Pax transactivation domain-interacting protein is required for preserving hematopoietic stem cell quiescence via regulating lysosomal activity

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Abstract

Hematopoietic stem cells (HSC) maintain lifetime whole blood hematopoiesis through self-renewal and differentiation. In order to sustain HSC stemness, most HSC reside in a quiescence state, which is affected by diverse cellular stress and intracellular signal transduction. How HSC accommodate those challenges to preserve lifetime capacity remains elusive. Here we show that Pax transactivation domain-interacting protein (PTIP) is required for preserving HSC quiescence via regulating lysosomal activity. Using a genetic knockout mouse model to specifically delete Ptip in HSC, we find that loss of Ptip promotes HSC exiting quiescence, and results in functional exhaustion of HSC. Mechanistically, Ptip loss increases lysosomal degradative activity of HSC. Restraining lysosomal activity restores the quiescence and repopulation potency of *Ptip*$^{-/-}$ HSC. Additionally, PTIP interacts with SMAD2/3 and mediates transforming growth factor-β signaling-induced HSC quiescence. Overall, our work uncovers a key role of PTIP in sustaining HSC quiescence via regulating lysosomal activity.

Introduction

Hematopoietic stem cells (HSC) maintain life-long blood homeostasis through self-renewal and differentiation into all lineages of blood cells. In order to sustain their stemness property for a lengthy period of time, HSC need to reside in a quiescent state.[1] Under stress or upon perturbations, HSC are activated and exit quiescence to a cycling state. HSC activation is also accompanied by alterations in multiple aspects, such as metabolism and protein synthesis. Thus, a fundamental question in this field is how HSC preserve their quiescence. Recent studies demonstrate that HSC are sensitive to metabolic perturbations.[2-10] HSC in quiescence are thought to require the minimal metabolic activity that is met by glycolysis.[10, 11] Lysosomes are recognized as centers for degradation and clearance in the cell but also as recycling centers that provide a reservoir of nutrients.[12] Lysosomes contain over 60 hydrolases, and macromolecules delivered to lysosomes, such as lipids, carbohydrates, proteins, and nucleic acids as well as defective organelles, are digested by these acid hydrolases into the fundamental units, which can be reused in biosynthesis.[12] Recent studies identified that lysosomes are critical in balancing HSC quiescence *versus* activation by regulating HSC metabolism.[13-16] Lysosomes are abundant and large in quiescent HSC, indicating the buildup of undigested materials; conversely, they are few, small and highly active in activated HSC. Repression of lysosomal activity in HSC enlarges lysosomes, suppresses glucose uptake, reverts activated HSC to quiescence, and enhances the competitive repopulation ability of primed HSC by over 90-fold *in vivo*.[13] These works demonstrate that lysosomal function in quiescent HSC is key to their stem cell capacity.[13,14,16,17] However, how the lysosomal function is regulated remains elusive.

The transforming growth factor-β (TGFβ) family of cytokines constitutes a multifunctional signaling circuitry, and plays pivotal functions in regulating cell fate and behavior

in all tissues of the body.[18] TGFβ signaling maintains a pool of quiescent HSC.[19] Neutralization of TGFβ *in vitro* releases early hematopoietic stem progenitor cells (HSPC) from quiescence,[20-22] which is mediated by upregulation of cyclin-dependent kinase inhibitors, such as $p57^{Kip2}$. Several other mechanisms may also account for TGFβ-mediated HSC quiescence. Our previous study indicates that elevated TGFβ signaling contributes to bone marrow (BM) failure in Fanconi anemia (FA) by impairing HSC function. Inhibition of TGFβ signaling improves the survival of FA cells and rescues the proliferative and functional defects of HSC derived from FA mice and FA patients.[23] However, the underlying mechanisms of how TGFβ signaling regulates HSC quiescence still needs to be explored.

Pax transactivation domain-interacting protein (PTIP) is a unique subunit for the MLL3 and MLL4 complexes. PTIP is essential for thymocyte development, humoral immunity and class-switch recombination of B lymphocytes.[24-26] Our recent work indicates that PTIP governs NAD^+ metabolism by regulating CD38 expression to drive macrophage inflammation.[27] PTIP is required to maintain the integrity of the BM niche by promoting osteoclast differentiation.[28] However, the role of PTIP in HSC is unknown. Here we uncover a key function of PTIP in coordinating TGFβ signaling to regulate lysosomal activity and sustain HSC quiescence.

Methods

Mice

C57BL/6J (CD45.2) background Ptipflox/flox mice were obtained from Biocytogen. Scl-CreER mice were a gift provided by Dr. Junke Zheng's group from Shanghai Jiaotong University. For induction of Cre-ER recombinase, mice were administered tamoxifen by intraperitoneal injection. All experimental mice were a mix of male and female 6-10-week-old mice. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Medical Research Institute, Wuhan University.

Flow cytometry analysis and sorting

Total BM cells were isolated from mice's femur and tibia. HSC were stained with biotin-conjugated lineage markers, then stained with APC-eFlour780-anti-streptavidin, PE-anti-c-Kit, APC-anti-Sca-1, PE-Cy5-anti-CD135, PE-Cy7-anti-CD48, FITC-CD150, PE-CF594-anti-CD41. BD sorters FACSAria III; BD analyzers FACSCelesta, FACSLSRFortessaX20, and Beckman CytoFlex were used. Experimental details are provided in the *Online Supplementary Appendix*.

Colony formation assay

Sorted mouse HSC were plated into methylcellulose medium (M3434) according to the manufacturer's protocols. Colonies were scored after 10-12 days. Where indicated, media were supplemented with 100 μM leupeptin (Leu) and 5 ng/mL TGFβ1.

Mouse competitive reconstitution analysis

$5x10^5$ donor BM cells obtained from 8-12-week-old donor *Ptip*$^{-/-}$ or wild-type (WT) mice, were mixed with $5x10^5$ competitor cells from CD45.1 mice, and transplanted into lethally irradiated (10 Gy) CD45.1 recipients followed by an analysis of repopulation and multiple lineages of donor-derived cells at 4, 8, 12, 16 weeks after transplantation. After 16 weeks, $1x10^6$ donor-derived BM cells obtained from the primary recipient mice along with the same number of competitor cells were transplanted into lethally irradiated (10 Gy) secondary CD45.1 recipient mice.

Immunofluorescence

Sorted $Lineage^-Sca1^+c\text{-}Kit^+$ (LSK) cells were fixed with 4% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), then permeabilized in PBS + 0.5% Triton X-100 for 15 minutes, and blocked for 1 hour (h) in 1% bovine serum albumin (BSA). Fixed and permeabilized cells were then incubated with primary antibodies in PBS + 1% BSA overnight at 4°C; washed and stained with fluorescence-conjugated secondary antibodies for 1 h at room temperature (RT); washed slides were sealed with a mounting medium with DAPI; single cell images were captured by a Zeiss LMS880 Airyscan confocal microscope using a 63/100 X objective and analyzed with ImageJ/Fiji.

Western blotting

Cells were lysed in RIPA and loaded per lane onto SDS PAGE gels. After transfer, nitrocellulose membranes were blocked and incubated overnight at 4°C with the primary antibody. After washes in Tris-buffered saline with Tween 20, membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies, then washed and subsequently incubated with ECL Western Blotting Substrate (Bio-Rad), exposure with X-Ray Super RX Films (Fujifilm).

Statistical analyses

The experiment data were analyzed using two-way Student's *t*-test. For the comparison of different specimens, the unpaired *t*-test was used. Asterisks indicate $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$.

Results

Loss of *Ptip* results in hematopoietic stem cell activation

We first examined the expression level of PTIP in different hematopoietic hierarchy cells from normal human single-

cell RNA-sequencing data.[29] Interestingly, we observed a higher expression of *PTIP* mRNA in HSC when compared with HSPC and GMP (*Online Supplementary Figure S1A*). Next, we sorted different HSC and HSPC populations from mouse BM to confirm the expression level of PTIP at different hematopoietic stratum. Through quantitative real-time polymerase chain reaction (qRT-PCR), we found that *Ptip* displayed a higher expression level in HSC populations including long-term and short-term HSC (LT-HSC and ST-HSC), compared to those in HSPC populations (Figure 1A). This data drove us to investigate the role of PTIP in HSC. We first generated a loss-of-function model by crossing *Mx1*-Cre transgenic mice with $Ptip^{flox/flox}$ mice (*Online Supplementary Figure S1B*). The Cre recombinase is under the control of the *Mx1* promoter, which is expressed both in hematopoietic cells and BM endothelium. Flow cytometry analysis showed that deletion of Ptip caused a dramatic decrease in frequency and total number of different HSPC (*Online Supplementary Figure S1C-F*), implying that PTIP is required for stem cell maintenance. Moreover, *Mx1-Cre*; $Ptip^{flox/flox}$ mice presented with enlarged spleens and whitish limb bones compared to $Ptip^{flox/flox}$ mice, which means that deletion of PTIP in the *Mx1* system induced strong extramedullary hematopoiesis (*Online Supplementary Figure S1C*), consistent with a previous report.[28]

As previous study indicates that PTIP is required for the integrity of the BM niche to sustain normal hematopoiesis,[28] we attempted to specifically delete *Ptip* in HSC in order to avoid the perturbation from the BM niche. We introduced the *Scl-Cre*ER transgenic mice and crossed with $Ptip^{flox/flox}$ mice (*Online Supplementary Figure S2A*). The expression of tamoxifen-inducible recombinase is under the control of the stem cell leukemia (*Scl*) stem-cell enhancer, and tamoxifen-dependent recombination specifically occurs in more than 90% of HSC.[30] The resultant *Scl-CreER;*$Ptip^{flox/flox}$ mice and control mice $Ptip^{flox/flox}$ (WT) were treated with tamoxifen for 4 weeks to induce *Ptip* knockout in HSC (Figure 1B). By performing qRT-PCR and western blotting (WB), we confirmed the deletion of Ptip in BM-derived HSPC ($Lin^-c\text{-}Kit^+$ cells) and LT-HSC, respectively (Figure 1C; *Online Supplementary Figure S2B*). Hereafter, for simplicity, tamoxifen-treated *Scl-CreER;*$Ptip^{flox/flox}$ mice will be referred to as $Ptip^{-/-}$ mice and tamoxifen-treated $Ptip^{flox/flox}$ control mice as WT mice.

Interestingly, we found that the total cell numbers of spleen and BM in $Ptip^{-/-}$ mice were not significantly changed compared to WT control mice (*Online Supplementary Figure S2C*). Unlike to the splenomegaly upon Ptip deletion observed in *Mx1-Cre;* $Ptip^{flox/flox}$ mice, the spleen size from $Ptip^{-/-}$ mice did not change (*data not show*), suggesting that specific deletion of PTIP in LT-HSC does not induce extramedullary hematopoiesis. Intriguingly, *Ptip* deficiency did not clearly affect the total numbers of different lineage cells in peripheral blood (PB), including white blood cells, lymphcytes, granulocytes, monocytes, platelets and red blood cells (*Online Supplementary Figure S2C*). Following further analysis of these mature cells in PB by flow cytometry, we found that, myeloid cells and lymphocytes were not significantly affected upon Ptip deletion, except for $CD8^+$ T cells showing a statistically obvious decrease in $Ptip^{-/-}$ mice (*Online Supplementary Figure S2D*). Ptip deletion also did not significantly change the frequencies of these lineage cells in the spleen and BM, except a modest decrease of B cells and $CD8^+$ T cell in the BM (*Online Supplementary Figure S2D*), which might be related with the role of PTIP in lymphocyte development.[24-26]

In order to explore the effect of Ptip loss in HSC, we further investigated HSPC populations in the BM from WT and $Ptip^{-/-}$ mice. Interestingly, we found that the frequency and total number of LT-HSC ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^+$ cells) were clearly increased (about 2.3-fold) in $Ptip^{-/-}$ mice at 4 weeks after tamoxifen treatment, when compared with WT control (Figure 1D; *Online Supplementary Figure S2E, F*). An increase of MPP2 population was also observed in $Ptip^{-/-}$ mice, but Ptip loss did not affect other stem progenitor populations (Figure 1D; *Online Supplementary Figure S2E, F*). Next, we attempted to explore why these phenotypic LT-HSC are increased upon Ptip deletion. We analyzed the cell cycle of HSC, and found that the percentage of $Ptip^{-/-}$ LT-HSC in G0 phase was markedly lower than that of WT control (37.2% *vs.* 68.5%), while the percentage of $Ptip^{-/-}$ LT-HSC in G1 phase was increased compared to WT LT-HSC (56.3% *vs.* 24%) (Figure 1E), indicating that PTIP loss promotes HSC exiting quiescence. Interestingly, there was no obvious difference in cycling cells between WT and $Ptip^{-/-}$ groups, suggesting a retention of $Ptip^{-/-}$ HSC in the G1 phase. Moreover, we did not observe a significant difference in apoptotic HSC from WT and $Ptip^{-/-}$ mice (Figure 1F). Taken together, our data indicate that PTIP is required for preserving the quiescence state of HSC.

PTIP deficiency impairs hematopoietic stem cell function

In order to illuminate whether the function of HSC is affected with *Ptip* deletion, we first performed a colony forming unit (CFU) assay by sorting HSC from the BM of WT and $Ptip^{-/-}$ mice and seeded them into an M3434 semi-solid medium. As expected, loss of Ptip clearly impaired the clonogenic ability of HSC, as $Ptip^{-/-}$ HSC showed less colony numbers when compared with WT HSC (Figure 2A). We next conducted a competitive repopulation assay to determine whether Ptip affects the self-renewal capacity of HSC. The same numbers of total BM cells from WT and $Ptip^{-/-}$ mice with an equal number of CD45.1 helper cells were transplanted into lethally irradiated CD45.1 recipients, and these chimeric mice were analyzed using flow

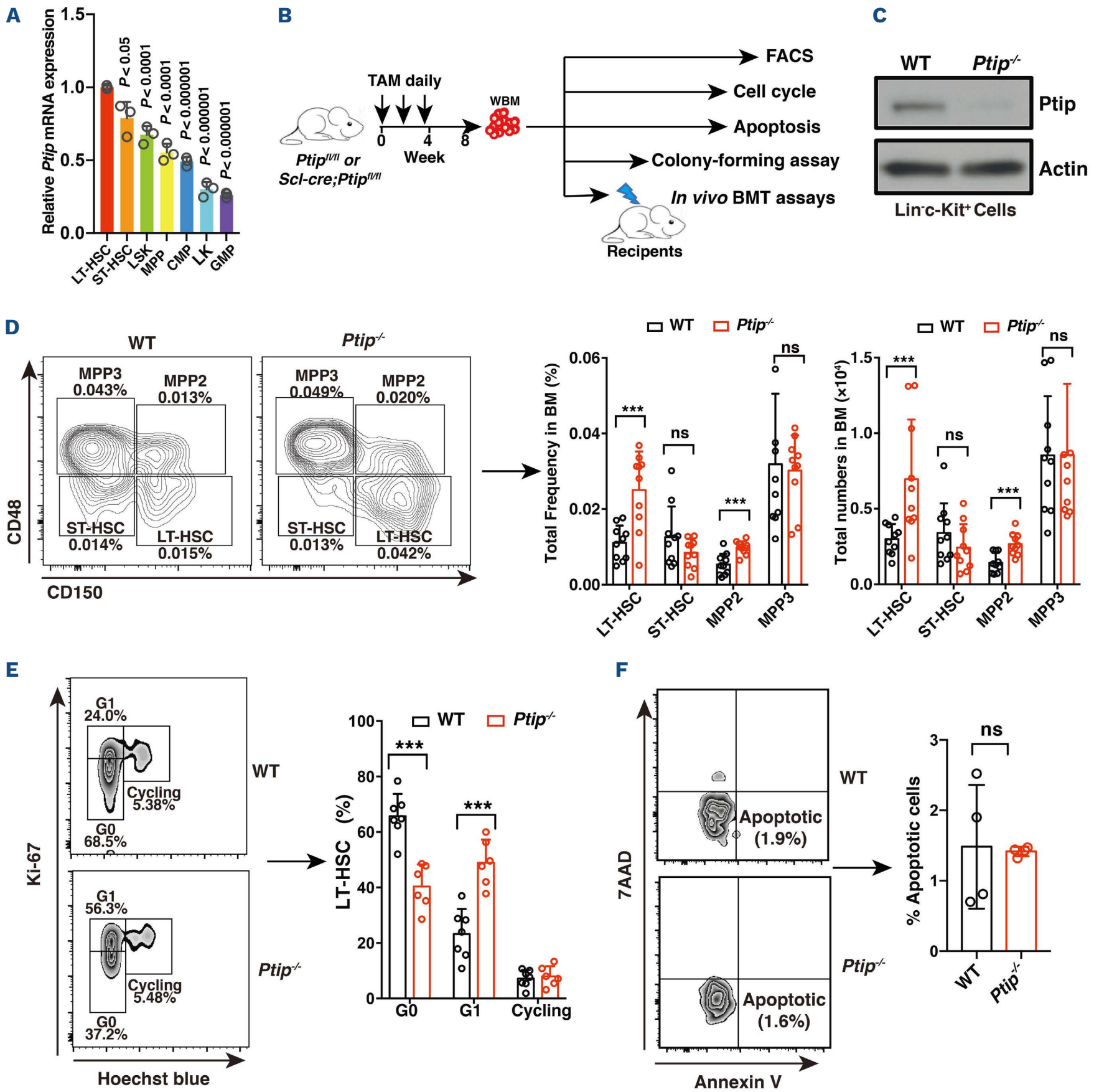


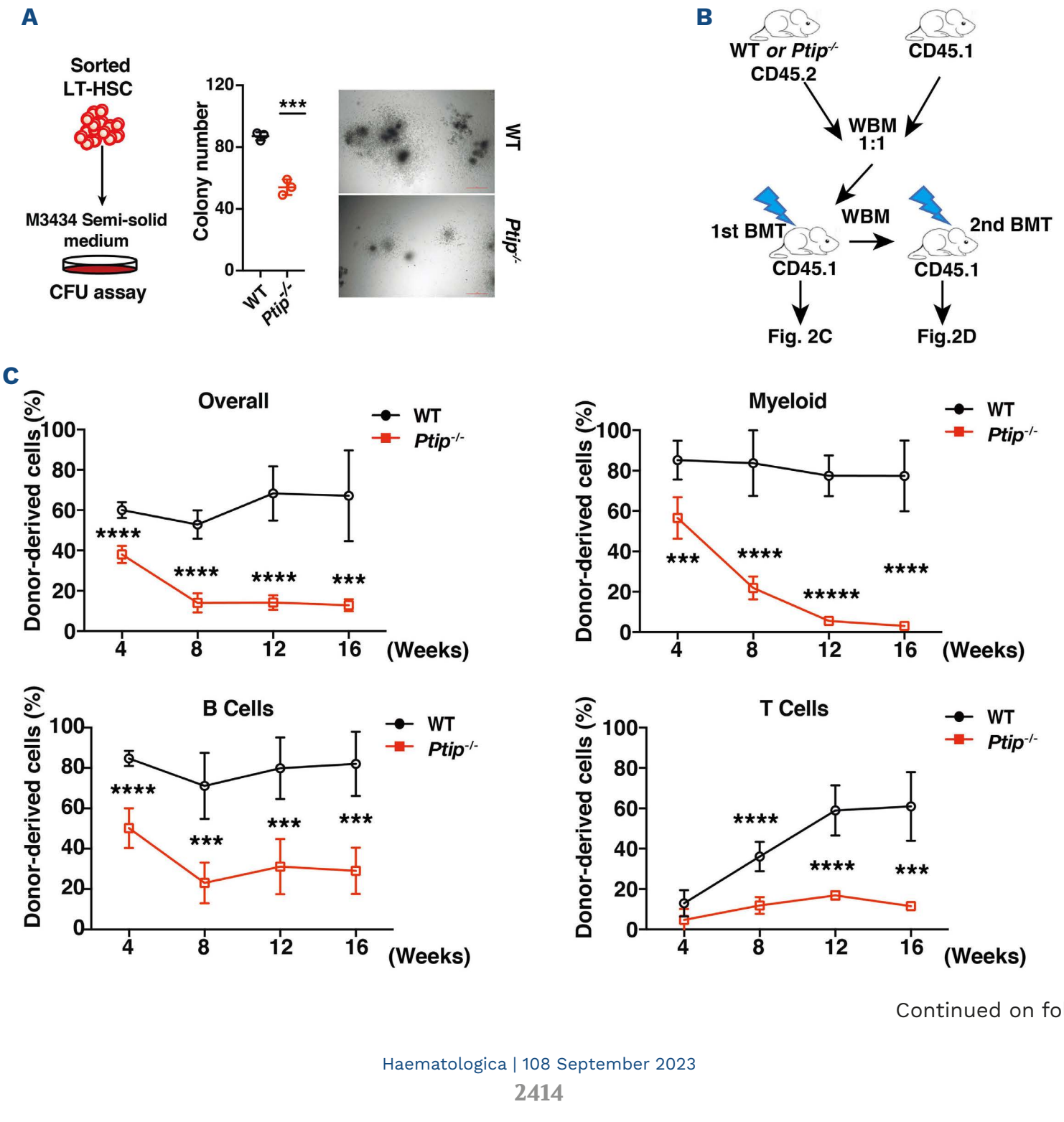
Figure 1. Loss of *Ptip* leads to hematopoietic stem cell expansion and activation. (A) Quantitative real-time polymerase chain reaction analysis showing the mRNA expression of *Ptip* in normal hematopoietic stem cell (HSC) and hematopoietic stem progenitor cell (HSPC) populations (B) experimental scheme for (C-F). (C) Immunoblotting showing PTIP expression in *c-kit*⁺ cells after tamoxifen induction. (D) Percentages and total numbers of different stem cell populations in bone marrow (BM) at 4 weeks after tamoxifen treatment. The upper panel shows the representative flow cytometry (FACS) plots. The lower panel plots percentages of wild-type (WT) and *Ptip*$^{-/-}$ cells in each stem cell population (N=10). (E) Flow cytometry analysis of cell cycle phase of long-term HSC (LT-HSC) from WT and *Ptip*$^{-/-}$ mice BM harvested at 4 weeks after tamoxifen treatment. The left pa7nel shows the representative flow cytometry plots. Right panel plots percentages of WT and *Ptip*$^{-/-}$ cells in each stage of the cell cycle (N=6). (F) Flow cytometry analysis of apoptosis of LT-HSC from WT and *Ptip*$^{-/-}$ mice BM harvested at 4 weeks after tamoxifen treatment. The left panel shows the representative flow cytometry plots. Right panel plots percentages of WT and *Ptip*$^{-/-}$ LT-HSC (N=4). ns: not significant; **P*<0.05, ***P*<0.01, ****P*<0.001 (*t*-test). Error bars denote mean ± standard error of the mean. BMT: BM transplant.

cytometry over 4-16 weeks after BM transplantation (Figure 2B, C). We found that the percentages of *Ptip*$^{-/-}$ donor-derived total cells (CD45.2⁺), myeloid cells (Gr-1⁺, Mac1⁺), T cells (CD3e⁺), and B cells (B220⁺) were dramatically lower than those of WT group (Figure 2C; *Online Supplementary S3A*), suggesting the impaired reconstitution abil-

ity of *Ptip*$^{-/-}$ HSC. At 16 weeks, we directly compared the fractions of WT and *Ptip*$^{-/-}$ donor-derived stem and progenitor populations, including LT-HSC, ST-HSC, MPP2, MPP3, MPP4, LSK, LMPP, CMP, GMP, MEP, and CLP. As expected, the frequencies and numbers of all the populations were significantly lower in *Ptip*$^{-/-}$ group (*Online Supplementary Figure S3B, C*). In order to test the long-term function of HSC, we performed a secondary transplantation (Figure 2B, D). This defect was further exacerbated upon secondary transplantation, as shown by almost undetectable donor-derived cells in the *Ptip*$^{-/-}$ group (Figure 2D). In order to assess whether *Ptip* loss affects BM homing, we performed a BM homing assay. BM cells from WT and Ptip$^{-/-}$ mice were transplanted into lethally irradiated CD45.1 mice, and CD45.2 donor-derived cells in the BM were detected 24 hours post transplantation. Interestingly, we did not observe a significant difference between the WT and *Ptip*$^{-/-}$ groups (\), suggesting that loss of *Ptip* does not impair BM homing of donor cells. Together, these results indicate that PTIP is critical for maintaining HSC function.

PTIP affects lysosomal activity of hemtopoietic stem cells

In order to comprehensively understand the regulatory role of PTIP in HSC maintenance, we conducted RNA-sequencing assays to compare gene expression profiles of HSC from WT and *Ptip*$^{-/-}$ mice. A total of 1,128 genes were found to be differentially expressed by at least 2-fold (P<0.01) (*Online Supplementary Figure S4A*). Gene Ontology enrichment analysis showed that the upregulated genes in *Ptip*$^{-/-}$ HSC were related to nucleic acid metabolic processes, regulation of RNA metabolic processes, the Wnt sigaling pathway, and kinase activity (*Online Supplementary Figure S4B*). The downregulated genes in *Ptip*$^{-/-}$ HSC were genes enriched in the regulation of inflammatory response, reactive oxygen species metabolic processes, apoptotic cell clearance, lysosome, and autophagy (*Online Supplementary Figure S4B*). Gene Set Enrichment Analysis (GSEA) also showed that genes related to lysosome, TGFβ pathways, and hematopoietic cell lineage were significantly downregulated in *Ptip*$^{-/-}$ HSC (Figure 3A;



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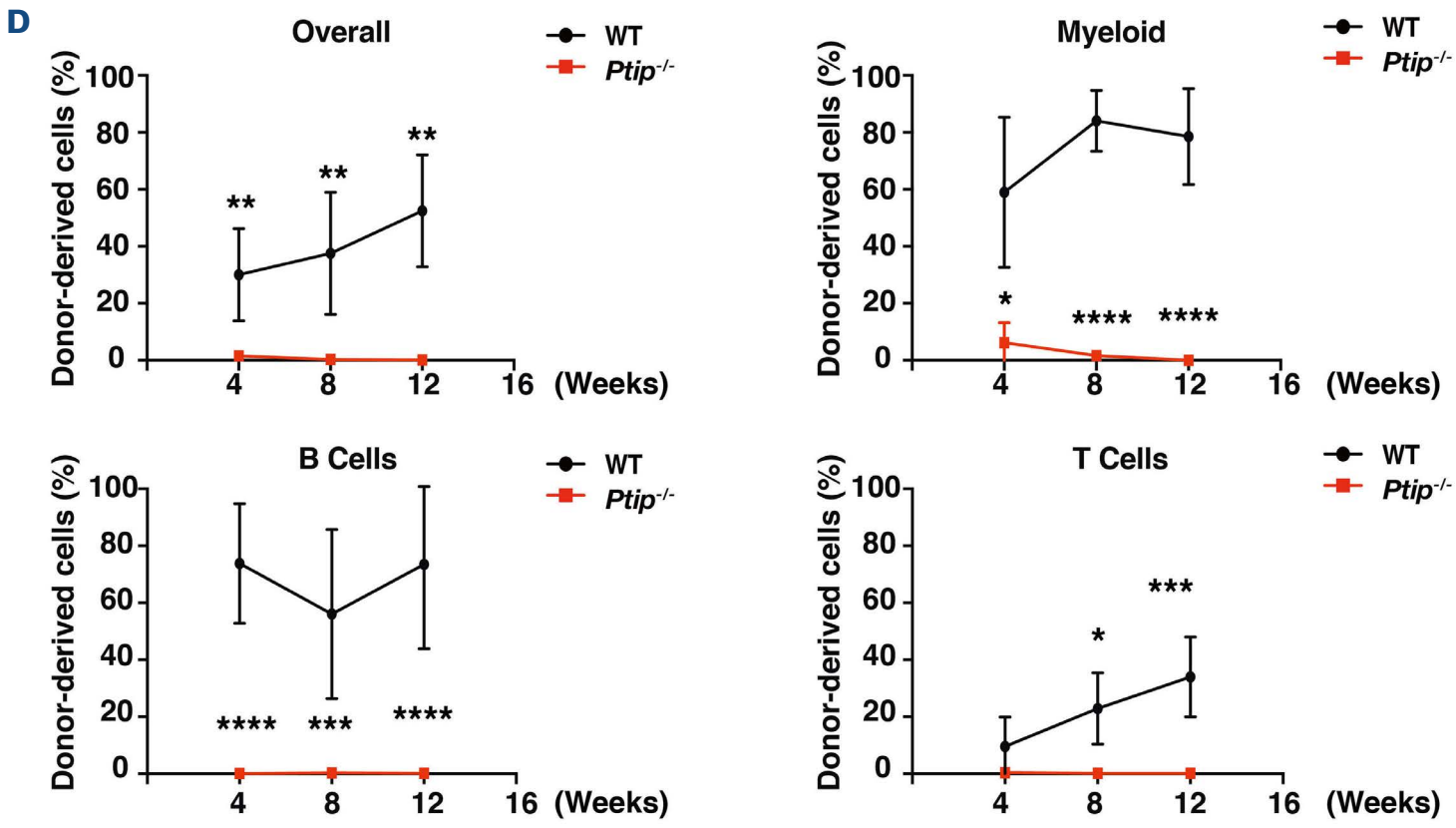

Figure 2. Loss of *Ptip* impairs hematopoietic stem cell long-term competitive repopulation ability *in vivo*. (A) Colony-forming unit (CFU) assay of long-term hematopoietic stem cells (LT-HSC) cells from wild-type (WT) and *Ptip*$^{-/-}$ mice bone marrow (BM) at 4 weeks after tamoxifen treatment (N=3). (B) Competitive repopulation assay. (B) The experimental set up. (C) Flow cytometry analysis for different donor-derived lineage cells in peripheral blood (PB) of recipient mice at 4, 8, 12, and 16 weeks after BM transplant (BMT) (N=9-10). (D) Secondary BMT showing impaired long-term self-renewal of WT and *Ptip*$^{-/-}$ HSC. Flow cytometry analysis for different donor-derived lineage cells in PB of recipient mice at 4, 8, 12, and 16 weeks after BMT (N = 5-6). ns: not significant; *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001, *****P<0.000001 (t-test). Error bars denote mean ± standard error of the mean.

Online Supplementary Figure S4C). In contrast, GSEA showed an enrichment of genes involved in the cell cycle pathway in *Ptip*$^{-/-}$ HSC (*Online Supplementary Figure S4C*). As shown in the *Online Supplementary Figure S4D*, we observed an obvious upregulation of *Mycn, Myc* and *Cdk6*, and downregulation of cell cycle inhibitors including *p21, p27* and *p57*.

Previous studies have shown that biological processes involving lysosomes are critical for HSC quiescence and activation.[14,15,31] Given the enrichment of gene sets of lysosomal-related pathways in *Ptip*$^{-/-}$ HSC, we next sought to assess whether Ptip affects lysosome function. Interestingly, *Ptip*$^{-/-}$ HSC exhibited an obvious decrease of *Tfeb*, a master transcriptional regulator of lysosomal biogenesis. Downregulation of genes encoding lysosomal enzymes, such as *Smpd1, Gns, Ctsh*, and *Ctsb* was also observed in *Ptip*$^{-/-}$ HSC (Figure 3B), suggesting that lysosomal biogenesis is less efficient in *Ptip*$^{-/-}$ HSC compared to WT HSC. We further assessed the lysosomes in HSC by staining the lysosomal marker lysosome membrane protein 1 (LAMP1). As shown in Figure 3C, we observed fewer lysosomes in *Ptip*$^{-/-}$ HSC compared to WT HSC, which is in line with a previous study showing that lysosomes are relatively scarce in activated HSC.[13] We further assessed the density of lysosomes in HSC. Consistently, we found that *Ptip*$^{-/-}$ HSC showed fewer lysosomes in HSC when compared to WT control (Figure 3D).

Lysosomes are acidic organelles, and their activity is often closely related to acidification. While quiescent HSC show slow lysosomal-degradative potential, activated HSC exhibit rapid lysosomal degradation with a higher activity.[13] Also, a previous study indicates that phosphorylation-activated mTORC1 translocation to the lysosome directly regulates H^+ transport of the vATPase proton pump.[32] Therefore, we assessed lysosomal activity in *Ptip*$^{-/-}$ HSC. First, we measured lysosomal activity directly by co-staining lysosomal biomarker LAMP2 with mTOR. We found that, when compared to WT HSC, the puncta of LAMP in *Ptip*$^{-/-}$ HSC was decreased, while the puncta of mTOR was increased and the co-localization of mTOR and Lamp2 was also increased (Figure 3E), indicating higher lysosomal proton influx. Next, we detected the potential activity of lysosomes in HSC using LysoSensor, a more pH-sensitive probe to characterize lysosomal activity. As expected, we found that deletion of Ptip increased lysosomal proton influx and leads to higher lysosomal acidification (Figure 3F). Taken together, our data suggest that PTIP affects lysosomal activity of HSC.

Restraining lysosomal activity restores the quiescence and repopulation potency of *Ptip*$^{-/-}$ hemtopoietic stem cells

We further investigated the effect of increased lysosomal activity on HSC function. Leu is known as a protease in-

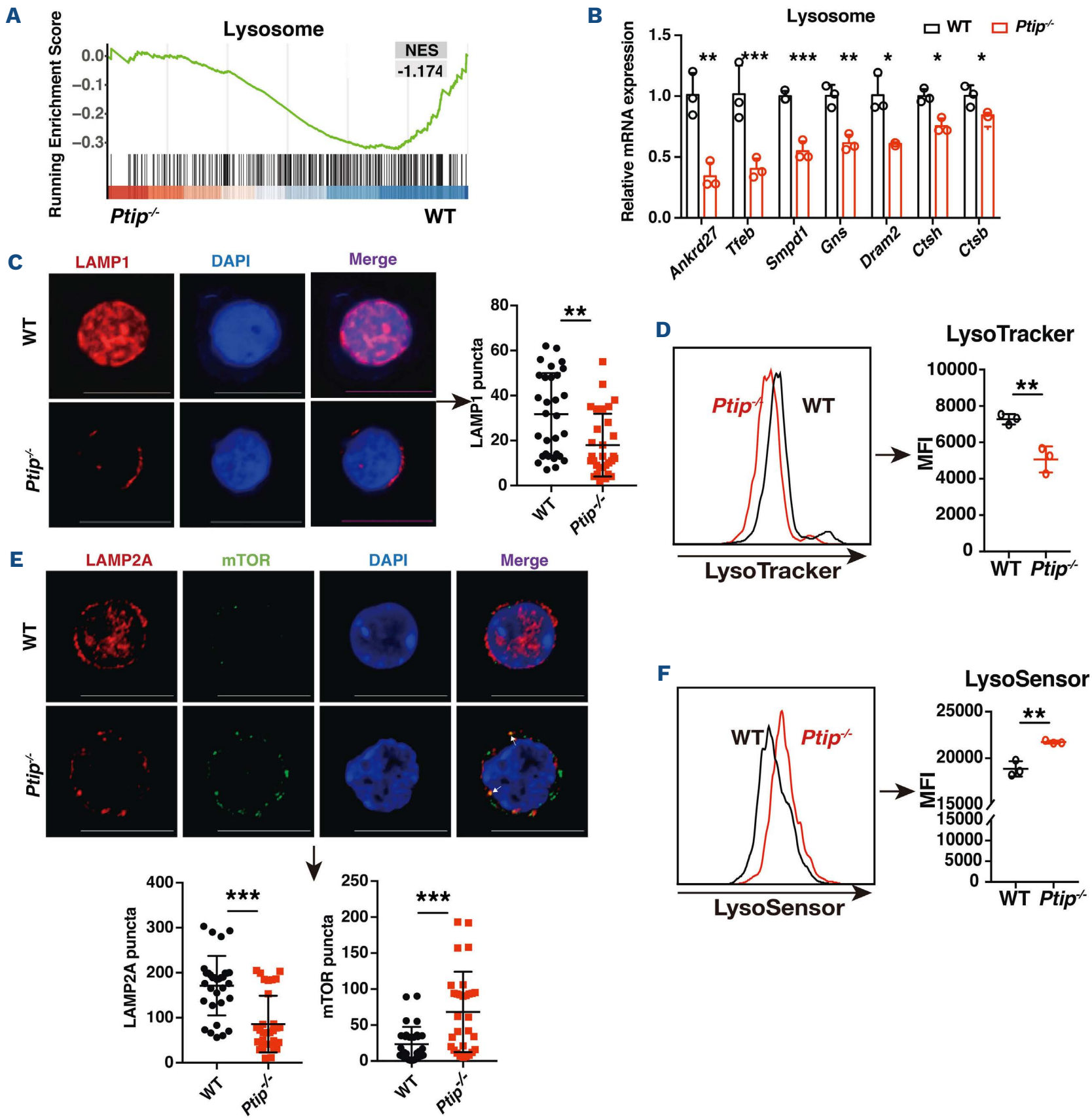

Figure 3. PTIP regulates lysosomal activity. (A) Gene sets enrichment analysis (GSEA) plot showing enrichment of gene sets of the lysosome. (B) Quantitative real-time polymerase chain reaction analysis validation of the effect of Ptip on the expression levels of lysosome targets: *Ankrd27*, *Tfeb*, *Smpd1*, *Gns*, *Dram2*, *Ctsh*, and *Ctsb.* (C) Representative immunofluorescent confocal images of LAMP1 and DAPI (left; bar, 10μm) and quantification (right; N=3). (D) Flow cytometry analysis of LysoTracker green of long-term hematopoietic stem cells (LT-HSC) from wild-type (WT) and *Ptip*$^{-/-}$ mice bone marrow (BM) harvested at 4 weeks after tamoxifen treatment. The left panel shows the representative flow cytometry plots. Right panel plots mean fluorescence intensity (MFI) of WT and *Ptip*$^{-/-}$ cells (N=3). (E) Representative immunofluorescent confocal images of LAMP2, mTOR and DAPI (left; bar, 10μm; arrow shows co-localization) and quantification (right; N=3). (F) Flow cytometry analysis of LysoSensor green of LT-HSC from WT and *Ptip*$^{-/-}$ mice BM harvested at 4 weeks after tamoxifen treatment. The left panel shows the representative flow cytometry plots. Right panel plots MFI of WT and *Ptip*$^{-/-}$ cells (N=3). ns: not significant; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.00001, (*t*-test). Error bars denote mean ± standard error of the mean.

hibitor that can inhibit enzymatic activity within lysosomes.[33] We first treated HSC from WT mice with Leu, and observed the increased size of lysosomes, indicating a buildup of undigested material (*Online Supplementary Figure S4E*). This data is in line with previous work,[13] confirming the specificity and efficacy of Leu in inhibiting lysosomal degradation. We next inhibited the lysosomal activity with Leu in sorted HSC from *Ptip*$^{-/-}$ mice. Interestingly, we

found that Leu treatment clearly increased the *in vitro* clonogenic potential of *Ptip*$^{-/-}$ HSC (Figure 4A). Next, we assessed whether inhibition of lysosomal activity rescues the cell cycle state and reconstitution ability of *Ptip*$^{-/-}$ HSC *in vivo*. We treated WT and *Ptip*$^{-/-}$ mice with Leu by intraperitoneal injection,[34,35] and then analyzed HSC, cell cycle and the *in vivo* repopulation ability (Figure 4B). As expected, we found that Leu treatment markedly decreased the proportion and numbers of LT-HSC in *Ptip*$^{-/-}$ mice (Figure 4C). Further, we found that the percentage of quiescent LT-HSC in *Ptip*$^{-/-}$ mice was significantly increased by approximately 24% after Leu treatment (Figure 4D). Thus, these data suggest that inhibition of lysosomal activity can effectively block the activation of HSC caused by Ptip loss.

In order to further explore the effect of lysosomal activity on HSC function, we performed a competitive repopulation assay using whole BM cells from WT and *Ptip*$^{-/-}$ mice

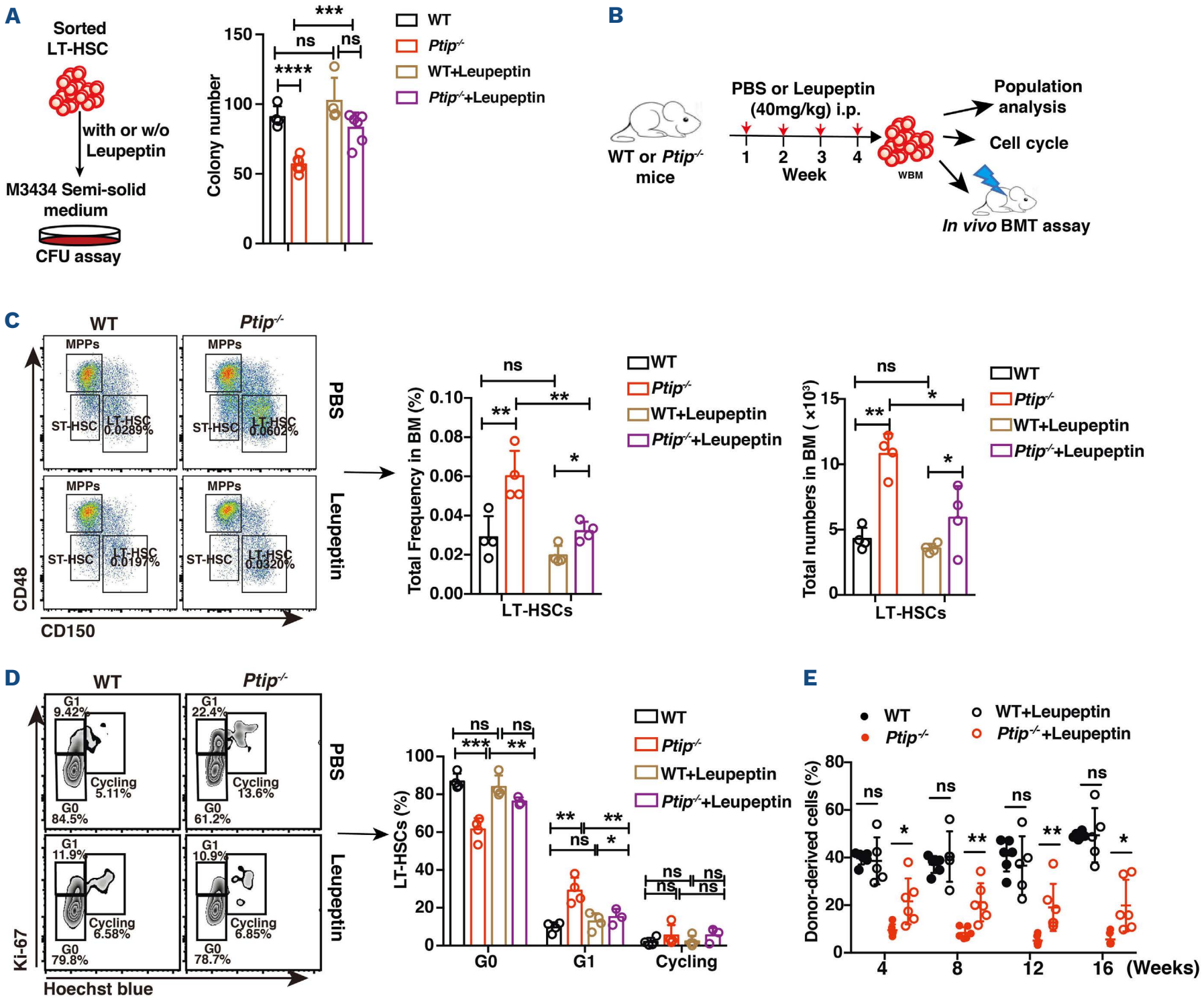

Figure 4. Restraining lysosomal activity restores hematopoietic stem cell competitive repopulation capacity. (A) Colony-forming unit (CFU) assay showing the rescue of phenotype by leupeptin (Leu) or phosphate-buffered saline (PBS) control on long-term hematopoietic stem cells (LT-HSC) (N=4-6). (B) Schematic of lysosomal inhibition by intraperitoneal injection with Leu or PBS control. (C) Percentages and total numbers of different stem cell populations in bone marrow (BM) after lysosomal inhibition by Leu or PBS control. The upper panel shows the representative flow cytometry plots. The lower panel plots percentages and total numbers of wild-type (WT) and *Ptip*$^{-/-}$ cells in each stem cell population (N=4). (D) Flow cytometry analysis of cell cycle phase of LT-HSC from WT and *Ptip*$^{-/-}$ mice in BM after lysosomal inhibition by Leu or PBS control. The left panel shows the representative flow cytometry plots. Right panel plots percentages of WT and *Ptip*$^{-/-}$ cells in each stage of the cell cycle (N=3-4). (E) Competitive repopulation assay. Whole BM of WT and *Ptip*$^{-/-}$ mice (CD45.2) were treated *in vivo* with Leu or PBS control, after which cells from each group were injected into lethally irradiated recipient (CD45.1) mice along with 3x10^5 CD45.1 helper cells (N=5-6). ns: not significant; ***P*<0.01, ****P*<0.001, *****P*<0.00001 (*t*-test). Error bars denote mean ± standard error of the mean.

with or without Leu treatment WT, *Ptip*$^{-/-}$ mice (Figure 4B, E). Donor-derived CD45.2 cells in PB were detected at 4, 8, 12, and 16 weeks. Interestingly, the percentages of donor-derived cells in PB were significantly higher in Leu-treated relative to untreated *Ptip*$^{-/-}$ groups (Figure 4E). About a 3.57-fold higher percentage of donor-derived cells in PB was observed in Leu-treated relative to untreated *Ptip*$^{-/-}$ groups at 16 weeks (Figure 4E). Taken together, our results show that inhibition of lysosomal activity effectively restores the quiescence and function of *Ptip*$^{-/-}$ HSC.

Ptip coordinates TGFβ signaling in regulating hematopoietic stem cell quiescence

TGFβ signaling pathway is known as a key regulator of HSC quiescence and function.[19] Given that the findings that Ptip deletion leads to the alteration of the TGFβ signaling pathway in HSC (*Online Supplementary Figure S4C, D*), we hypothesized that PTIP may mediate the function of TGFβ signaling in HSC maintenance. We first employed single-cell RNA-sequencing data[36] for correlation analysis of PTIP. Interestingly, correlation analysis showed that PTIP was highly correlated with the TGFβ signaling pathway, lysosomes and cell cycle (Figure 5A). Especially, PTIP expression exhibited a positive correlation with the expression of SMAD3 and TFEB, and a negative correlation with CDK6 (*Online Supplementary Figure S5A*). In addition, we found that PTIP interacted with SMAD2/3 under endogenous and exogenous conditions (Figure 5B; *Online Supplementary Figure S5B*). Thus, these data suggest that PTIP may cooperate with the TGFβ signaling pathway to maintain the quiescent state of HSC.

We next investigated whether PTIP cooperates with TGFβ signaling in regulating HSC maintenance. As expected, we observed an obvious increase and decrease in the phosphorylation level of p-Smad2/3 in BM cells upon TGFβ-1 and LY364947 treatment, respectively (*Online Supplementary Figure S5C*), indicating its efficiency in activating and inhibiting TGFβ signaling. In addition, co-immunoprecipitation assays showed that the interaction of PTIP with SMAD3 was promoted by TGFb-1 treatment, but was blocked by inhibition of TGFβ with LY364947 (*Online Supplementary Figure S5D*), which suggests that TGFβ signaling regulates the interaction of PTIP with SMAD3. We then investigated whether PTIP participates in TGFβ-regulated HSC proliferation in WT and *Ptip*$^{-/-}$ mice treated with LY364947 (Figure 5C). Interestingly, inhibition of TGFβ signaling by LY364947 significantly increased the total number of HSC in the WT control group, but did not clearly affect HSC in *Ptip*$^{-/-}$ mice (Figure 5C). We also found that TGFβ signaling inhibition promotes the transition of quiescent HSC to activated HSC in WT mice, but not in *Ptip*$^{-/-}$ mice (*Online Supplementary Figure S5E*). Thus, these data prompted us to further assess whether TGFβ signaling activation could rescue the defects of HSC due to PTIP deletion. We performed serial CFU assays using LT-HSC treated with active TGFβ1 *in vitro* (Figure 5D). As expected, when comparing to WT HSC, *Ptip*$^{-/-}$ HSC displayed impaired clonogenic ability in first-round CFU plating, which was partially rescued by TGFβ1 treatment in the second and third CFU replating (Figure 5D). Thus, this data suggests that TGFβ signaling could partially rescue the function of *Ptip*$^{-/-}$ HSC. In addition, we also found that TGFβ1 treatment downregulated the expression levels of *Cdk6, Myc*, and *Mycn*, and upregulated the expression of *p21, p27* and *p57* in HSPC (*Online Supplementary Figure S5F*). We further examined lysosomal activity and the expression of lysosomal-associated genes. Interestingly, we found that TGFβ1 treatment reversed the increased lysosomal activity caused by Ptip deletion (Figure 5E). Meanwhile, TGFb1 treatment also restored the expression levels of lysosome-related genes, including *Dram2, Ctsd, Ctsb*, and *Gm2a* (*Online Supplementary Figure S5G*), which suggests that TGFβ signaling is involved in regulating lysosomal activity of HSC. Taken together, these results indicate that PTIP cooperates with TGFβ signaling pathway in maintaining HSC quiescence.

Discussion

HSC need to reside in a quiescent state in order to maintain their function during the whole lifetime. Therefore, how HSC preserve their quiescence is a fundamental scientific question in this field. Here, we uncover a crucial role of the histone methylation regulator PTIP in regulating lysosomal activity and coordinating TGFβ signaling to sustain HSC quiescence and function.

Our findings clarify the intrinsic and key role of PTIP in regulating HSC function and normal hematopoiesis. Previous study showed that deletion of PTIP in HSC and HSPC disrupts the microenvironment in the BM by blocking osteoclast differentiation, leading to a reduction of the BM HSPC pool and extramedullary hematopoiesis.[28] In our study, we generated *Scl-CreER;Ptip*$^{flox/flox}$ mice. In this strain, the expression of tamoxifen-inducible Cre-ER recombinase is under the control of the stem cell leukemia (*Scl*) stem-cell enhancer.[30] It is known that tamoxifen-dependent recombination occurs in more than 90% of adult long-term HSC, whereas the targeted proportion within mature progenitor populations is significantly lower.[30] Thus, this HSC-SCL-Cre-ER mice provides us with a valuable tool to investigate the role of PTIP in HSC avoiding the interruption from an altered BM niche due to PTIP deletion. Interestingly, our findings suggest that PTIP is required for preserving HSC in quiescence, and PTIP loss promotes quiescent HSC entry into G1 phase. However, the lack of an obvious difference in cycling cells upon PTIP deletion suggests a retention of *Ptip*$^{-/-}$ HSC in the G1

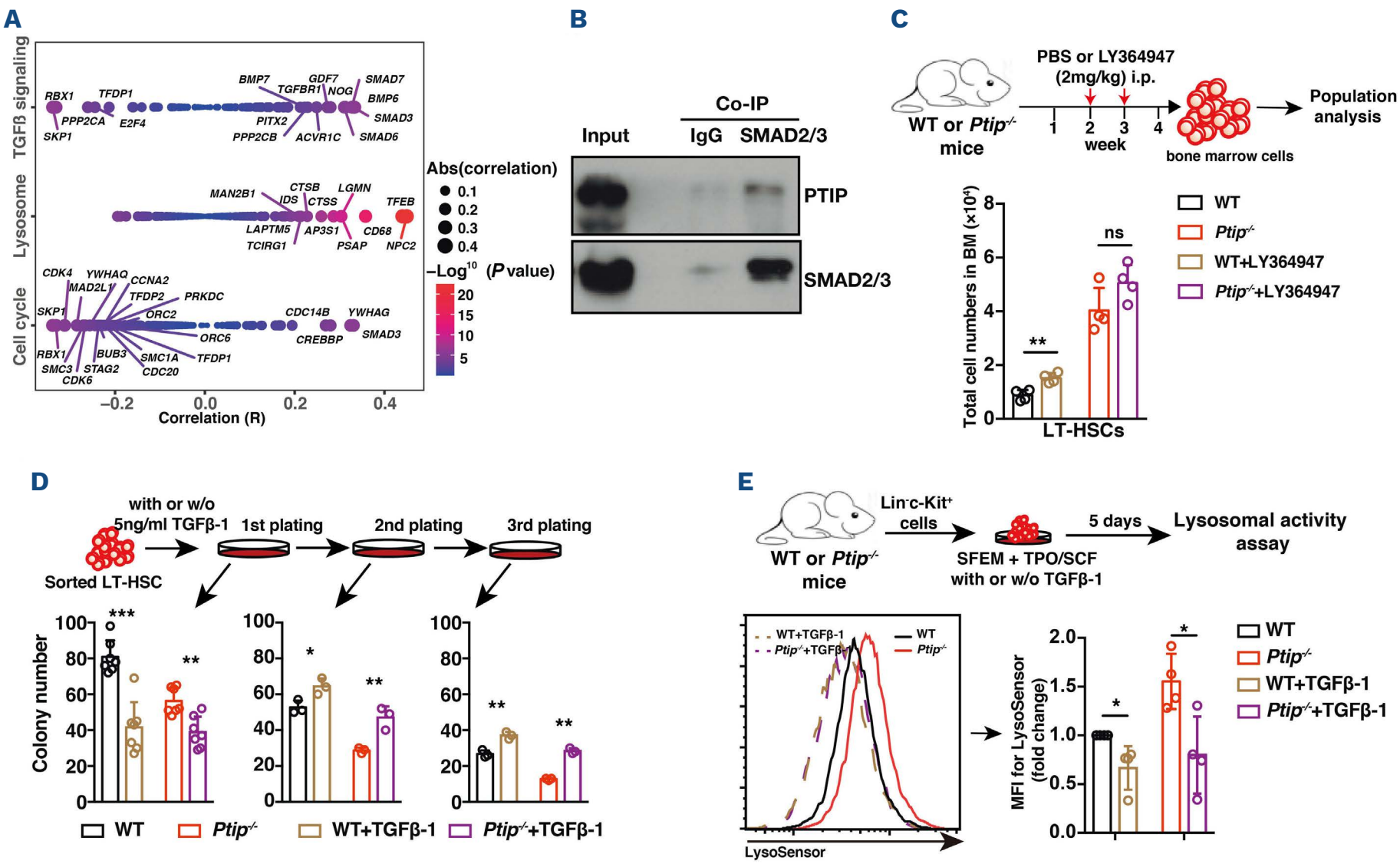

Figure 5. Ptip coordinates TGFβ signaling in regulating hematopoietic stem cell quiescence. (A) Pearson's correlation between *PTIP* and TGFβ signaling, Lysosome and cell cycle targets from single-cell RNA-sequencing data (GSE157591). (B) Co-immunoprecipitation (CoIP) of SMAD2/3 and PTIP from OCI-AML3 cell line extract following incubation with SMAD2/3 or rabbit IgG antibodies. Immunoprecipitated immunoblotted with the indicated antibodies. (C) The effect of TGFβ signaling inhibition on hematopoietic stem cells (HSC). Upper panel: scheme for LY364947 injection to wild-type (WT) and *Ptip*$^{-/-}$ mice after tamoxifen administration; Lower panel: total cell numbers of long-term HSC (LT-HSC) in WT and *Ptip*$^{-/-}$ mice with or without LY364947 treatment. (D) Serial colony-formation unit (CFU) assay using LT-HSC from WT and *Ptip*$^{-/-}$ mice. Cells were treated with phosphate-buffered saline (PBS) and TGFβ1 at 1st plating separately (N=7); 2,000-3,000 cells from last colony formation were used for 2nd and 3rd plating (N=3). (E) Lysosomal activity assay for TGFβ1 treatment on hematopoietic stem progenitor cells (HSPC). Scheme for TGFβ1 treatment (upper). Bone marrow-derived Lin^{-}c-Kit^{+} cells from WT and *Ptip*$^{-/-}$ mice were treated with or without 5 ng/ml TGFβ1 in an *ex vivo* SFEM medium. The lower panel shows the representative flow cytometry plots and mean fluorescence intensity (MFI) of WT and *Ptip*$^{-/-}$ cells (N=3). ns: not significant; *P<0.05 **P<0.01, ***P<0.001 (t-test). Error bars denote mean ± standard error of the mean.

phase. Cell cycle progression is regulated by cyclin-CDK complex, and G1-S phase transition is associated with CDK4/6 and Cyclin D and the related inhibitors p21 and p27.[37] Thus, G1 arrest of *Ptip*$^{-/-}$ HSC might correlate with the upregulation of p21 and p27. Together, our work here clearly reveals a critical role of PTIP in HSC maintenance. Our study identifies PTIP as a key factor for regulating lysosomal activity in HSC. Recent studies indicate that lysosomes play an important role in balancing HSC quiescence *versus* activation.[13-16] Quiescent HSC display more and larger lysosomes with slower degradative potential, while activated HSC have fewer and smaller lysosomes with relative higher activity. However, how the lysosomal function is regulated in HSC remains elusive. Interestingly, we find that PTIP loss causes increased lysosomal activity, and subsequently results in activation of HSC. Repression of lysosomal activity enhances the competitive repopulation ability of *Ptip*$^{-/-}$ HSC. Our data indicate that PTIP is involved in regulating lysosomal activity in HSC. As the expression of TFEB, the master transcriptional regulator of lysosome biogenesis, is altered by PTIP, it is necessary to further investigate how PTIP regulates the expression of TFEB in the future. Interestingly, we find that PTIP interacts with SMAD2/3, and mediates the function of TGFβ signaling in HSC quiescence. Previous studies showed that interplay between TGFβ signaling and cell metabolism is thought to be instrumental in maintaining homeostasis.[38] TGFβ signaling regulates lysosome function and involves in lysosome-associated physiological processes.[39-41] Therefore, it would be of great interest to uncover whether TGFβ signaling is involved in PTIP-mediated lysosomal activity regulation in HSC.

Disclosures

No conflicts of interest to disclose.

Contributions

TZ and HZ conceived the project. TZ, MC, and HZ designed the experiments and analyzed the data. TZ, MC, YL, YC performed the experiments with the help of JW, TZ, WT and YW. TZ, YL and YC performed bioinformatic analyses with the help of TZ, GH, WL. TZ, MC, and YL performed mouse experiments with the help of GH, RY, and ZG. YL, JW and TZ constructed the RNA-sequencing library. Other researchers in the lab (PW, JH, JW, YW) helped with experiments. YC, ZG, and TZ performed the statistical analysis, and TZ, ZL, and HZ wrote the manuscript. HZ supervised the study.

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Data-sharing statement

RNA-sequencing data are available from the corresponding author upon request.

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