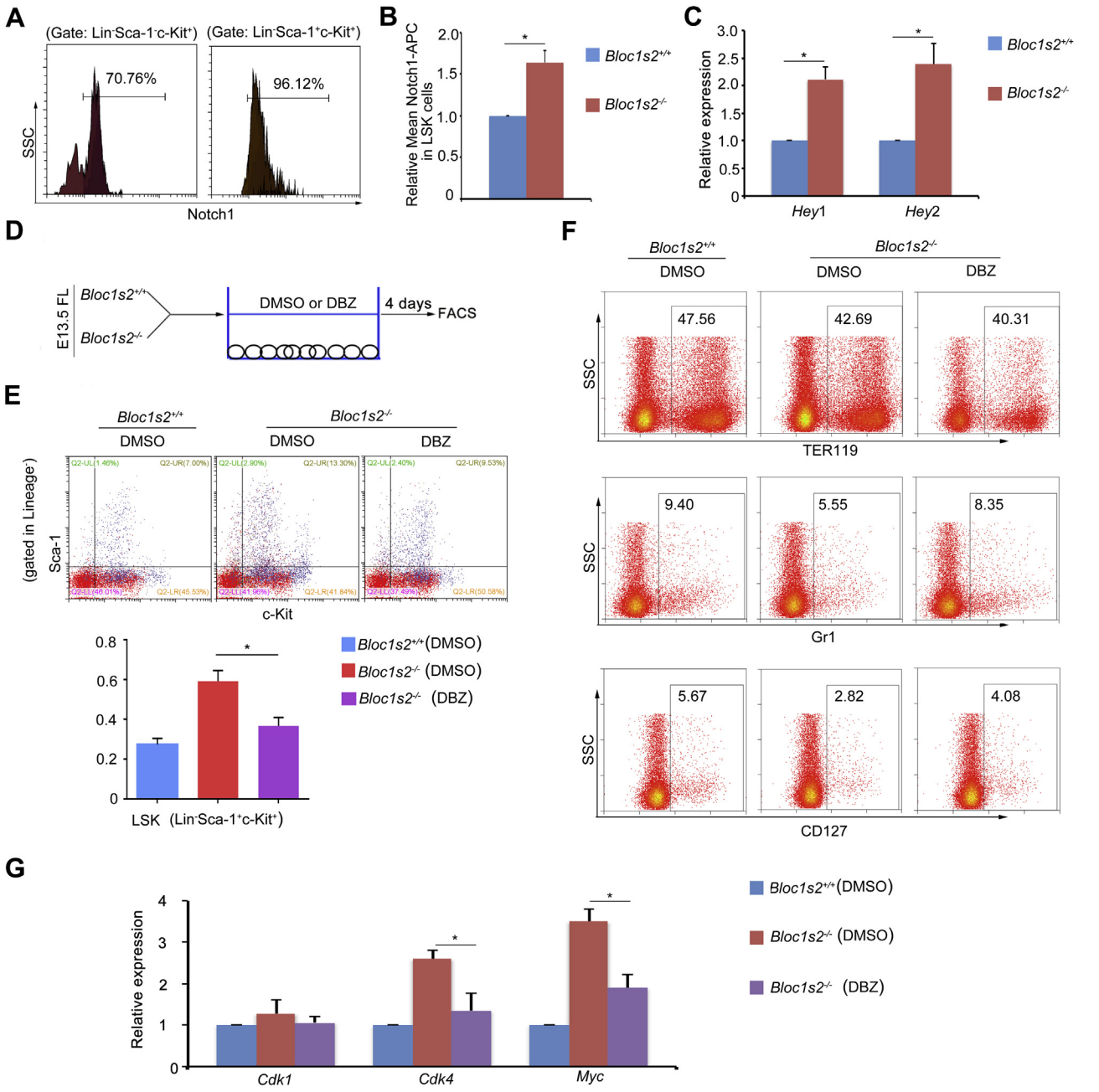


**Figure 2.** Inhibition of Notch signaling rescued the phenotypic HSC expansion in BLOS2 deletion FL. (**A**) Representative FACS profiles of Notch1 expression in LSK cells and $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$ cells from E13.5 FL. (**B**) Quantification of Notch1 expression in sorted LSK cells from *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL ($n = 2$ embryos for each group, three independent experiments). (**C**) qRT-PCR analyses of *Hey1* and *Hey2* in LSK cells from *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL ($n = 2$ embryos for each group, three independent experiments). (**D**) Experimental design for *in vitro* rescue assay by treatment with the Notch inhibitor DBZ in $6 \times 10^4$ cells in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL. (**E**) FACS analysis of LSK cells after treatment with DMSO or DBZ for 4 days. Representative FACS profiles are shown at the top and quantification data at the bottom ($n = 2$ embryos for each group, three independent experiments). (**F**) Representative FACS profiles of $TER119^{+}$, $Gr1^{+}$, and $CD127^{+}$ cells after treatment with DMSO or DBZ for 4 days ($n = 2$ embryos for each group, three independent experiments). (**G**) qRT-PCR analysis of cell cycle-related gene expression in *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ E13.5 FL cells treated with DMSO or DBZ. All graphs are mean ± SEM. *$p < 0.05$. (Color version available online.)

decreased self-renewal potential of the *Bloc1s2*$^{-/-}$ HSCs (Fig. 1C).

To further define the hematopoietic phenotype, the cellular compositions of E13.5 FL in *Bloc1s2*$^{-/-}$ mice were analyzed. There was no obvious alteration in the proportion of $TER119^{+}$ erythrocytes between *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ embryos, but significant reductions were observed in myeloid and lymphoid lineage cells (Fig. 1D).

To investigate whether *Bloc1s2* affects HSC differentiation directly, a correlation readily observed in the *in vivo* analysis, we evaluated the differentiation potential of LSK cells by *in vitro* CFU culture assays. LSK cells sorted from E13.5 *Bloc1s2*$^{+/+}$ FL gave rise to various types of colonies, including CFU-GM, BFU-E, and CFU-GEMM. In contrast, LSK cells sorted from E13.5 *Bloc1s2*$^{-/-}$ FL exhibited a severely reduced number of colonies (Fig. 1E). To further confirm the impaired differentiation ability in *Bloc1s2*$^{-/-}$ mice, $6 \times 10^4$ cells from E13.5 *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ FL were cultured with various hematopoietic cytokines (Fig. 1F). After 7 days, cells were harvested and analyzed for differentiated cells. The occurrence of myeloid and lymphoid cells was decreased sharply in *Bloc1s2*$^{-/-}$ group, whereas the proportion of erythrocytes was unchanged (Fig. 1G). These results suggest that BLOS2 is essential for hematopoietic differentiation.

Our recent study indicated that BLOS2 regulates Notch signaling through lysosomal trafficking to control neural stem cell and HSC development [12]. Notch signaling is essential for embryonic HSC emergence in the AGM [15]. Notch activation is sufficient to facilitate the formation of AGM-derived HSCs [16], whereas several recent studies showed that elevated Notch signaling during the endothelial-to-hematopoietic transition inhibits HSC development in the AGM [17,18]. However, the exact stage-specific requirement for this signaling pathway in the FL remains unclear. We next detected Notch1 expression in FL LSK and hematopoietic progenitor cells. Notch1 was highly enriched in LSK cells compared with Lin$^-$hematopoietic progenitors, indicating that Notch signaling might play a role in LSK cells (Fig. 2A). To further explore the underlying mechanisms of BLOS2-mediated FL HSC homeostasis, we investigated whether Notch signaling was affected in the absence of *Bloc1s2* in the FL. FACS analysis of LSK cells exhibited higher Notch1 expression in *Bloc1s2*$^{-/-}$ LSK cells (Fig. 2B), which is consistent with our previous findings in the AGM [12]. qRT-PCR analysis further confirmed that *Bloc1s2*$^{-/-}$ LSK cells displayed increased expression of Notch target genes (Fig. 2C). To determine whether hyperactivation of Notch signaling in *Bloc1s2*$^{-/-}$ LSK cells accounted for abnormal HSC proliferation and subsequent functional defects, we treated the *Bloc1s2*$^{+/+}$ and *Bloc1s2*$^{-/-}$ FL cells with the small-molecule Notch inhibitor DBZ (Fig. 2D). DBZ treatment partially rescued hyperproliferation of LSK cells (Fig. 2E) and the decrease of myeloid and lymphoid cells (Fig. 2F) and reversed the increased expression of cell-cycle genes (Fig. 2G). These data support that both the phenotypic expansion and differentiation defect of *Bloc1s2*$^{-/-}$ HSCs were attributed to hyperactivation of Notch signaling. Although a recent study showed that Notch signaling is independent of pre-HSC maturation in the AGM region [19], our data suggest that the balanced control of Notch activity by BLOS2 is essential for HSC maintenance in the FL.

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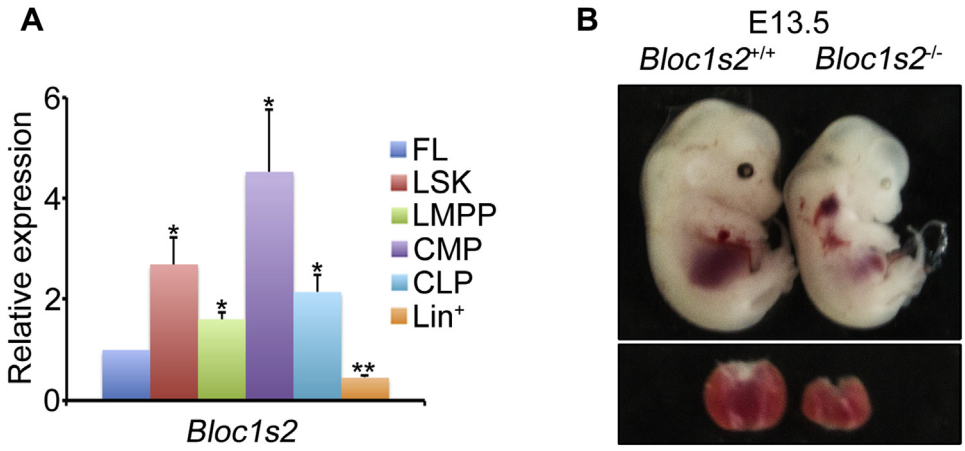
## Conflict of interest disclosure

The authors declare no competing financial interests.

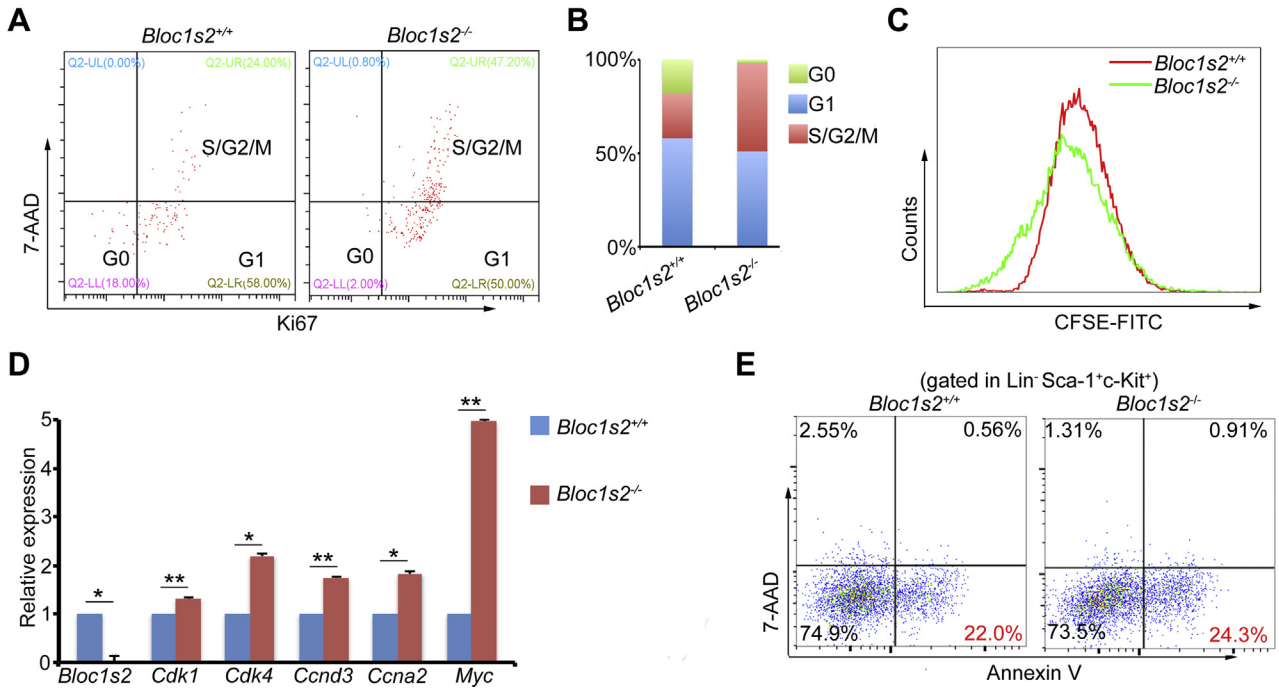
## References

1. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature. 2010;464:108–111.
2. Dzierzak E, Speck NA. Of lineage and legacy: The development of mammalian hematopoietic stem cells. Nat Immunol. 2008;9:129–136.
3. Sugiyama D, Tsuji K. Definitive hematopoiesis from endothelial cells in the mouse embryo: A simple guide. Trends Cardiovasc Med. 2006;16:45–49.
4. Khan JA, Mendelson A, Kunisaki Y, et al. Fetal liver hematopoietic stem cell niches associate with portal vessels. Science. 2016;351:176–180.
5. Zhao Y, Zhou J, Liu D, et al. ATF4 plays a pivotal role in the development of functional hematopoietic stem cells in mouse fetal liver. Blood. 2015;126:2383–2391.
6. Rybtsov S, Ivanovs A, Zhao S, Medvinsky A. Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. Development. 2016;143:1284–1289.
7. Walter D, Lier A, Geiselhart A, et al. Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. Nature. 2015;520:549–552.
8. Karamitros D, Patmanidi AL, Kotantaki P, et al. Geminin deletion increases the number of fetal hematopoietic stem cells by affecting the expression of key transcription factors. Development. 2015;142:70–81.
9. Vincent-Fabert C, Platet N, Vandevelde A, et al. PLZF mutation alters mouse hematopoietic stem cell function and cell cycle progression. Blood. 2016;127:1881–1885.
10. Jones M, Chase J, Brinkmeier M, et al. Ash1l controls quiescence and self-renewal potential in hematopoietic stem cells. J Clin Invest. 2015;125:2007–2020.
11. Starcevic M, Dell'Angelica EC. Identification of snapin and three novel proteins (BLOS1, BLOS2, and BLOS3/reduced pigmentation) as subunits of biogenesis of lysosome-related organelles complex-1 (BLOC-1). J Biol Chem. 2004;279:28393–28401.
12. Zhou W, He Q, Zhang C, et al. BLOS2 negatively regulates Notch signaling during neural and hematopoietic stem and progenitor cell development. Elife. 2016;5:e18108.
13. He Q, Zhang C, Wang L, et al. Inflammatory signaling regulates hematopoietic stem and progenitor cell emergence in vertebrates. Blood. 2015;125:1098–1106.
14. Tario JD Jr, Humphrey K, Bantly AD, Muirhead KA, Moore JS, Wallace PK. Optimized staining and proliferation modeling methods for cell division monitoring using cell tracking dyes. J Vis Exp. 2012;70:e4287.
15. Kumano K, Chiba S, Kunisato A, et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity. 2003;18:699–711.

16. Hadland BK, Varnum-Finney B, Poulos MG, et al. Endothelium and NOTCH specify and amplify aorta-gonad-mesonephros-derived hematopoietic stem cells. J Clin Invest. 2015;125:2032–2045.
17. Tang Y, Bai H, Urs S, Wang Z, Liaw L. Notch1 activation in embryonic VE-cadherin populations selectively blocks hematopoietic stem cell generation and fetal liver hematopoiesis. Transgenic Res. 2013;22:403–410.
18. Zhang P, He Q, Chen D, et al. G protein-coupled receptor 183 facilitates endothelial-to-hematopoietic transition via Notch1 inhibition. Cell Res. 2015;25:1093–1107.
19. Souilhol C, Lendinez JG, Rybtsov S, et al. Developing HSCs become Notch independent by the end of maturation in the AGM region. Blood. 2016;128:1567–1577.




**Supplementary Figure E1.** Bloc1s2 expression in E13.5 FL and comparison of E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ embryos. (**A**) mRNA levels of Bloc1s2 were examined by qRT-PCR and normalized to whole E13.5 FL (n= 2 embryos for each group, 3 independent experiments). All graphs are mean ± s.e.m, *$p<0.05$, **$p<0.01$. (**B**) Photographs of the whole embryo and FL of E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ mice.




**Supplementary Figure E2.** Loss of Bloc1s2 promotes HSC aberrant proliferation in E13.5 FL. (**A**) Representative FACS profiles showing the cell-cycle analysis of E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ LSK cells. (**B**) The percentage of the cell-cycle distribution of E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ LSK cells (n = 2 embryos for each group, 3 independent experiments). (**C**) CFSE dilution assays using equal amount of LSK cells (5000) from Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ E13.5 FL treated with CFSE for 72 hours. (**D**) qRT-PCR analysis of cell-cycle-related gene expression in E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ LSK cells. All graphs are mean ± s.e.m, *$p<0.05$, **$p<0.01$. (**E**) 7-AAD and Annexin V staining of LSK cells from the E13.5 Bloc1s2$^{+/+}$ and Bloc1s2$^{-/-}$ FL to determine the apoptosis status.

**Supplementary Table E1.** The PCR primers used in qRT-PCR assay

| Gene | Primer name | qRT-PCR primer sequence (5′→3′) |
|---|---|---|
| *Hey1* | *Hey1* F | GCGCGGACGAGAATGGAAA |
| | *Hey1* R | TCAGGTGATCCACAGTCATCTG |
| *Hey2* | *Hey2* F | AAGCGCCCTTGTGAGGAAAC |
| | *Hey2* R | GGTAGTTGTCGGTGAATTGGAC |
| *Gapdh* | *Gapdh* F | TCCCACTCTTCCACCTTCGATGC |
| | *Gapdh* R | GGGTCTGGGATGGAAATTGTGAGG |
| *Cdk1* | *Cdk1* F | CTCGGCTCGTTACTCCACT |
| | *Cdk1* R | TCCACTTGGGAAAGGTGTTC |
| *Cdk4* | *Cdk4* F | AACTGATCGGGACATCAAGG |
| | *Cdk4* R | CAGGCCGCTTAGAAACTGAC |
| *Cdkn1a* | *Cdkn1a* F | GTACTTCCTCTGCCCTCTG |
| | *Cdkn1a* R | TCTGCGCTTGGAGTATAGA |
| *Myc* | *Myc* F | TTGGAAACCCCGCAGACAG |
| | *Myc* R | GCTGTACGGAGTCGTAGTCG |
| *Ccnd3* | *Ccnd3* F | CATGGCAGTTGCGGGAGT |
| | *Ccnd3* R | TTTTGACCAAAGCCTGCCG |
| *Ccna2* | *Ccna2* F | GAGAATGTCAACCCCGAAAA |
| | *Ccna2* R | GCAGTGACATGCTATCGTT |