Glutamylation of deubiquitinase BAP1 controls self-renewal of hematopoietic stem cells and hematopoiesis

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All hematopoietic lineages are derived from a limited pool of hematopoietic stem cells (HSCs). Although the mechanisms underlying HSC self-renewal have been extensively studied, little is known about the role of protein glutamylation and deglutamylation in hematopoiesis. Here, we show that carboxypeptidase CCP3 is most highly expressed in BM cells among CCP members. CCP3 deficiency impairs HSC self-renewal and hematopoiesis. Deubiquitinase BAP1 is a substrate for CCP3 in HSCs. BAP1 is glutamylated at Glu651 by TTLL5 and TTLL7, and BAP1-E651A mutation abrogates BAP1 glutamylation. BAP1 glutamylation accelerates its ubiquitination to trigger its degradation. CCP3 can remove glutamylation of BAP1 to promote its stability, which enhances Hoxa1 expression, leading to HSC self-renewal. Bap1E651A mice produce higher numbers of LT-HSCs and peripheral blood cells. Moreover, TTLL5 and TTLL7 deficiencies sustain BAP1 stability to promote HSC self-renewal and hematopoiesis. Therefore, glutamylation and deglutamylation of BAP1 regulate HSC self-renewal and hematopoiesis.

Introduction

Many blood components, including erythrocytes, neutrophils, and megakaryocytes, are short-lived and are constantly regenerated. All hematopoietic lineages are derived from a limited pool of hematopoietic stem cells (HSCs), which represents one of the most canonical adult stem cells (Orkin and Zon, 2008). At the very top of hematopoietic hierarchy is the long-term HSC (LT-HSC). LT-HSCs possess pluripotency to generate all blood cells throughout their lifetime, and they keep predominantly quiescent at G0 phase (Wilson et al., 2008). Oxidative stress (Tothova et al., 2007), infection (Sato et al., 2009), and aging (Flach et al., 2014; Takubo et al., 2010) can activate HSCs to enter the cell cycle and replenish blood cells, but excessive mobilization burdens HSCs and renders them exhausted. LT-HSCs give rise to daughter stem cells through self-renewal, as well as downstream short-term HSCs and multipotent progenitors (MPPs), along with progressively bereaving self-renewal ability (Rossi et al., 2012). Thus, HSCs are essential to guarantee lifelong hematopoiesis.

Protein posttranslational modifications, such as methylation, phosphorylation, ubiquitination, and sumoylation, have been reported to participate in regulating hematopoiesis (Cimmino et al., 2017; Liu et al., 2014; Nakagawa et al., 2015; Zhu et al., 2011). Glutamylation is another posttranslational modification that was initially identified on tubulins (Edd´e et al., 1990). By adding glutamate side chains onto the γ-carboxyl groups of glutamic acid residues of the target proteins, glutamylation alters charge characteristics, protein–protein interaction, stability, and activity of the modified targets. As a reversible process of glutamylation, a group of tubulin tyrosine ligase-like (TTLL) enzymes add the glutamate side chains (Janke et al., 2005), while members of the cytosolic carboxypeptidase (CCP) family of enzymes remove them (Rogowski et al., 2010). Given that TTLLs and CCPs are reversible-modification enzyme members with unique distributions, they might harbor nonredundant roles in the regulation of cellular processes by orchestrating glutamylation and deglutamylation of target proteins (Janke, 2014). Besides glutamylation of tubulin, several other target proteins have been recently identified to be glutamylated (van Dijk et al., 2008). We previously reported that Mad2 can be glutamylated to modulate megakaryocyte maturation (Ye et al., 2014). IL-7Rα is glutamylated to regulate the development of group 3 innate lymphoid cells (ILC3s; Liu et al., 2017a). Cyclic GMP-AMP...
synthase (cGAS) can be glutamylated to inhibit its synthase activity during DNA virus infections (Xia et al., 2016). However, how glutamylation regulates HSC self-renewal is still unclear.

BAP1 is a deubiquitinase that is involved in many cellular processes, including transcription regulation, cell cycle, proliferation, DNA damage, and cell death (Bononi et al., 2017). BAP1 mutations have been reported to be implicated in oncogenesis of several malignancies (Pilarski et al., 2014). Moreover, cancer-derived BAP1 mutations that abolish autodeubiquitination and promote its cytoplasmic sequestration abolish its function as a tumor suppressor (Mashtalir et al., 2014). Cytoplasmic BAP1 can promote its cytoplasmic sequestration abolish its function as a derived BAP1 mutations that abolish autodeubiquitination and BAP1 stability to promote HSC self-renewal and hematopoiesis.

Results

CCP3 deficiency impairs hematopoiesis and HSC self-renewal

We previously demonstrated that CCP6 deficiency in mice causes underdeveloped megakaryocytes and dysfunctional platelets (Ye et al., 2014). To further explore how glutamylation regulated hematopoiesis, we tested expression levels of Cps in mouse bone marrow (BM). We found that only Cps3 was most highly expressed in BM cells (Fig. 1 A). Of note, Cps3-deleted (Cps3−/−) mice displayed splenomegaly and increased spleen weight (Fig. 1 B). Moreover, Cps3−/− mice showed disordered structure of splenic white pulp, suggesting extramedullary hematopoiesis (Fig. S1 A). In addition, Cps3−/− mice decreased cell counts of erythrocytes, myeloid cells, and lymphocytes in peripheral blood (Fig. 1 C, Fig. S1 B, and Table S1). By contrast, CCP4-deficient mice displayed normal spleen and cell numbers of blood lineages we tested (Fig. 1 B and Fig. S1, A and B). Consequently, Cps3−/− mice showed reduced BM cellularity compared with Cps3+/+ mice (Fig. 1 D and Fig. S1 C).

Blood cells can be replenished a short time after being damaged. To determine this regeneration capacity in Cps3−/− mice, we used 5-fluorouracil (5-FU) to eliminate proliferating cells and enforce hematopoietic regeneration. We observed that peripheral white blood cell (WBC) numbers in Cps3−/− mice were dramatically reduced after 5-FU treatment, and they failed to replenish blood cells and BM cells (Fig. S1, D and E). Furthermore, Cps3−/− mice could not survive three rounds of continuous 5-FU treatment, while WT mice could (Fig. S1 F). We used carboxypeptidase inhibitor phenanthroline (Rogowski et al., 2010) to suppress CCP3 activity in WT mice during 5-FU treatment. We noticed that phenanthroline treatment showed phenotypes similar to Cps3−/− mice (Fig. S1, G and H), suggesting CCP3 enzymatic activity is indispensable for hematopoietic regeneration capacity.

HSCs dominate the ultimate source of blood cell regeneration. We found that Cps3 was most highly expressed in HSCs compared with other lineages (Fig. S1 I). Of note, Cps3−/− mice showed decreased total numbers and total frequencies of LSKs (Lin−Sca-1+c-Kit+), MPPs (Lin−Sca-1+c-Kit+CD48−CD150+), and LT-HSCs (Lin−Sca-1+c-Kit+CD48+CD150+; Fig. 1 E and Fig. S1 J). HSCs give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) and further differentiate into mature blood components hierarchically (Xia et al., 2015). Consequently, Cps3−/− BM cells produced fewer colonies compared with those of Cps3+/− littermate control mice (Fig. 1 G). Moreover, Cps3−/− BM cells generated much lower numbers of colonies in secondary replating formation (Fig. 1 H). To determine in vitro differentiation and maintenance ability of Cps3-deficient LT-HSCs, we performed serial plating CFU assays using Cps3−/− and Cps3+/− LT-HSCs. CFU formations of primary plating were comparable between Cps3+/+ and Cps3−/− LT-HSCs (Fig. 1 I). However, Cps3−/− LT-HSCs produced much fewer CFUs in successive plating (Fig. 1 I), suggesting Cps3−/− LT-HSCs disrupt maintenance and self-renewal ability. A single LT-HSC can undergo population expansion for a certain time after being cultured with cytokine cocktail (Himburg et al., 2010). We found that Cps3−/− LT-HSCs lost their expanding and regenerating ability through single-cell culture assay (Fig. 1 J). In addition, phenanthroline treatment impeded WT LT-HSC expansion, and the CCP enzyme agonist CoCl2 (Berezniuk et al., 2012) remarkably increased their expansion. However, with phenanthroline or CoCl2 treatment, expansion abilities of Cps3−/− LT-HSCs were unchanged, suggesting a specific role of CCP3 in the regulation of LT-HSC expansion (Fig. 1 J). Of note, Cps3−/− LT-HSCs failed to be recovered after 5-FU treatment (Fig. S1 L). Finally, we observed that Cps3−/− mice had fewer quiescent LT-HSCs and greater number of cycling HSCs (Fig. 1 K). Of note, Cps3−/− LT-HSCs did not show apparent cell death (Fig. 1 L). Collectively, CCP3 deficiency impairs hematopoiesis and HSC self-renewal.

CCP3 intrinsically regulates HSC self-renewal

We next sought to determine whether CCP3 deficiency-mediated impairment of hematopoiesis was intrinsic or extrinsic. We transplanted CD45.2+ Cps3−/− or Cps3+/− BM cells into lethally irradiated CD45.1+ recipients (Fig. 2 A). Engraftment of Cps3−/− BM cells decreased numbers of peripheral blood cells and BM cells (Fig. 2, B and C). Then we performed LT-HSC transplantation. We found that homing efficiency of Cps3−/− LT-HSCs was comparable to Cps3+/− LT-HSCs (Fig. S2 A). However, Cps3−/− LT-HSC transplantation generated fewer peripheral blood cells and BM cells and failed to maintain LT-HSC self-renewal and hematopoiesis, whereas transplantation of Cps3+/− LT-HSCs could produce normal numbers of peripheral blood cells and BM cells and maintain normal long-term hematopoiesis as well (Fig. 2, D and E; and Fig. S2, B and C).

We also performed competitive BM transplantation assays. We transplanted a 1:1 mixture of CD45.1+ WT and CD45.2+ Cps3−/− or Cps3+/− BM cells into lethally irradiated recipient mice (Fig. 2 F). Engraftment of Cps3−/− BM cells kept long-term
Figure 1. Ccp3 deficiency impairs hematopoiesis and HSC pool. (A) mRNAs of BM cells from Ccp family (Agtpbp1, called Ccp1 here; Agbl2, called Ccp2 here; Agbl3, called Ccp3 here; Agbl4, called Ccp4 here; Agbl5, called Ccp5 here; and Agbl4, called Ccp6 here) were analyzed by quantitative real-time PCR (qPCR). Results were normalized to expression of endogenous Actb gene (n = 5). (B) Photographs of spleens from Ccp3+/+, Ccp3−/−, Ccp4+/+, and Ccp4−/− mice (left) and relative spleen weight (spleen/body) of respective mice (right). n = 6. (C) Peripheral blood cells from Ccp3+/+ and Ccp3−/− mice were counted by flow cytometry. (D) BM cell numbers in a femur of Ccp3+/+ and Ccp3−/− mice were counted (n = 6). (E) Left: LSKs (Lin−Sca-1−c-Kit+), MPPs (Lin−Sca-1−c-Kit+CD48−CD150−), and LT-HSCs (Lin−Sca-1−c-Kit+CD48−CD150+) from Ccp3+/+ and Ccp3−/− mice were analyzed by flow cytometry. Right: Total numbers of indicated cells in a femur were calculated (n = 6). (F) Left: CMP cells (Lin−Sca-1−c-Kit+CD34+CD16/32−) and CLP cells (Lin−CD127+Sca-1lowc-Kitlow) from Ccp3+/+ and Ccp3−/− mice were assayed by flow cytometry. Right: Total numbers of the indicated cells in a femur were calculated (n = 6). (G) CFUs of granulocyte colonies (G), macrophage colonies (M), granulocyte-macrophage colonies (GM), and granulocyte, erythroid, macrophage, and megakaryocyte colonies (GEMM), erythroid colonies (E), and megakaryocyte colonies (Mk) were scored 10 d after plating of Ccp3+/+ and Ccp3−/− BM cells (n = 6). (H) 1×10^4 BM cells from Ccp3+/+ and Ccp3−/− mice were plated for 7-d cultures, and 1×10^4 cells were replated for the next round of cultures. Colonies were counted every 7 d (n = 6). (I) 1×10^4 Ccp3+/+ and Ccp3−/− LT-HSCs were sorted and plated for 7-d cultures, and then 2,000 cells were replated for a second and third round of plating. Colonies were counted every 7 d (n = 6). (J) Ccp3+/+ and Ccp3−/− LT-HSCs were sorted for single-cell culture and counted after 7 d. CCP3 agonist CoCl2 (10 µM) or inhibitor phenanthroline (1 µM) was added, respectively (n = 12). (K) Flow cytometry analysis of cell cycle of Ccp3+/+ and Ccp3−/− LT-HSCs. Percentages of different cell cycle phases were calculated (n = 4). (L) Left: BM cells from Ccp3+/+ and Ccp3−/− mice were stained with PI and Annexin V to analyze cell apoptosis. Right: Percentages of apoptotic cells (PI−, AnnexinV+) were calculated (n = 4). Results are shown as means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-tailed Student’s t test. Data in A–C and G–L are representative of three independent experiments. Data in D–F are pooled from three independent experiments.
Figure 2. **CCP3 is an intrinsic factor to regulate HSC self-renewal.** (A) Schematic diagram of BM transplantation. $1 \times 10^6$ BM cells from $Ccp3^{+/+}$ and $Ccp3^{-/-}$ mice (CD45.2) were transplanted into lethally irradiated CD45.1 mice, followed by analysis 16 wk after BM transplantation. (B) Peripheral WBCs derived from transplanted $Ccp3^{+/+}$ and $Ccp3^{-/-}$ BM cells were counted 16 wk after BM transplantation ($n = 6$). (C) Left: H&E staining of BM sections from femurs of $Ccp3^{+/+}$ and $Ccp3^{-/-}$ BM transplanted recipient mice. Scale bars, 50 µm. Right: BM cells were counted 16 wk after BM transplantation ($n = 6$). (D) $1 \times 10^2$ LT-HSCs from $Ccp3^{+/+}$ and $Ccp3^{-/-}$ mice were sorted and mixed with $5 \times 10^5$ BM helpers (CD45.1+CD45.2+), and then transplanted into lethally irradiated CD45.1 mice. Peripheral WBCs were analyzed by flow cytometry every 4 wk ($n = 5$). (E) Donor-derived BM cells (left) and LT-HSCs (right) were analyzed by flow cytometry 16 wk after LT-HSC transplantation ($n = 5$). (F) Schematic diagram of competitive BM transplantation. $1 \times 10^6$ $Ccp3^{+/+}$ or $Ccp3^{-/-}$ BM cells (CD45.2) were mixed with $1 \times 10^6$ competitors (CD45.1) and cotransplanted into lethally irradiated mice (CD45.1+CD45.2+). Peripheral WBCs were analyzed by flow cytometry every 4 wk. Donor-derived LT-HSCs were analyzed by flow cytometry 16 wk after transplantation. (G) Percentages of peripheral WBCs derived from $Ccp3^{+/+}$ or $Ccp3^{-/-}$ BM (CD45.2) cells and competitors (CD45.1) were analyzed by flow cytometry every 4 wk ($n = 6$). (H) Left: Flow cytometry analysis of LT-HSCs 16 wk after competitive BM transplantation. Right: Ratios of $Ccp3^{+/+}$ or $Ccp3^{-/-}$ LT-HSCs to competitors were calculated ($n = 6$). (I) CCP3-wt or enzymatic inactive CCP3 (CCP3-mut) were overexpressed in $Ccp3^{-/-}$ LT-HSCs (CD45.2) with pMYs retrovirus. $1 \times 10^2$ GFP+ cells were sorted and mixed with $5 \times 10^5$ BM helpers for BM transplantation. Peripheral WBCs were analyzed by flow cytometry 16 wk after transplantation ($n = 5$). Results are shown as means ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Two-tailed Student’s t test. Data are representative of three independent experiments.
balance with their competitors, whereas transplantation of \( \text{Ccp3}^-/- \) BM cells was gradually reduced during competition (Fig. 2 G). 16 wk after transplantation, transplanted \( \text{Ccp3}^-/- \) BM cells had reduced counts of peripheral blood cells and LT-HSCs compared with their WT competitors (Fig. 2, G and H). These results indicate that CCP3 is an intrinsic factor in regulating HSC self-renewal and hematopoiesis.

It has been reported that Glu540-to-Ala mutation abolishes deglutamylation activity of CCP3 (Tort et al., 2014). To examine whether CCP3 deficiency-mediated impairment of hematopoiesis was dependent on CCP3 enzymatic activity, we generated the Glu540-to-Ala mutation construct (CCP3-mut) and overexpressed it into \( \text{Ccp3}^-/- \) LT-HSCs, followed by reconstitution assay (Fig. S2 D). Through BM transplantation assay, we found that engraftment of LT-HSCs with CCP3-wt overexpression could restore hematopoiesis, whereas transplantation of LT-HSCs with CCP3-mut overexpression still abrogated hematopoiesis (Fig. 2 I). These data suggest that enzymatic activity of CCP3 is required for the regulation of hematopoiesis.

**BAP1 is a substrate for CCP3**

To further determine the molecular mechanism of CCP3-mediated hematopoiesis, we analyzed lysates of \( \text{Ccp3}^-/- \) or \( \text{Ccp3}^+/- \) LSK cells by immunoblotting with a glutamylation-specific antibody: GT335. The antibody GT335 specifically recognizes the branch points of glutamate side chains and detects all glutamylation forms of target proteins (Ye et al., 2018). After Western blot analysis, one band around 95 kD appeared in the lane of CCP3-deficient LSK lysates (Fig. 3 A). This band was Western blot analysis, one band around 95 kD appeared in the lane of CCP3-deficient LSK lysates, followed by reconstitution assay (Fig. S2 D). Through BM transplantation assay, we found that engraftment of LT-HSCs with CCP3-wt overexpression could restore hematopoiesis, whereas transplantation of LT-HSCs with CCP3-mut overexpression still abrogated hematopoiesis (Fig. 2 I). These data suggest that enzymatic activity of CCP3 is required for the regulation of hematopoiesis.

**BAP1 is glutamylated at Glu651 by TTLL5 and TTLL7**

Nine glutamylase members of the TTLL family have been recently identified (Garnham et al., 2015). We wanted to explore which TTLL members catalyzed BAPI in HSCs. We tested expression levels of these nine TTLL members in HSCs and mature lineage cells. We observed that \( \text{TTll5} \) and \( \text{TTll7} \) were most highly expressed in LSKs, especially in LT-HSCs (Fig. 4 A). We then expressed mouse TTLL5 and TTLL7 in HEK293T cells and incubated them with recombinant mouse BAPI for in vitro glutamylaton assay. We noticed that BAPI was highly glutamylated by TTLL5 and TTLL7 (Fig. 4, B and C). By contrast, CCP3 could remove the glutamylation of BAPI (Fig. 4, B and C). These results indicate that BAPI is glutamylated by TTLL5 and TTLL7, whose glutamylation is removed by CCP3.

Glutamylation has been found to be modified on the glutamate-rich stretches and acidic environment at the acceptor sites (van Dijk et al., 2008). We analyzed conservative amino acid sequences of BAPI and screened out conserved glutamic acid residues for mutations (Fig. S3 B). Through in vitro glutamylaton assay, we identified that only the Glu651-to-Ala mutation of BAPI (BAPI-E651A) abolished its glutamyla-tion, indicating that BAPI is catalyzed by TTLL5 and TTLL7 at Glu651 (Fig. 4 D and Fig. S3, C and D). We next generated \( \text{TTll5}^-/- \) and \( \text{TTll7}^-/- \)deficient mice, as well as BAPI-E651A knock-in mutant mice via CRISPR-Cas9 technology (Fig. S3, E-G). As expected, we found that glutamylation of BAPI was dramatically reduced in TTLL5- and TTLL7-deficient LSKs, and BAPI failed to undergo glutamylaton in BAPI-E651A knock-in mutant mice (Fig. 4 E). These data confirm that BAPI is glutamylated by TTLL5 and TTLL7 at Glu651.

Of note, we observed that BAPI-E651A mice displayed an increased percentage and number of LT-HSCs and consequently increased numbers of peripheral blood cells and BM cells (Fig. 4, F and G). As expected, more LT-HSCs were kept in G0 phase in BAPI-E651A mice (Fig. 4 H). Through serial LT-HSC transplantation, we noticed that engraftment of BAPI-E651A LT-HSCs could produce more LT-HSCs and peripheral blood cells (Fig. 4, I and J). We conclude that glutamylation negatively regulates BAPI function in the regulation of HSC maintenance and hematopoietic reconstruction ability.

**BAP1 glutamylaton enhances its ubiquitination for degradation**

It has been reported that the ubiquitin-conjugating enzyme UBE2O interacts with BAPI to catalyze its ubiquitination (Mashtalir et al., 2014). We found that BAPI protein level in \( \text{Ccp3}^-/- \) LSKs was markedly lower than that in \( \text{Ccp3}^+/- \) cells (Fig. 5 A). However, BAPI mRNA levels were comparable in \( \text{Ccp3}^-/- \) and \( \text{Ccp3}^+/- \) LSKs (Fig. 5 B). These data suggest that BAPI glutamylaton might facilitate its degradation. With cycloheximide (CHX) treatment, BAPI in \( \text{Ccp3}^-/- \) LSKs was rapidly degraded, whereas BAPI in \( \text{Ccp3}^+/- \) LSKs was more stable (Fig. 5 C). Of note, the proteasome inhibitor MG132 could impede BAPI degradation (Fig. 5 C). These data suggest that BAPI glutamylaton facilitates its degradation. In addition, the interaction between BAPI and UBE2O was confirmed by coimmunoprecipitation assay (Fig. 5 D). Moreover, BAPI glutamylaton enhanced their...
interaction (Fig. 5 D). In parallel, BAP1 glutamylation surely promoted its UBE2O-mediated ubiquitination (Fig. 5 E). Consistently, BAP1 glutamylation only enhanced K48-linked ubiquitination, but not K63-linked ubiquitination (Fig. 5 F). More importantly, K48-linked ubiquitination of BAP1 was much more accumulated in Ccp3−/− LSKs than in Ccp3+/+ BM LSKs (Fig. 5 G).

However, BAP1 in Bap1E651A LSKs was still stable following CHX treatment (Fig. 5 H). Altogether, BAP1 glutamylation promotes its interaction with UBE2O and accelerates K48-linked ubiquitination of BAP1 for degradation.

BAP1 facilitates Hoxa1 expression, which is required for HSC self-renewal

We next conducted transcriptome analysis between Ccp3+/+ and Ccp3−/− LT-HSCs through RNA sequencing. Of differential gene changes of Ccp3+/+ and Ccp3−/− LT-HSCs, 601 genes were downregulated and 532 genes were upregulated (Fig. S4 A). Hematopoiesis and lymphocyte differentiation-related genes were downregulated (Fig. S4 B). Through gene set enrichment analysis (GSEA), HSPC-related gene sets were enriched in Ccp3−/− LT-HSCs, whereas cell cycle–related gene sets were enriched in Ccp3-deficient LT-HSCs (Fig. S4 C). Since Ccp3−/− cells showed reduced BAP1 protein levels, we supposed that some differential genes might overlap between Ccp3−/− HSCs and BAP1-deficient HSCs. In fact, Ccp3−/− HSCs displayed overlapping differential genes with BAP1-deficient LSKs (Fig. S4 C). Transcription factors (TFs) were essential to HSC function, and we found that many TFs were differentially regulated in Ccp3−/− versus Ccp3+/+ LT-HSCs (Fig. 6 A). The top 10 downregulated TFs in Ccp3−/− HSCs were further verified via quantitative PCR (qPCR; Fig. S4 D). We then silenced these 10 TFs in HSCs by shRNAs and followed by single-cell culture assay (Fig. S4 E). We observed that Hoxa1 depletion most significantly impaired HSC expansion (Fig. 6 B). Hoxa1 is a member of the homeobox (Hox) gene encoding TFs, which plays a critical role in development and tumor progression (Bach et al., 2010; Makki and Capecchi, 2010; Wang et al., 2015). Of note, we observed that only Hoxa1 was remarkably downregulated in Ccp3−/− HSCs, while other HoxA genes were unchanged between Ccp3+/+ and Ccp3−/− LT-HSCs (Fig. S4 F). We next wanted to determine how BAP1 regulated Hoxa1 expression. Through chromatin immunoprecipitation (ChIP), we found that BAP1 accumulated onto Hoxa1 promoter (Fig. 6 C). BAP1 overexpression in HSCs facilitated Hoxa1 transcription, whereas overexpression of BAP1 with Hoxa1...
Figure 4. **BAP1 is glutamylated at Glu651 by TTLL5 and TTLL7.** (A) mRNA levels of Ttll family member genes were examined in LT-HSCs, MPPs, LSKs, and lineage positive cells by real-time qPCR. Results were normalized to endogenous Actb gene (n = 4). (B and C) Flag-tagged TTLL5 (B) or TTLL7 (C) and Myc-tagged CCP3 were cotransfected into HEK293T cells for 48 h. Cell lysates were incubated with recombinant GST-BAP1 at 37°C for 2 h, followed by incubating with GST beads at 4°C for 1 h and immunoblotting. Protein glutamylation was tested with GT335 antibody. (D) Flag-tagged TTLL5 was transfected into HEK293T cells for 48 h. Cell lysates were incubated with recombinant WT BAP1 or various indicated mutants at 37°C for 2 h, followed by incubating with GST beads at 4°C for 1 h and Western blotting for the precipitate. Protein glutamylation was examined with GT335 antibody. (E) WT, Ttll5−/−, Ttll7−/−, and Bap1E651A LSK cell lysates were immunoprecipitated with anti-BAP1 antibody, followed by Western blotting with GT335 antibody. (F) Left: Flow cytometry analysis of LT-HSCs in WT and Bap1E651A mice. Right: The percentage of LT-HSCs in BM cells per femur was calculated (n = 6). (G) Cell numbers of peripheral WBCs, BM cells, and LT-HSCs in WT and Bap1E651A mice (n = 6). (H) Cell cycle analysis of WT and Bap1E651A LT-HSCs (n = 5). (I and J) 1 × 10^5 LT-HSCs from WT and Bap1E651A mice (CD45.2) were sorted and mixed with 5 × 10^5 helpers (CD45.1) and then cotransplanted into lethally irradiated (CD45.1) mice. 16 wk after transplantation, 1 × 10^2 LT-HSCs from donors (CD45.2) were sorted and mixed with 5 × 10^4 helpers (CD45.1) for secondary transplantation. Donor-derived peripheral WBCs (CD45.2) were analyzed by flow cytometry every 4 wk (n = 5). Donor-derived LT-HSCs (CD45.2) were analyzed by flow cytometry 16 wk after each transplantation (n = 5). Results are shown as means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-tailed Student’s t test. Data in F–H are pooled from three independent experiments. Data in A–E, I, and J are representative of three independent experiments. IP, immunoprecipitation.
promoter binding region deletion had no such effect (Fig. 6 D and Fig. S4 G). By contrast, BAP1 depletion decreased Hoxa1 expression. Additionally, BAP1 overexpression in Ccp3−/− HSCs rescued Hoxal expression (Fig. 6 E and Fig. S4 H). Consistently, CCP3 deficiency or BAP1 depletion caused the Hoxa1 promoter to be more resistant to DNase I digestion, and BAP1 overexpression

Figure 5. BAP1 glutamylation facilitates its K48-linked ubiquitination for its degradation. (A) 10⁶ LSK cells sorted from Ccp3+/+ and Ccp3−/− mice were probed by Western blotting with anti-BAP1 antibodies. (B) Bap1 mRNA levels in Ccp3+/+ and Ccp3−/− LSKs were detected by real-time qPCR (n = 4). (C) 10⁶ LSKs from Ccp3+/+ and Ccp3−/− mice were treated with CHX (20 µg/ml) and MG132 (10 µM). Left: At different time points, equal amounts of cells were sampled and protein levels were analyzed by Western blotting with anti-BAP1 antibody. Right: Percentages of remaining protein amounts were normalized to the initial WT cell amount and calculated as means ± SD (n = 3). (D) Flag-tagged UBE2O, Myc-tagged BAP1, and HA-tagged TTLL5 vectors were cotransfected into HEK293T cells for 48 h. Cell lysates were incubated with anti-Myc antibody for immunoprecipitation, followed by Western blotting. (E) His-tagged ubiquitin, Myc-tagged BAP1, Flag-tagged UBE2O, and HA-tagged TTLL5 vectors were cotransfected into HEK293T cells for 48 h and assayed as in D. (F) Myc-tagged BAP1, Flag-tagged UBE2O, HA-tagged TTLL5, and His-tagged WT ubiquitin or its mutant form vectors (Ub-K48R or Ub-K63R) were cotransfected into HEK293T cells for 48 h and assayed as in D. (G) 10⁶ Ccp3+/+ and Ccp3−/− LSK cell lysates were incubated with anti-BAP1 antibody for immunoprecipitation, followed by Western blotting with K48-linked specific ubiquitination antibody or GT335 antibody. (H) 10⁶ LSKs from WT and Bap1f565A mice were treated with CHX (20 µg/ml) and MG132 (10 µM). Left: At indicated time points, an equal amount of cells was sampled and protein levels were analyzed by Western blotting with anti-BAP1 antibody. Percentages of remaining protein amounts were normalized to the initial WT cell amount and calculated as means ± SD (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-tailed Student’s t test. Data are representative of three independent experiments. IP, immunoprecipitation.

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Figure 6. BAP1 facilitates Hoxa1 expression that is required for HSC self-renewal. (A) Heat map of upregulated and downregulated (greater than threefold change) expression values of TFs from RNA-seq data of Ccp3+/+ and Ccp3−/− LT-HSCs. The top 10 downregulated TFs in Ccp3−/− LT-HSCs are shown. (B) LT-HSCs were infected with LMP retrovirus carrying respective shRNA, followed by single-cell culture. Cells were calculated 7 d later (n = 8). (C) Enrichment assessment of BAP1 on indicated regions of Hoxa1 promoter in cultured HSCs. Enrichments were detected by qPCR with the indicated primers and normalized to IgG enrichment value (n = 4). (D) LT-HSCs were sorted and infected with lentivirus containing BAP1, Cas9, and the indicated sgRNAs, targeting the BAP1 binding region of Hoxa1 promoter, followed by single-cell culture. 7 d later, partition of cells from each well was performed with PCR and TA clone for DNA sequencing. Cells from the corresponding wells that successfully deleted the indicated region (ΔP#1 or ΔP#2) were collected for qPCR. Hoxa1 expression
values were normalized to endogenous Actb gene (n = 5). (e) LT-HSCs were infected with LMP retrovirus carrying shRNA to knock down BAP1. BAP1 was overexpressed in Cbp3−/− LT-HSCs with pMYS retrovirus, followed by qPCR to detect Hoxa1 expression (n = 4). (f) BAP1 was knocked down in WT LT-HSCs or overexpressed in Cbp3−/− LT-HSCs. 10^6 cells were lysed and nuclei were extracted for DNase I digestion. Undigested DNA was extracted and analyzed by qPCR with Hoxa1 promoter–specific primer. Results were normalized respective to 0 U DNase I–treated samples (n = 4). (g) BAP1-wt or BAP1-mut with Ttll5 or Ttll7 was co-overexpressed in Tll5+/−, Tll7+/− LT-HSCs with pMYS retrovirus, followed by qPCR to detect Hoxa1 expression (n = 4). (h) WT, Cbp3+/−, and Bap1E651A LSKs were probed by Western blotting with the indicated antibodies. (i) Enrichment assessment of BAP1 on the BAP1 binding region of Hoxa1 promoter in WT, Cbp3−/−, and Bap1E651A LSKs, followed by qPCR, and values normalized to IgG enrichment value (n = 4). (j) 10^6 WT, Cbp3−/−, and Bap1E651A LSKs were co-extracted for nuclear extraction and DNase I digestion. Remaining DNA was extracted and analyzed by qPCR with Hoxa1 promoter–specific primer. Results were normalized respective to 0 U DNase I–treated samples (n = 4). (k and l) Hoxa1 was knocked out in LT-HSCs from Cas9 knock-in mice through infection of lentivirus carrying Cre and indicated sgRNA, and then 1 × 10^7 GFP+ LT-HSCs were sorted and cotransplanted with 5 × 10^5 helpers (CD45.1) into lethally irradiated CD45.1 mice. 16 wk after BM transplantation, LT-HSCs were detected by flow cytometry (left), and absolute numbers of BM and peripheral blood cells and could not maintain their self-renewal (Fig. 6, M and N). Transplantation of Hoxa1-overexpressing Cbp3−/− LT-HSCs could restore the normal numbers of HSCs and peripheral blood cells compared with engraftment of WT LT-HSCs (Fig. 6 O and Fig. S4 O). These results indicate that Hoxa1 is required for the maintenance of HSC self-renewal.

**Discussion**

HSCs maintain self-renewal with a sophisticated mechanism to provide lifelong hematopoiesis. However, the mechanism underlying HSC self-renewal is still elusive. In this study, we...
showed that CCP3 is most highly expressed in BM cells among CCP members. CCP3 deficiency impairs HSC self-renewal and hematopoiesis. BAP1 is a substrate for CCP3 in LT-HSCs. BAP1 is catalyzed at Glu651 by TTLL5 and TTLL7, and BAP1-E651A mutation abrogates BAP1 glutamylation. BAP1 glutamylation accelerates its ubiquitination to trigger its degradation. CCP3 can remove glutamylation of BAP1 to promote its stability, which enhances Hoxa1 expression leading to HSC self-renewal. Bap1E651A mice produce higher numbers of LT-HSCs and peripheral blood cells, and Bap1E651A LT-HSCs were more quiescent. Moreover, TTLL5 and TTLL7 deficiencies sustain BAP1 stability to promote HSC self-renewal and hematopoiesis (Fig. S5 C).

Protein glutamylation is catalyzed by a family of polyglutamylases, also called TTLLs (Janke et al., 2005).
well-known substrates of glutamylation are tubulins. Tubulins are glutamylated at their acidic, glutamate-rich C termini, which are the binding sites for most microtubule (MT)-associated proteins (Janke and Bulinski, 2011). Thus, tubulin glutamylation generates functionally divergent MTs by regulating the affinity between MT-associated proteins and MTs (van Dijk et al., 2008). Through regulating MT character, glutamylation is therefore proposed to be involved in MT-associated cellular processes, including stability of centrosomes, motility of cilia and flagella, and neurite outgrowth, as well as neurodegeneration (Bosch Grau et al., 2013; Rogowski et al., 2010). Given that TTLLs have different expression patterns in diverse tissues and show nonredundant functions, more novel substrates of glutamylation need to be defined. We recently reported that TTLL4 and TTLL6 are highly expressed in megakaryocytes and catalyze glutamylation of Mad2 to modulate megakaryocyte maturation (Ye et al., 2014). IL-7Rα can be catalyzed by TTLL4 and TTLL13, and IL-7Ra glutamylation initiates TF Sall3 expression in common helper-like innate lymphoid progenitors, leading to development of ILC3 cells (Liu et al., 2017a). In this study, we demonstrated that TTLL5 and TTLL7 are most highly expressed in HSCs and catalyze BAP1 glutamylation to facilitate its ubiquitylation for degradation, which regulates HSC self-renewal and hematopoiesis.

Protein glutamylation is also a reversible modification, whose glutamylation is removed by a family of carboxypeptidases, also called CCPs (Janke et al., 2005). It has been reported that CCP family members catalyze deglutamylation of tubulins and display enzymatic specificities (Rogowski et al., 2010). We previously showed that CCP6 hydrolyzes Mad2 glutamylation to modulate megakaryocyte maturation (Ye et al., 2014). We also reported that CCP2 is highly expressed in common helper-like innate lymphoid progenitors, the progenitor of ILCs, and catalyzes IL-7Ra deglutamylation, leading to ILC3 development (Liu et al., 2017a). We recently demonstrated that cGAS can be glutamylated by TTLL4 and TTLL6, whose glutamylation can be hydrolyzed by CCP5 and CCP6 (Xia et al., 2016). Glutamylation and deglutamylation of cGAS tightly regulate immune responses to DNA virus infections. Herein, we showed that CCP3 is most highly expressed in BM cells and hydrolyzes BAP1 glutamylation to modulate HSC self-renewal and hematopoiesis. These findings suggest that different tissue and cell type distributions of TTLLs and CCPs exert unique roles in the regulation of different physiological processes.

BAP1 is a member of the ubiquitin C-terminal hydrolase subfamily of deubiquitylating enzymes, which is associated with multiprotein complexes to regulate various cellular processes (Carbone et al., 2013). BAP1 mutations are implicated in various malignancies, including myelodysplastic syndrome patients (Carbone et al., 2013). BAP1 catalytic mutation has been identified in myelodysplastic syndrome patients (Carbone et al., 2013). In addition, BAP1-deficient mice also manifest myeloid transformation (Dey et al., 2012). It has been reported that BAP1 locates at the nucleus and interacts with TTs or multiprotein complexes to regulate transcription initiation and elongation (Carbone et al., 2013; Yu et al., 2010). However, how BAP1 regulates HSC self-renewal and hematopoiesis is still unknown. In actuality, we found elevated enrichment of H2AK19Ub and H3K27me3 on the Hoxal promoter in Cep3+/− LSKs, suggesting that BAPI could indirectly regulate Hoxal expression through effects on H2AK19Ub and other chromatin marks in HSCs. We are still exploring the molecular mechanism by which BAPI glutamylation modulates Hoxal expression in a direct or indirect manner in HSCs. TTLL5- and TTLL7-mediated BAPI glutamylation promotes its interaction with ubiquitin-conjugating enzyme UBE2O to facilitate K48-linked ubiquitination for its degradation. TTLL5 and TTLL7 deficiencies maintain BAPI stability, and stable BAPI further promotes Hoxal expression, leading to enhancement of HSC self-renewal and hematopoiesis. As expected, Hoxal deletion causes reduced numbers of LT-HSCs and peripheral blood cells. In summary, glutamylation and deglutamylation of BAPI play a critical role in the regulation of HSC self-renewal and hematopoiesis.

Materials and methods

Antibodies and reagents

The following commercial antibodies were used: mouse hematopoietic lineage eFluor 450 cocktail (eBioscience; 22-777S), PerCP-Cy5.5-anti-CD45.1 (eBioscience; 45–0453), FITC-anti-CD45.2 (eBioscience; 11–0454), FITC–anti–IL-7Ra (eBioscience; 11–1271), APC–anti-Ly6A/E (Sca-1, eBioscience; 17–5981), PE–anti-CD117 (c-Kit, eBioscience; 12–1171), PE–Cy7–anti-CD16/32 (eBioscience; 25–0161), APC–eFluor 780–anti-CD48 (eBioscience; 47–0481), PE–Cy7–anti-CD150 (eBioscience; 25–1502), eFluor 450–anti-CD3 (eBioscience; 48–0031), PE–anti-CD19 (eBioscience; 12–0193), FITC–anti–Gr-1 (eBioscience; 11–5931), Alexa Fluor 700–anti-CD34 (eBioscience; 56–0341), FITC–anti–Ki-67 (eBioscience; 7B11), and FITC–anti–Annexin V (eBioscience; VQA-33). Anti-CCP3 (I6990–1–AP) antibody was purchased from Proteintech. Anti-CCP4 (T-17), anti-GST (6G9C6), anti-Myc (9E10), anti-Flag (M1), anti-β-actin (SP124), and anti-His (6A7T8) antibodies were bought from Sigma-Aldrich. GT335 antibody was obtained from AdipoGen. BAPI (DIW9B), K48 linkage–specific polyubiquitin (D9D5), ubiquitinyl-histone H2A (Lys119; D271), and tri-methyl-histone H3 (Lys27; C36B11) antibodies were obtained from Cell Signaling Technology. TTLL5 (ARP75440_P050) antibody was obtained from Aviva Systems Biology. TTLL7 (NIN2) antibody was bought from GeneTex. Hoxal (BA3730–2) antibody was bought from Boster Biological Technology. Paraformaldehyde, CHX, MG132, phenanthroline, 5-FU, and DAPI were purchased from Sigma-Aldrich. CoCl2 was from Sinopharm Chemical Reagent. EDTA-free protease inhibitor cocktail and DNase I were purchased from Roche Molecular Biochemicals. West Pico and West Fentmo plus chemiluminescent substrate were purchased from SageBrightness.

Generation of knockout and Bap1ES551A knock-in mice by CRISPR-Cas9 technology

Ccp1- and Ccp6-deficient mice were described previously (Ye et al., 2014). Cep2−, Cep3−, Cep4−, and Cep5-deficient mice were generated using CRISPR-Cas9 approaches as described (Xia et al., 2016). For generation of TTLL5- and TTLL7-deficient mice, vector pSTI374-NLS-flag-linker-Cas9 (Addgene plasmid #44758)
expressing Cas9 and pUC57-sgRNA (Addgene plasmid #51132) expressing small guide RNAs (sgRNAs) targeting Tll15 and Tll17 genes were constructed (Table S2). Mixtures of Cas9 mRNA (100 ng/µl) and sgRNA (50 ng/µl) were microinjected into the cytoplasm of C57BL/6 fertilized eggs, followed by transfer to the uterus of pseudo-pregnant ICR-background female mice, from which viable founder mice were obtained. Genotyping of KO mice with indicated primers (Table S3) was performed as previously described (Zhu et al., 2014). We chose frameshift mutation by PCR screening and TA clone for sequencing and then confirmed knockout efficiency by Western blot. For generation of Bap1E651A mice, the genome locus of Bap1 gene was knocked in with BAP1-E651A mutation via a CRISPR-Cas9 approach (Ye et al., 2018). A mixture of Cas9 mRNA, sgRNA, and BAP1-E651A donor templates was microinjected into the cytoplasm of C57BL/6 fertilized eggs and transferred into the uterus of pseudo-pregnant ICR females. BAP1-E651A mutants were identified by PCR screening and TA clone for DNA sequencing. The gRNA sequence for Bap1E651A was up, 5’-GCCCTTAAGTTA TACAATGT-3’; down, 5’-CAGCTGTCCCTGGGAGTAGA-3’. Gt(ROSA) 26Sortm1(CAG-xstp-xas9, -EGFP) F63b mice were purchased from The Jackson Laboratory (Platt et al., 2014). For deletion of Hoxa1, LT-HSCs were Gt(ROSA)26Sortm1(CAG-xstp-xas9,-EGFP) F63b knock-in mice were sorted and infected with lentivirus containing the indicated sgRNA and Cre recombinase expression. Then infected LT-HSCs were mixed with 5 × 10^5 helper cells and transplanted into lethally irradiated recipient mice (CD45.1). 1 mo later, GFP^+ BM cells were sorted from the recipient mice to confirm Hoxa1 deficiency with Western blot. GFP^+ LT-HSCs were sorted for further transplantation. All the mice we used were C57BL/6 background and ~3 mo old. We used littermates with the same age and gender for each group. We performed three independent experiments of each mouse from at least three mice for each group. Animal use and protocols were approved by the Institutional Animal Care and Use Committees at the Institute of Biophysics, Chinese Academy of Sciences.

**Histology analysis**

Mouse spleens were fixed in 4% paraformaldehyde for 12 h at room temperature. Mouse femurs were fixed in PBS buffer containing 10% formaldehyde for 12 h and then decalcified in decalcifying buffer (10% EDTA in PBS, pH 7.4) for 48 h, changing new decalcifying buffer every 24 h. Fixed tissues were washed twice using 70% ethanol and embedded in paraffin, followed by sectioning and staining with H&E according to standard laboratory procedures.

**Flow cytometry**

BM cells were flushed out from femurs with precooled PBS buffer and sifted through 70-µm cell strainers. RBCs were removed by suspending cells in RBC lysis buffer, followed by washing twice with PBS. BM cells were counted with a blood counting chamber at least three times and then checked again through flow cytometry with a FACSaria III instrument (BD Biosciences). For hematopoietic lineage analysis, 10^7 BM cells were incubated with fluorophore-conjugated antibodies at 4°C for 1 h and then washed twice. LSKs (Lin^− Sca-1^− c-Kit^− ), LT-HSCs (Lin^− Sca-1^− c-Kit^− CD48^+ CD150^− ), short-term HSCs (Lin^− Sca-1^− c-Kit^− CD48^+ CD150^+ ), MPPs (Lin^− Sca-1^− c-Kit^− CD48^− CD150^− ), HSPCs (Lin^− Sca-1^− c-Kit^− ), CMPs (Lin^− Sca-1^− c-Kit^− CD34^+ CD16/32^+ ), and CLPs (Lin^− CD127^-Sca-1^low-c-Kit^low^) were analyzed or sorted with a FACSaria III instrument. Lineage cocktail antibodies contained anti-B220, anti-CD3, anti-TER119, anti-Gr-1, anti-CD11b, anti-CD19, and anti-NK1.1. For peripheral WBC flow cytometric analysis, blood samples were collected through tail veins, and 10 µl of blood was incubated with 200 µl RBC lysis buffer at room temperature for 2 min, followed by washing with PBS twice. T cells (CD3^+ ), B cells (CD19^+ ), and myeloid cells (CD11b^+Gr-1^− ) were analyzed with a FACSaria III instrument. Data were analyzed using the FlowJo 7.6.1 software.

**In vitro colony-forming assay**

4 × 10^4 BM cells from WT or KO mice were mixed with cytokine-supplemented methylcellulose medium (Methocult, M3434; STEMCELL Technologies) and plated in 35-mm tissue-culture dishes. After 10 d of culture at 37°C in 5% CO2, granulocyte colonies, macrophage colonies, granulocyte-macrophage colonies, granulocyte, erythroid, macrophage, and megakaryocyte colonies, erythroid colonies, and megakaryocyte colonies were observed with an inverted microscope and assigned scores (Hou et al., 2015). For continuous replating colony-forming assay, 1 × 10^4 BM cells were initially plated in M3434 medium. Colonies were scored after 7-d cultures, and cells were resuspended and washed with PBS. Then, 1 × 10^4 cells were cultured again for a second or third replating (Moran-Crusio et al., 2011).

**BM transplantation**

BM cells from the indicated mice (CD45.2) were flushed out from femurs with precooled PBS buffer and sifted through 70-µm cell strainers. 1 × 10^6 BM cells were then transplanted into lethally irradiated (10 Gy) recipient mice (CD45.1) through tail veins. The indicated parameters were detected 16 wk after BM transplantation. For LT-HSC transplantation, 1 × 10^2 LT-HSCs were sorted and mixed with 5 × 10^6 BM helpers (CD45.1) and then transplanted into lethally irradiated recipient mice (CD45.1), followed by examination at the times mentioned. For competitive BM transplantation, 1 × 10^6 BM (CD45.2) cells were mixed with 1 × 10^6 BM competitors (CD45.1) to be transplanted into lethally irradiated recipient mice (CD45.1^−CD45.2^+). Peripheral WBCs were detected every 4 wk. LT-HSCs from different donors were identified with anti-CD45.1 and anti-CD45.2 antibodies through flow cytometry 16 wk after transplantation.

**In vitro single LT-HSC culture assay**

In vitro single LT-HSC culture assay was performed as described previously (Rathinam et al., 2011). In brief, we isolated LT-HSCs (Lin^− Sca-1^− c-Kit^− CD48^+ CD150^− ) by flow cytometry and sorted single cells into 96-well plates. Single LT-HSCs were cultured in vitro in the presence of the following recombinant cytokines: mouse stem cell factor (50 ng/ml), mouse thrombopoietin (10 ng/ml), mouse IL-3 (10 ng/ml), mouse IL-6 (10 ng/ml), and human Flt3L (50 ng/ml; all from Peprotech). Cells were cultured in IMDM supplemented with 10% (volume/volume) FCS, 2 mM...
L-glutamine, 1% (volume/volume) penicillin-streptomycin, and 1 mM nonessential amino acids. 3 d later, cells were transferred into 24-well plates with 1 ml fresh medium containing cytokines for further culture. After 7-d cultures, cells were counted with a blood counting chamber or analyzed by flow cytometry.

**Western blot**
10^6 sorted LSKs or cultured HSCs were lysed with RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM EDTA, and 50 mM Tris, pH 8.0, containing protease inhibitor cocktail) at 4°C for 30 min, followed by separation with SDS-PAGE. Samples were then transferred onto nitrocellulose membranes and incubated with primary antibodies in 5% BSA at room temperature for 2 h. After washing with Tris-buffered saline containing 0.1% Tween-20 three times, membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Chemiluminescent signals were generated using West Pico or West Femto plus enhanced chemiluminescent substrate.

**Immunoprecipitation assay**
HEK293T cells were transfected with the indicated plasmids and cultured for 48 h. For endogenic immunoprecipitation, 1 × 10^6 LSKs were sorted or LT-HSCs were cultured for expansion. Cells were lysed with radioimmunoprecipitation assay buffer at 4°C for 1 h. Lysates were incubated with the indicated antibodies for 2 h and immunoprecipitated with protein A/G agarose beads for 1 h, followed by SDS-PAGE separation and immunoblotting.

**Quantitative real-time PCR**
Cell populations were isolated by flow cytometry. Total RNAs were extracted with RNA MiniPrep kit (Tiangen Biotech) according to the manufacturer’s protocol. Then, cDNA was synthesized with M-MLV reverse transcription (Promega). mRNA transcripts were analyzed with the ABI 7300 qPCR system using specific primer pairs as listed in Table S3. Relative expressions were calculated and normalized to endogenous Actb expression.

**Recombinant protein expression**
cDNAs were cloned from a BM cDNA library. CCP3-wt, CCP3-mut, BAPI-wt, and indicated BAPI mutants were cloned into pGEX6p-1 plasmid for GST-tagged protein expression. Plasmids were transformed into *Escherichia coli* strain BL21 (DE3), followed by induction with 0.1 mM isopropyl β-D-1-thio-galactopyranoside at 16°C for 24 h. Cells were collected and lysed by an ultrasonic cell disruption system (Branson), followed by purification with GST resins.

**ChIP assay**
ChIP assay was described previously (Liu et al., 2017b). In brief, 1 × 10^6 LSKs or cultured HSCs were cross-linked with 1% formaldehyde at 37°C for 10 min. Then, cells were washed twice with PBS, lysed, and sonicated to get 300–500-bp DNA fragments. Lysates were incubated with 4 µg anti-BAPI antibody rolling overnight at 4°C. Salmon sperm DNA/protein agarose beads were added for DNA immunoprecipitation. After washing, DNA was eluted from beads and purified. DNA fragments were extracted and analyzed with qPCR. Primers used for ChIP are listed in Table S4.

**RNA interference and gene overexpression**
Target sequences for RNA interference were designed according to MSCV-LTRmiR30-PIG (LMP) system instructions. LMP vectors containing target sequences were constructed. shRNA sequences are listed in Table S5. For gene overexpression, the indicated genes were cloned into pMYs-IRES-GFP (pMYs) retrovirus vectors. LMP or pMYs vectors were cotransfected with packaging plasmid PCL122 into HEK293T cells for 48 h. Media containing virus particles were collected and ultracentrifugated at 25,000 rpm (82,700 g) for 2 h for viral concentration. Pellets were resuspended in IMDM, and viral titers were determined by infecting HEK293T cells with diluted viruses. LT-HSCs were sorted and incubated with viruses in the presence of 8 µg/ml polybrene, followed by centrifuging at 500 g for 2 h. After 36-h culture to allow gene expression, GFP+ cells were sorted for transplantation or further cultured for another 2 d to get enough cells for analyzing gene expression or other experiments as mentioned.

**DNase I accessibility assay**
DNase I digestion assay was performed as described previously (Liu et al., 2017a). In brief, nuclei were purified from 1 × 10^6 LSKs or cultured HSCs, according to the manufacturer’s protocol, with the Nuclei Isolation Kit (Sigma-Aldrich). Then, nuclei were resuspended with DNase I digestion buffer and treated with indicated units of DNase I (Sigma-Aldrich) at 37°C for 5 min. 2× DNase I stop buffer (20 mM Tris, pH 8.0, 4 mM EDTA, and 2 mM EGTA) was added to stop reactions. DNA was extracted and examined by qPCR.

**In vitro glutamylation assay**
Detailed protocol for in vitro glutamylation assay was previously described (Ye et al., 2014). In brief, CCP3, TTLL5, and TTLL7 were transfected into HEK293T cells for 48 h. Cells were harvested and lysed. Supernatants were incubated with GST-BAPI or indicated mutants at 37°C for 2 h. GST-BAPI was precipitated, followed by immunoblotting to detect glutamylation with GT335 antibody.

**Gene expression analysis**
10^4 LT-HSCs (Lin−Sca-1−c-Kit+CD48 CD150−) from Ccp3+/− and Ccp3−/− mice were sorted by flow cytometry. Total RNA was extracted with the RNA MiniPrep Kit and was qualified using Agilent 2100 for the construction of sequencing libraries. Libraries were sequenced on BGISEQ-500 using 50-bp single-end reads. Heatmap.2, ggplot2, and clusterProfiler in Bioconductor were used for generating heatmap, volcano plot, and gene ontology analyzing. GSEA v4.0.1 was used. RNA-seq data have been deposited under GEO accession no. GSE138298.

**Statistical analysis**
For statistical analysis, data were analyzed by Sigma Plot or GraphPad Prism 5.0. Two-tailed unpaired Student’s t test was used in this study. P values < 0.05 were considered significant.
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BAP1 glutamylation regulates HSC self-renewal

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Figure S1. Ccp3 deletion impairs HSC expansion. (A) H&E staining of spleen sections from Ccp3+/+ and Ccp3−/− mice (left) and Ccp4+/+ and Ccp4−/− mice (right). Scale bars, 50 µm. (B) Left: Peripheral blood smears with Wright’s staining of Ccp3+/+ and Ccp3−/− (top) and Ccp4+/+ and Ccp4−/− mice (bottom). Scale bars, 30 µm. Right: T cells (CD3+), B cells (CD19+), and myeloid cells (CD11b+Gr-1+) of peripheral blood from Ccp4+/+ and Ccp4−/− mice were analyzed with flow cytometry (n = 6). (C) H&E staining of BM sections from femurs of Ccp3+/+ and Ccp3−/− mice. Scale bars, 50 µm. (D and E) Ccp3+/+ and Ccp3−/− mice were injected i.p. with 5-FU (150 mg/kg). Peripheral blood cells were counted every 4 d (D), and BM cells were counted 2 wk after 5-FU treatment (E). n = 5. (F) Ccp3+/+ and Ccp3−/− mice were injected i.p. with 5-FU (150 mg/kg) every 7 d for three rounds, and survival rates were calculated (n = 7). (G and H) WT mice were injected i.p. with 5-FU (150 mg/kg) and then with phenanthroline (1.8 mg/kg) or PBS as a control every other day. Peripheral blood cells were counted every 4 d (G), and BM cells were counted 2 wk after 5-FU treatment (H). n = 4. (I) Ccp3 mRNA expression levels in indicated lineages were detected by qPCR. Results were normalized to endogenous Actb gene (n = 4). (J) Flow cytometry gating strategies for LSKs (Lin−Sca-1+c-Kit+), MPPs (Lin−Sca-1+c-Kit+CD48+CD150−), and LT-HSCs (Lin−Sca-1+c-Kit−CD48−CD150−) from Ccp3+/+ and Ccp3−/− mice. Total percentages of LSKs, MPPs, and LT-HSCs in a femur were counted (n = 6). (K) Flow cytometry gating strategies for HSCs (Lin−Sca-1+c-Kit−), CMPs (Lin−Sca-1+c-Kit−CD34+CD16/32−), and CLPs (Lin−CD127−Sca-1lowc-Kitlow; right) from Ccp3+/+ and Ccp3−/− mice. (L) Ccp3+/+ and Ccp3−/− mice were injected i.p. with 5-FU (150 mg/kg). 1 wk later, LT-HSCs were analyzed by flow cytometry (n = 4). Results are shown as means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-tailed Student’s t test. Data in J are pooled from three independent experiments. Data in A–I, K, and L are representative of three independent experiments.
Figure S2. **CCP3 plays an intrinsic role in HSC self-renewal.**

(A) LT-HSC homing assay. 2 × 10^3 LT-HSCs from Ccp3^+/- and Ccp3^-/- mice were sorted and stained with CellTrace violet and CellTrace CFSE, respectively, and then injected into lethally irradiated mice through tail vein injection. Homing of LT-HSCs in BM was analyzed by flow cytometry 18 h after transplantation, and homing efficiencies were calculated (n = 6).

(B) 1 × 10^2 LT-HSCs from Ccp3^+/- or Ccp3^-/- mice were sorted and mixed with 5 × 10^5 BM helpers (CD45.1^+CD45.2^+) and then cotransplanted into lethally irradiated CD45.1^+ mice (n = 5).

(C) Peripheral blood cells were analyzed by flow cytometry every 4 wk. Peripheral myeloid cells (CD11b^+Gr-1^+), T cells (CD3^+), and B cells (CD19^+) derived from Ccp3^+/- and Ccp3^-/- LT-HSCs were further detected.

(D) CCP3-wt or enzymatic inactive CCP3 (CCP3-mut) were overexpressed in Ccp3^-/- LT-HSCs with pMYs retrovirus. GFP^+ cells were sorted and CCP3-wt or CCP3-mut overexpression levels were analyzed by qPCR (n = 4). Results are shown as means ± SD, ***, P < 0.001. Two-tailed Student’s t test. Data are representative of three independent experiments.
Figure S3. **BAP1 is glutamylated at E651 by TTLL5 and TTLL7.** (A) Recombinant CCP3-wt and enzymatic inactive CCP3 (CCP3-mut) were immobilized in Affi-Gel 10 resin and went through BM cell lysates. Eluted fractions were resolved by SDS-PAGE and stained by silver staining. A differential band around 95 kD in the CCP3-mut lane was cut for mass spectrometry. Tandem mass spectrometry profiles of representative BAP1 peptide sequences are shown. (B) Schematic representation of eight putative glutamate-rich sites in BAP1. (C) Amino acid alignment of BAP1 from different species. Conserved sequences are highlighted in yellow. (D) Flag-tagged TTLL7 were transfected into HEK293T cells for 48 h. Cell lysates were incubated with recombinant WT BAP1 or various indicated mutants at 37°C for 2 h, followed by incubating with GST beads at 4°C for 1 h and Western blotting. Protein glutamylation was examined by immunoblotting with GT335 antibody. (E) Diagram of strategy for Ttll5 and Ttll7 knockout generation via CRISPR-Cas9 technology. 5-bp deletions of Ttll5 exon 2 and 1-bp insertion of Ttll7 exon 3, forming frameshift mutation, were identified by PCR and TA clone for DNA sequencing. (F) Western blot to confirm deficiency of TTLL5 and TTLL7. (G) Bap1E651A knock-in mice were generated by CRISPR-Cas9 approach. Glutamine 651 of BAP1 was mutated to alanine (Bap1E651A). Mutant mice were identified by PCR and TA clone for DNA sequencing. Data in A, D, F, and G are representative of three independent experiments.
BAP1 targets on Hoxa1 promoter to enhance Hoxa1 expression. (A) Volcano plot of differentially expressed genes in Ccp3+/- and Ccp3−/− LT-HSCs. Genes whose expression changed more than twofold and whose false-discovery rate q value was <0.05 were selected as differentially expressed genes. (B) GO analysis of differentially expressed genes in Ccp3+/- and Ccp3−/− LT-HSCs. (C) GSEA of genes in Ccp3+/- and Ccp3−/− LT-HSCs enriched in selected gene sets, which were related to HSC and progenitor (first), G1/S transition mitotic cell cycle (second), downregulated genes in BAP1-deficient LSKs (third), and upregulated genes in BAP1-deficient LSKs (fourth). Gene sets related to HSC and progenitor and G1/S transition mitotic cell cycle were from the Molecular Signatures Database. Gene sets related to downregulated and upregulated genes in BAP1-deficient LSKs were based on transcriptomic data from GEO accession no. GSE40541. (D) RNA were extracted from Ccp3+/- and Ccp3−/− LT-HSCs (n = 4), and the top 10 downregulated genes were further analyzed by qPCR. Results were normalized to expression of endogenous Actb gene. (E) The top 10 downregulated genes in Ccp3−/− LT-HSCs were knocked down by LMP microRNA-adapted retroviral system, followed by qPCR. Results were normalized to expression of empty vector infection (n = 4). (F) Genes in Hoxa cluster in Ccp3+/- and Ccp3−/− LT-HSCs were analyzed by qPCR. Results were normalized to expression of empty vector infection (n = 4). (G) Schematic of deletion of BAP1 binding region of Hoxa1 promoter with CRISPR-Cas9 technology. Indicated region deletions were identified by PCR and DNA sequencing. (H) LT-HSCs were infected with LMP retrovirus carrying shRNA against BAP1. BAP1 was overexpressed in Ccp3−/− LT-HSCs with pMYs retrovirus, followed by qPCR. Results were normalized to expression of empty vector infection (n = 4). (I) Enrichment assessment of H2AK119Ub on indicated regions of Hoxa1 promoter in Ccp3+/- and Ccp3−/− LT-HSCs. Enrichments were detected by qPCR with indicated primers and normalized to IgG enrichment value (n = 4). (J) Enrichment assessment of H3K27me3 on indicated regions of Hoxa1 promoter in Ccp3+/- and Ccp3−/− LT-HSCs. Enrichments were detected by qPCR with indicated primers and normalized to IgG enrichment value (n = 4). (K) BAP1-wt or BAP1-mut with TTLL5 or TTLL7 was co-overexpressed in Ttll5−/−; Ttll7−/− LT-HSCs with pMYs retrovirus, followed by qPCR. Results were normalized to expression of empty vector infection (n = 4). (L) sgRNA targeting Hoxa1 was designed according to an online tool and screened for efficiency. sgRNA targeting LacZ was used as a control. LT-HSCs sorted from Cas9 knock-in mice were infected with lentivirus including sgRNA and Cre recombinase expression, followed by cotransplantation with helper cells into lethally irradiated recipient mice. 1 mo later, GFP+ BM cells were sorted to analyze Hoxa1 protein levels through Western blotting. (M) Cell cycle analysis of Hoxa1+/- and Hoxa1−/− LT-HSCs (n = 6). (N) Apoptosis analysis of Hoxa1+/- and Hoxa1−/− LT-HSCs (n = 6). (O) Hoxa1 was overexpressed in Ccp3−/− LT-HSCs, followed by qPCR. Results were normalized to expression of WT LT-HSCs (n = 4). Results are shown as means ± SD. *** P < 0.001 (two-tailed Student’s t test). Data in M and N are pooled from three independent experiments. Data in D–F, H–L, and O are representative of three independent experiments. FDR, false-discovery rate; FC, fold change; NES, normalized enrichment score.
Figure S5. Ttll5 and Ttll7 deficiencies promote HSC self-renewal. (A) LSKs and LT-HSCs from WT, Ttll5−/−, Ttll7−/−, and Ttll5−/−;Ttll7−/− (DKO) mice were detected by flow cytometry, and percentages per femur were calculated (n = 6). (B) Left: Peripheral blood smears with Wright’s staining of WT, Ttll5−/−, Ttll7−/−, and DKO mice. The arrows indicate peripheral white blood cells. Right: Numbers of peripheral blood cells in indicated mice were calculated (n = 6). (C) Model of BAP1 glutamylation in regulating HSC self-renewal. Results are shown as means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two-tailed Student’s t test. Data in A and B are pooled from three independent experiments.

Table S1. Hematopoietic cell counts in peripheral blood of Ccp3+/+ and Ccp3−/− mice

| Parameter | Ccp3+/+ (n = 5) | Ccp3−/− (n = 5) | P value |
|-----------|----------------|----------------|---------|
| WBC (x10⁶/ml) | 22.3 ± 4.5 | 10.0 ± 1.6 | 0.001 |
| Lymph (x10⁶/ml) | 16.6 ± 3.9 | 6.5 ± 2.5 | 0.003 |
| Mon (x10⁶/ml) | 0.7 ± 0.1 | 0.5 ± 0.1 | 0.005 |
| Gran (x10⁶/ml) | 5.0 ± 0.7 | 2.9 ± 1.3 | 0.023 |
| RBC (x10⁹/ml) | 10.2 ± 1.4 | 7.7 ± 1.1 | 0.018 |
| HGB (mg/ml) | 157.0 ± 13.6 | 128.2 ± 19.7 | 0.043 |

Hematopoietic parameters were analyzed using an XFA6030 automated hemocytometer (Slpoo). Cell numbers and percentages were counted for each population. Data are shown as means ± SD. Lymph, lymphoid cells; Mon, monocytes; Gran, granulocyte; HGB, hemoglobin.
| Target gene | sgRNAs |
|-------------|---------|
| Ccp3        | 5’-GGAGTATCAGCTAGGAAGAT-3’ |
| Ccp4        | 5’-GCCTATACCTTCCCCAGCCCC-3’ |
| Tll5        | 5’-GGGATACCAGCTTAGTATTATG-3’ |
| Tll7        | 5’-GCCGGAACAAAGTTTGGAAT-3’ |
| Bap1<sup>E651A</sup>-up | 5’-GCCCTAAGGTATACTG-3’ |
| Bap1<sup>E651A</sup>-down | 5’-CAGCTGTCTTGGGCAATG-3’ |
| Hoxa1       | 5’-ATCCTTGCGAGTGCGAGCTC-3’ |
| LacZ        | 5’-TGCGAATACGCCACGGCT-3’ |

sgRNAs were designed according to an online tool ([http://crispr.mit.edu/](http://crispr.mit.edu/)) and purchased from Sangon. sgRNA targeting LacZ was used as a control.
| Primers       | Sequences                             |
|--------------|---------------------------------------|
| Ccp3 KO (Forward) | 5'-TCAGCTGATTCTATTTGGTACCC-3'         |
| Ccp3 KO (Reverse) | 5'-TGACCTCACAGTGGATGCC-3'             |
| Ccp4 KO (Forward) | 5'-AGGCTGTGTGCTTACCTTATC-3'           |
| Ccp4 KO (Reverse) | 5'-AGAGATCATCAGTGGACTGAAAC-3'         |
| Tdl5 KO (Forward) | 5'-GCCAAGTGAGGTAGGAGA-3'              |
| Tdl5 KO (Reverse) | 5'-GGTCTACACAGATCCCTCT-3'             |
| Tdl7 KO (Forward) | 5'-GTACCAGTTCGTAGCTTTAACC-3'          |
| Tdl7 KO (Reverse) | 5'-CTCCAGAACCCTACACTGCTT-3'           |
| Bap1E651A (Forward) | 5'-CTTGAGTGGAGAGAAGTACTC-3'           |
| Bap1E651A (Reverse) | 5'-ATAGTTGTGGGTCCTTCGCTG-3'           |
| Hoxa1 KO (Forward) | 5'-ATGGAGGAAGTGAGAAAGTTGGC-3'         |
| Hoxa1 KO (Reverse) | 5'-TGGTGGTGGGGCGAGCTGATCTG-3'         |
| Ccp1 qPCR (Forward) | 5'-TGGAAAGCTATCAGCCCTGG-3'            |
| Ccp1 qPCR (Reverse) | 5'-GAGCTGGCGTCTGAGAGATG-3'            |
| Ccp2 qPCR (Forward) | 5'-TCGAGAACCCCGAGAACTCTT-3'           |
| Ccp2 qPCR (Reverse) | 5'-TGCTCCTCTCCCAATCTCTC-3'            |
| Ccp3 qPCR (Forward) | 5'-TGACTTGAGGTAGGAGGATC-3'            |
| Ccp3 qPCR (Reverse) | 5'-GGGAAGAATGGGTCACCAATAG-3'          |
| Ccp4 qPCR (Forward) | 5'-CCACGAGTGCTATCAACTTCCC-3'          |
| Ccp4 qPCR (Reverse) | 5'-TGACTTGAGGTAGGAGGATC-3'            |
| Ccp5 qPCR (Forward) | 5'-CTGCTCATTCTCGTCTCTCAGG-3'          |
| Ccp5 qPCR (Reverse) | 5'-ATCGAGTCTCTCCCAATCTCTC-3'          |
| Ccp6 qPCR (Forward) | 5'-AGGAGCAATGATAACAGA-3'              |
| Ccp6 qPCR (Reverse) | 5'-GGTACCACCTTTCAAGAAGAAG-3'          |
| Bap1 qPCR (Forward) | 5'-CTCCAGGTTGAAAGATTC-3'              |
| Bap1 qPCR (Reverse) | 5'-GAGTGGCAAAAGAGATTGGGAA-3'          |
| Hoxa1 qPCR (Forward) | 5'-CCCTGGAGATGATGTGCCAG-3'            |
| Hoxa1 qPCR (Reverse) | 5'-AGCAACCAGTCTGAGCCACG-3'            |
| Myog qPCR (Forward) | 5'-AGGACATGAGTGCCCCTGACC-3'           |
| Myog qPCR (Reverse) | 5'-AGGCTTTGGAACCAGAGAGC-3'            |
| Nkx2-1 qPCR (Forward) | 5'-GACATATGGAGGACAGAGC-3'             |
| Nkx2-1 qPCR (Reverse) | 5'-CGGCGTCTCCTACTGTTGAA-3'            |
| Aire qPCR (Forward) | 5'-AGCACATGGGACCTTTCGTC-3'            |
| Aire qPCR (Reverse) | 5'-ATAGTGACCTGTCCTCCTT-3'             |
| Pax7 qPCR (Forward) | 5'-TCAAGCCAGAGACAGACCTGT-3'           |
| Pax7 qPCR (Reverse) | 5'-TAGGCTGTCCCGTCTCCAC-3'             |
| Tal2 qPCR (Forward) | 5'-GTCCCAGCTCTCCTAGCAAGA-3'           |
| Tal2 qPCR (Reverse) | 5'-CACCGCTCCCTGGTATTTG-3'             |
| Hey1 qPCR (Forward) | 5'-TAAACGGAACGACTGCTGGA-3'            |
| Hey1 qPCR (Reverse) | 5'-TCGTTGGAGACATGGAACAC-3'            |
| Foxc1 qPCR (Forward) | 5'-AGTGCTGTGTTAAGAGCGAGG-3'           |
| Foxc1 qPCR (Reverse) | 5'-ATGATGGTCCACACCTGGA-3'             |
| Sox18 qPCR (Forward) | 5'-GCTGACACCGCGGTCTCATT-3'            |
| Sox18 qPCR (Reverse) | 5'-TGGCATCTTTAGGCCCACAC-3'            |
| Primers | Sequences |
|---------|-----------|
| Gli2 qPCR (Forward) | 5'-GGTGTTGACCTATTGCGCTGA-3' |
| Gli2 qPCR (Reverse) | 5'-TGCACTATTGATTGCGCTGA-3' |
| 18S (Forward) | 5'-AACCGTTGAAACCCATT-3' |
| 18S (Reverse) | 5'-CATCAAATCGTATAGGGCGC-3' |
| Actb (Forward) | 5'-GGCTGTATTGCCCTATCGC-3' |
| Actb (Reverse) | 5'-GCAAGTGGTTAAGAATCGC-3' |
| Ttll1 qPCR (Forward) | 5'-GAAGTGGGTCACTGACATTGAG-3' |
| Ttll1 qPCR (Reverse) | 5'-ACGTTGCGAATGTCTTGGCA-3' |
| Ttll2 qPCR (Forward) | 5'-GAGTTCACACCCCTGACATTC-3' |
| Ttll2 qPCR (Reverse) | 5'-GCATTGTACCTACCCACGAGT-3' |
| Ttll4 qPCR (Forward) | 5'-TGGATGAGAACCTGAAACCCT-3' |
| Ttll4 qPCR (Reverse) | 5'-TGGGGCTGCTGGAACTAGA-3' |
| Ttll5 qPCR (Forward) | 5'-ACTCCCCAGCTCCCATCTG-3' |
| Ttll5 qPCR (Reverse) | 5'-GGGGCATTGTCAGGAACGG-3' |
| Ttll6 qPCR (Forward) | 5'-CTAACTGCCGGTATGACAGCG-3' |
| Ttll6 qPCR (Reverse) | 5'-AGTAGTCGGTCCAATAGAGAGTC-3' |
| Ttll7 qPCR (Forward) | 5'-CTCTGCCTCAAGATGGGGTTA-3' |
| Ttll7 qPCR (Reverse) | 5'-GTTCCGGCAACATTAGCTGTAA-3' |
| Ttll9 qPCR (Forward) | 5'-TGGAGTGTCGAAAGGAAAAGA-3' |
| Ttll9 qPCR (Reverse) | 5'-TGCTCATCCATGTAGGTGTGG-3' |
| Ttll11 qPCR (Forward) | 5'-CCTGACCAACTACTCCCTGAA-3' |
| Ttll11 qPCR (Reverse) | 5'-GGGATGTCTGACTGGTAGAAAAC-3' |
| Ttll13 qPCR (Forward) | 5'-GGCCTGAAGGAAGTAGGGGA-3' |
| Ttll13 qPCR (Reverse) | 5'-CATGCCAGGGAAGTGGTTGA-3' |
| Hoxa2 qPCR (Forward) | 5'-TCAGAATTTGAGCGAGAGATTGG-3' |
| Hoxa2 qPCR (Reverse) | 5'-GTCGAGGTCTTGATTGATGAACT-3' |
| Hoxa3 qPCR (Forward) | 5'-TGAGCGATCTACGGTGGCTA-3' |
| Hoxa3 qPCR (Reverse) | 5'-GAGGCAAAGGTGGTTCACCC-3' |
| Hoxa4 qPCR (Forward) | 5'-GAAAGCACAAACTCACAGCCC-3' |
| Hoxa4 qPCR (Reverse) | 5'-GTCTCGGGTTTACTTAGGGAAG-3' |
| Hoxa5 qPCR (Forward) | 5'-CTCATTTTGCGGCTGCAACTCC-3' |
| Hoxa5 qPCR (Reverse) | 5'-CTCCATGCGATTACGCGTGA-3' |
| Hoxa6 qPCR (Forward) | 5'-CAGCGAATCTACGGTGGCTA-3' |
| Hoxa6 qPCR (Reverse) | 5'-GAGGCAAAGGTGGTTCACCC-3' |
| Hoxa7 qPCR (Forward) | 5'-GACCCTGAGTTTGCTGACTGCT-3' |
| Hoxa7 qPCR (Reverse) | 5'-ACGTTTCTCCACTTGCTC-3' |
| Hoxa9 qPCR (Forward) | 5'-GGGCCATTGAGGCTAAGCTG-3' |
| Hoxa9 qPCR (Reverse) | 5'-ACAAAGTGCTGTGCAAGCGC-3' |
| Hoxa10 qPCR (Forward) | 5'-GGCTAGCTTTGCGATGCCGTA-3' |
| Hoxa10 qPCR (Reverse) | 5'-GCTGGTGGTCTCGTGAAGGG-3' |
| Hoxa11 qPCR (Forward) | 5'-CTCAGCTTGGGGGCTGAAAG-3' |
| Hoxa11 qPCR (Reverse) | 5'-GGCTGATGGCGTACTTCTC-3' |
| Hoxa13 qPCR (Forward) | 5'-GGAAAGCTATCGGCGCTG-3' |
Table S3. Sequences of primers used for genotyping and qPCR (Continued)

| Primers                | Sequences                                               |
|------------------------|---------------------------------------------------------|
| Hoxa13 qPCR (Reverse)  | 5’-GAGCTGGGCTCTGAGGATG-3’                              |

Primers were designed with Primer 5 and purchased from Sangon.

Table S4. Sequences of primers used in chIP assays

| Loci                  | Sequences                                               |
|-----------------------|---------------------------------------------------------|
| -200 ~ 0 (Forward)    | 5’-TCCAAGTCAGCTCCGGG-3’                                |
| -200 ~ 0 (Reverse)    | 5’-GAATGTACAGTGGCGAAGAAG-3’                            |
| -400 ~ -200 (Forward) | 5’-AGAGATTTCGGCCCAACAGA-3’                             |
| -400 ~ -200 (Reverse) | 5’-GCCTCAGTTGGAGCCTGGG-3’                              |
| -600 ~ -400 (Forward) | 5’-TCTGGGCAAGTCCCTCTA-3’                               |
| -600 ~ -400 (Reverse) | 5’-GCGGAAATCTCTTCCTGGG-3’                              |
| -800 ~ -600 (Forward) | 5’-TTCCCAAGAGCTGGGTTCTGTA-3’                           |
| -800 ~ -600 (Reverse) | 5’-GGACGTGGCCAGAGATTTGA-3’                             |
| -1000 ~ -800 (Forward)| 5’-AGCATGCTCTGGGCTCTCTTA-3’                            |
| -1000 ~ -800 (Reverse)| 5’-GAACCCAGCTCTCTGGGAA-3’                             |
| -1200 ~ -1000 (Forward)| 5’-TGCTCCCTCCACCTGCG-3’                              |
| -1200 ~ -1000 (Reverse)| 5’-GCCGACCACCTGCACCTTGA-3’                            |
| -1400 ~ -1200 (Forward)| 5’-CCAAGCTTAGATGGTATCAG-3’                             |
| -1400 ~ -1200 (Reverse)| 5’-GGTGTTGGACCUGCAG-3’                                |
| -1600 ~ -1400 (Forward)| 5’-TGCTGCTCTCTGGGCTCTCT-3’                            |
| -1600 ~ -1400 (Reverse)| 5’-AGATGTACAGTCACTCTAAGTGC-3’                         |
| -1800 ~ -1600 (Forward)| 5’-TCACTCCCCGAAATGCTGGT-3’                            |
| -1800 ~ -1600 (Reverse)| 5’-TCATAGGAGGGAAGGAGGACACTGC-3’                       |
| -2000 ~ -1800 (Forward)| 5’-GAGAGAGCACCCTAACCCG-3’                             |
| -2000 ~ -1800 (Reverse)| 5’-ACCCACTCTTCCGGGGAGTGATAT-3’                        |

Primers were designed with Primer 5 and purchased from Sangon.
| shRNA target gene | Sequences |
|------------------|-----------|
| Myog #1          | 5’-AGGAATTAGCTGACTCCTTAA-3' |
| Hoxa1 #1         | 5’-CGGCCCTGGCCACGTATAAATAA-3' |
| Hoxa1 #2         | 5’-AGCCACGTATAATAACTCCTTAA-3' |
| Nkx1-1 #1        | 5’-CCGTTCCACAGAAGAGAAAT-3' |
| Nkx1-1 #2        | 5’-GCCATGTCCCAGAACAAGCAT-3' |
| Aire #1          | 5’-CCCTTCTCTCTTGGAGAAGAAAT-3' |
| Aire #2          | 5’-CGACCTGGAGTCCCTCCTCAA-3' |
| Pax7 #1          | 5’-CGTCCAGGCTGTCTGTTGTAAC-3' |
| Pax7 #2          | 5’-GGCTCTTCAAGGCTGTGACAA-3' |
| Tal1 #1          | 5’-CGGACAACCTGCTCTGACATA-3' |
| Tal1 #2          | 5’-CCCTGTCTTCTGCTCTTGTTAT-3' |
| Hey1 #1          | 5’-ACGACGAGACCGAATCTCAA-3' |
| Hey1 #2          | 5’-CCGCCACTATGCTCAAATGTTAA-3' |
| Foxc1 #1         | 5’-CGGCCCTCCTCCTAGTAAAT-3' |
| Foxc1 #2         | 5’-CCCTATATGCTGAACTTTTA-3' |
| Sox18 #1         | 5’-ACGAATTGAGCGATCTGCTCAA-3' |
| Gli2 #1          | 5’-GGCCACAAAACCCCTCTGACTA-3' |
| Gli2 #2          | 5’-GGCCAGATACGAGATATAAT-3' |
| Bap1 #1          | 5’-CCGTCTGTGATGATGATGATA-3' |
| Bap1 #2          | 5’-CCACGTACCTTCTGAGAAA-3' |

Target sequences for RNA interference were designed according to MSCV-LTRmiR30-PIG system instructions.