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

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Supplementary Data

Supplementary methods

FACS Analysis and Sorting

Total BM cells were isolated from mice's femur and tibia. In brief, two million BM cells were lysed with RBC to remove red blood cells. For HSC, cells 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. For CLP, LSKs were additionally stained with CD135 and FITC-anti-CD127. For LK subpopulations, LSKs were additionally stained with FITC-anti-CD34 and PE-Cy7-anti-CD16/32. For mature cells, BM and PB cells were stained with APC-anti-CD4, FITC-anti-CD8, PE-anti-CD11b, APC-Cy7-Gr-1, and PE-Cy5-anti-B220. BD sorters FACSAria III; BD analyzers FACSCelesta, FACSLSRFortessaX20, and Beckman CytoFlex were used.

***In vivo* treatment assays**

*Tgfbr*1 inhibitor LY364947 (S2805; Selleckchem) was intraperitoneally injected at 2mg/kg. Lysosome protease inhibitor Leupeptin (L2884; Sigma) was intraperitoneally injected at 40mg/kg.

TGFβ1 in vitro treatment assays

Lin$^-$c-kit$^+$ cells isolated from WT and Ptip mice were cultured in StemSpan (StemCell Technologies) supplemented with mTPO and mSCF and treated with or without TGFβ1 5 days, and the cells were used calculated for total cell numbers, or harvested for FACS or WB.

Genotyping and Quantitative RT-PCR (RT-qPCR)

For genotyping, Mice's tails were lysed in lysis buffer with Proteinase K, then genotyped by PCR. For Ptip genotyping primers were Forward: AGACAGCAGTCCCGAATCTGAGC; Reverse: CAGCAGTAGAAGGTTTCCAAACAT. Scl-CreER genotyping primers were Forward: GAGTGATGAGGTTCGCAAGA; Reverse: CTACACCAGAGACGGAAATC. For RT-qPCR analysis, Total RNA from HSC/HSPCs was purified using TRIzol (Life Technologies) according to the manufacturer's instructions. Total RNA was reversely transcribed using the ReverTra Ace qPCR RT Kit (TOYOBO). The levels of specific RNAs were measured using Bio-Rad real-time PCR machine and Fast SYBRGreen PCR mastermix according to the manufacturer's instructions. Primer sequences are listed in Supplementary Information Table S1. The 2 -ΔΔCt method was used to normalize expression to Gapdh and actin for cells.

RNA sequencing (RNA-seq) and data analysis

For RNA-seq, LT-HSCs were sorted from WT and Ptip-/- mice bone marrow. Total RNA was harvested using Trizol reagent. RNA was reverse transcribed with oligo dT primers and template-switching oligos, followed by amplification by PCR of 12-15 cycles with ISPCR oligos. The PCR program was as follows: 98℃ for 3 min; 10-14 cycles of 98℃ 20 s, 67℃ 15 s, and

72℃ 6 min; 72℃ 5 min and 4℃ cold. The amplified cDNAs were purified using AMPure XP beads to abolish primer dimers. After purification, 50 ng cDNAs were fragmented using the TruePrep DNA Library Prep Kit. Then the fragments were amplified with N5 primers and N7 primers. The PCR program was as follows: 72℃ 3min, 98℃ 30 s; 5-9 cycles of 98℃ 15 s, 60℃ 30 s, and 72℃ 3 min; 72℃ 5 min and 4℃Cold. After PCR, samples were purified using 1.0 volumes of AMPure XP beads, the cDNA libraries were ready for sequencing on an Illumina Hiseq X Ten sequencing platform. For RNA-seq analysis, reads were mapped to mouse genome version 38 (GRCm38) with Hisat2（v2.1.0）, and FeatureCounts (v1.6.4）was used to calculate counts from bam files. DESeq2(v1.26.0) was employed for data normalization and differential expression analysis of RNA-seq counts.

Statistical analyses

All the experiments were analyzed using a two-way Student's t-test. For the comparison of different specimens, the unpaired t-test was used. For the comparison of different treatments within the same specimen, the paired t-test was used. The log-rank test was used to compare survival curves. P values of less than 0.05 were considered statistically significant. In the figures, asterisks indicate *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ns means no significance.

Supplementary Figure legends

Figure S1. Conditional knockout Ptip in Mx1-Cre induced model leads to strong extramedullary hematopoiesis.

(A) Single-cell RNA-seq data showing PTIP expression in healthy donor-derived bone marrow cells. Each dot represents one cell (GSE116256).

(B) Scheme for generating Mx1-Cre; Ptip conditional knockout mice.

(C) Gross morphology of spleens and tibias.

(D) qRT-PCR analysis showing the efficiency of Ptip deletion in LT-HSC cells at 4 weeks after pIpC treatment.

(E) Representative fluorescence-activated cell sorting plots showing the gating strategy for different stem and progenitor cell population in BM from WT and *Ptip*$^{-/-}$ mice at 4 weeks after pIpC treatment.

(F) Percentages and total numbers of different stem cell populations in bone marrow at 4 weeks after pIpC treatment from WT and *Ptip*$^{-/-}$ mice (n = 4).

Figure S2. Specific knockout Ptip in LT-HSC does not arouse extramedullary hemopoiesis and leads to HSC expansion

(A) Scheme for generating Scl-CreER^{+}; Ptip$^{flox/flox}$ conditional knockout mice.

(B) qRT-PCR analysis showing the efficiency of Ptip deletion in LT-HSC cells at 4 weeks after Tamoxifen treatment.

(C) Total cell number counting of BM and SP (left panel: BM (n=10); right panel: SP (n=5)), and blood count analysis in the PB of WT and *Ptip*$^{-/-}$ mice at 4 weeks after tamoxifen treatment (n = 13-14).

(D) Percentages of different populations of mature lineage cells in PB, BM, and SP of WT and *Ptip*$^{-/-}$ mice at 4 weeks after tamoxifen treatment (PB and BM, n = 10; SP, n = 4-5).

(E) Frequencies of different stem and progenitor cell populations in BM from WT and *Ptip*$^{-/-}$ mice at 4 weeks after tamoxifen treatment (n=10).

(F) Total cell numbers (low) of different stem and progenitor cell populations in BM from WT and *Ptip*$^{-/-}$ mice at 4 weeks after tamoxifen treatment (n=10).

Figure S3. Loss of Ptip reduces HSC long-term competitive activity in vivo.

(A) Representative peripheral blood analysis of the chimeric mice was performed by flow cytometry after the bone marrow transplantation from WT and *Ptip*$^{-/-}$ mice at 16 weeks.

(B and C) Percentage and total numbers of donor-derived stem cell (left) and progenitor (right) compartments in the bone marrow of recipients at 16 weeks after BMT.

(D) Homing assay showing the comparable homing capability of WT and *Ptip*$^{-/-}$ BM. Flow cytometry analysis for different donor-derived cells in BM of recipient mice at 24h after BMT (n = 5).

Figure S4. Loss of Ptip leads to an increase lysosomal degradative potential

(A) Volcano map showing differential expression of *Ptip* targets in WT and *Ptip*$^{-/-}$ LT-HSCs.

(B) Gene ontology (GO) enrichment analysis of terms enriched in different pathways with a significantly up- and down-regulated gene in *Ptip*$^{-/-}$ LT-HSCs.

(C) Gene sets enrichment analysis (GSEA) plot showing enrichment of gene sets of TGFβ signaling pathway, Hematopoietic cell lineage, and Cell cycle.

(D) qRT-PCR validation of the effect of Ptip on the expression levels of TGFβ targets: *Mycn, Myc, Cdk6, p21, p27,* and *p57*.

(E) Representative immunofluorescent confocal images of LAMP2 and DAPI (left; bar, 2μm; arrow shows co-localization) and quantification (right; n = 3; 153-193 individual cells).

Figure S5. Ptip coordinates TGFβ signaling in regulating HSC quiescence

(A) Pearson's correlation between PTIP and SMAD3 and CDK6 from single-cell RNA-seq data (GSE157591).

(B) Co-immunoprecipitation (CoIP) of HA-SMAD3 and Flag-PTIP from HEK-293 extract the following incubation with HA or Flag antibodies. Immunoprecipitated immunoblotted with the indicated antibodies.

(C) Immunoblotting showing p-Smad2, Smad2/3 expression in total BM cells upon TGFβ1(left) or LY354947(right) treatment.

(D) Co-immunoprecipitation (CoIP) of HA-SMAD3 and Flag-PTIP from HEK-293 extract the following incubation with HA or Flag antibodies. Cells were treated with TGFβ1 and LY364947 respectively. Immunoprecipitated immunoblotted with the indicated antibodies.

(E) Comparison of LT-HSC cell cycle stage (n =4) after TGFβ signaling inhibition with LY364947.

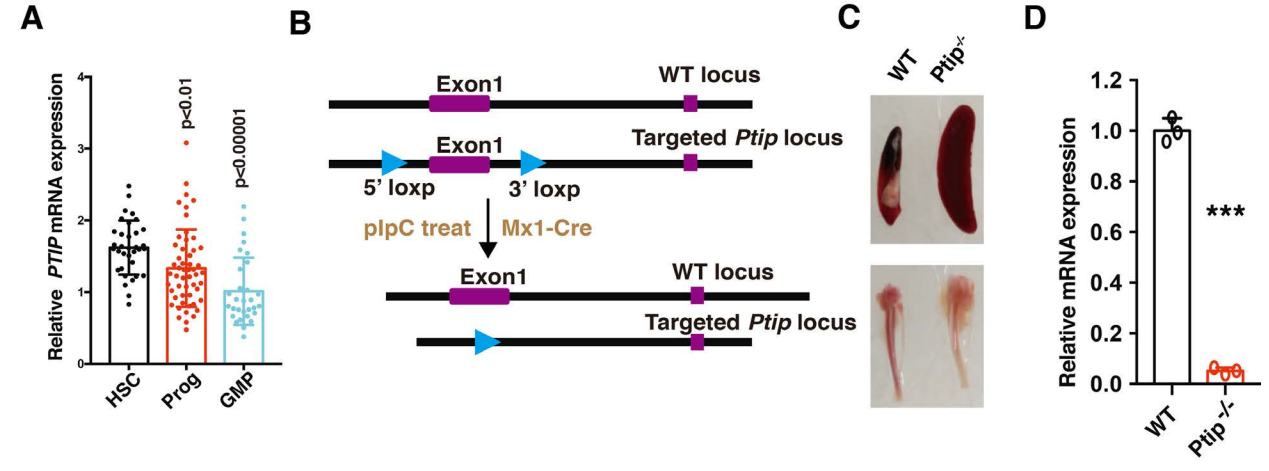
(F) qRT-PCR validation of the effect of *Ptip* on the expression levels of Lysosome targets: *Cdk6, c/n-Myc, p21, p27* and *p57* upon PBS and TGFβ1 treatment.

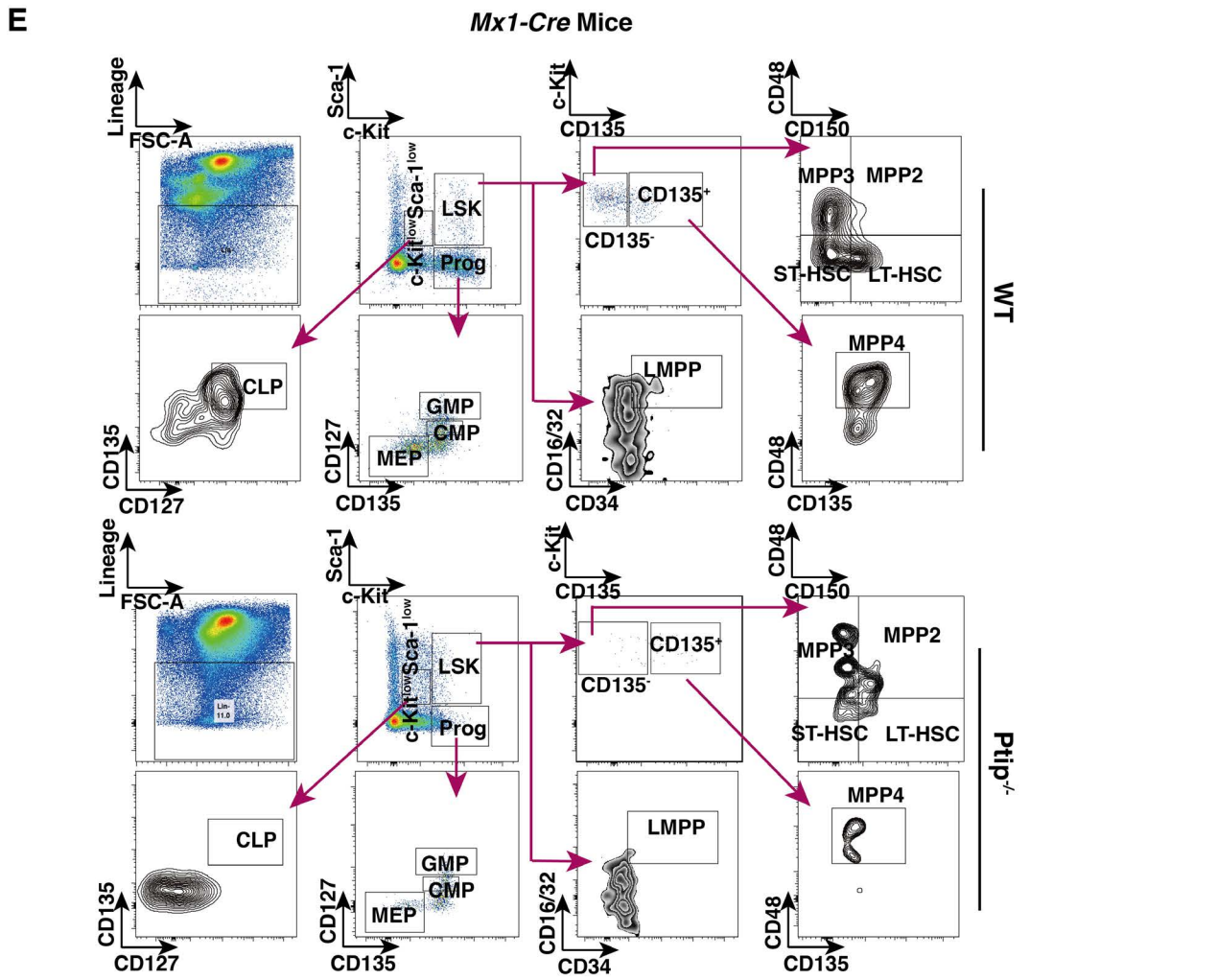
(G) qRT-PCR validation of the effect of *Ptip* on the expression levels of Lysosome targets: *Dram2, Ctsd, Gm2a* and *Ctsb* upon PBS and TGFβ1 treatment.

Supplementary Table S1 Primer sequences using in this study

Name	Sequence 5'-3'
Ptip-F	CCGAAGTTCCAGAGGAGCTA
Ptip-R	AATAACCTGCGGGTCGATGT
Cdk6-F	CTGGTGTGAGATGTTATCATT
Cdk6-R	TAGTCAGAGCAGGAAGTG
P21-F	AATCCTGGTGATGTCCGACC
P21-R	TTGCAGAAGACCAATCTGCG
P27-F	TATGGAAGAAGCGAGTCAGC
P27-R	GCGAAGAAGAATCTTCTGCAG
P57-F	AGGTAGCGAGGTGGATCTGTC
P57-R	GGCCTCTGATTCCCGAGGA
Tfeb-F	CCACCCCAGCCATCAACAC
Tfeb-R	CAGACAGATACTCCCGAACCTT
Myc-F	ATGCCCCTCAACGTGAACTTC
Myc-R	CGCAACATAGGATGGAGAGCA
Dram2-F	GCTGTCCTTGCCTTTAGTATGG
Dram2-R	AGATAACCAACAGTAGTCGGACC
Ankrd27-F	TGGCATTGTCCTAGTGCCTTG
Ankrd27-R	AGCCTTTCCATCTAAGGTCTGA
Smpd1-F	TGGGACTCCTTTGGATGGG
Smpd1-R	CGGCGCTATGGCACTGAAT
Gns-F	CGGTGTGCGGCTATCAGAC
Gns-R	CAGGGCATACCAGTAACTCCA
Ctsb-F	GAAGTCCCAGCTTGAATCGAAAGAA
Ctsb-R	TAGGAAGACAGGGTCAAAGGCTTGT
Mycn-F	ACCATGCCGGGGATGATCT
Mycn-R	AGCATCTCCGTAGCCCAATTC
Gapdh-F	TGGATTTGGACGCATTGGTC
Gapdh -R	TTTGCACTGGTACGTGTTGAT

Figure S1


A
Relative PTIP mRNA expression
p<0.01
p<0.00001
HSC
Prog
GMP
B
Exon1
WT locus
Exon1
Targeted Ptip locus
5' loxp
3' loxp
pIpC treat
Mx1-Cre
Exon1
WT locus
Targeted Ptip locus
C
WT
Ptip-/-
D
Relative mRNA expression
***
WT
Ptip -/-


E
Mx1-Cre Mice
Lineage
FSC-A
Sca-1
c-Kit
c-Kit
CD135
CD48
CD150
LSK
Prog
CD135+
CD135-
MPP3
MPP2
ST-HSC
LT-HSC
MPP4
CLP
GMP
CMP
MEP
LMPP
CD135
CD127
CD16/32
CD34
WT
Ptip-/-

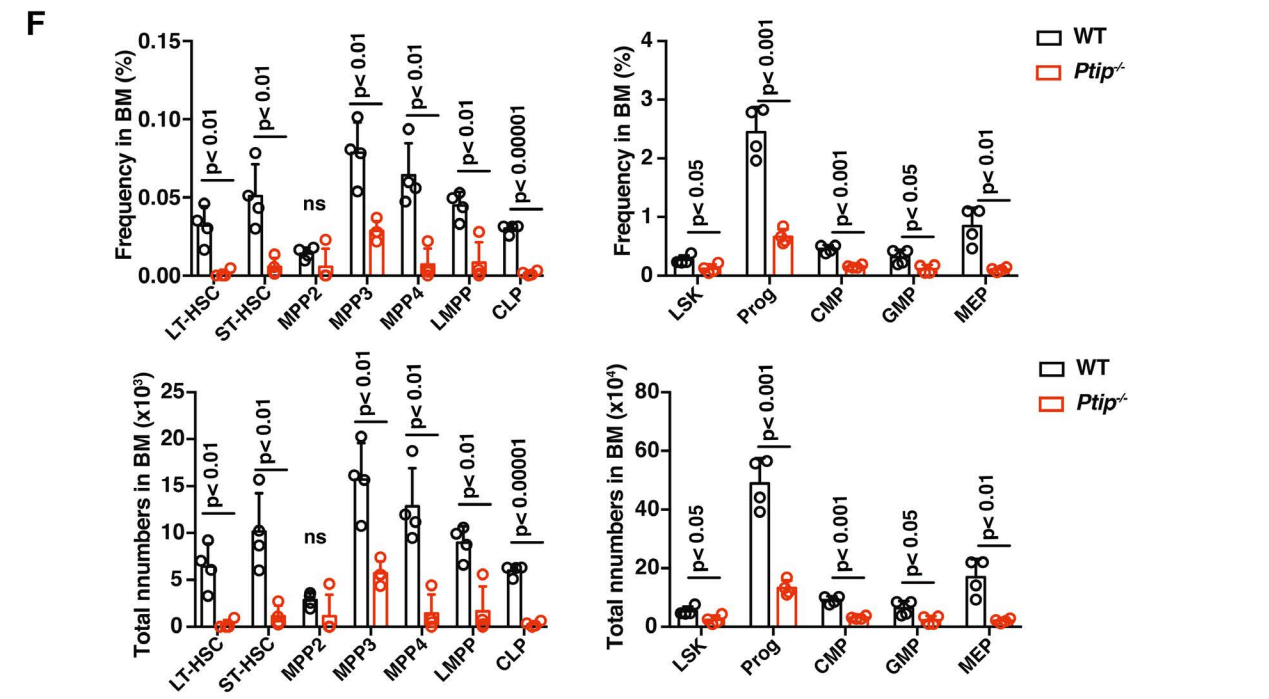

F
Frequency in BM (%)
Total nnumbers in BM (x10³)
Total nnumbers in BM (x10⁴)
WT
Ptip-/-
p< 0.01
p< 0.00001
ns
p< 0.05
p< 0.001
LT-HSC
ST-HSC
MPP2
MPP3
MPP4
LMPP
CLP
LSK
Prog
CMP
GMP
MEP

Figure S2

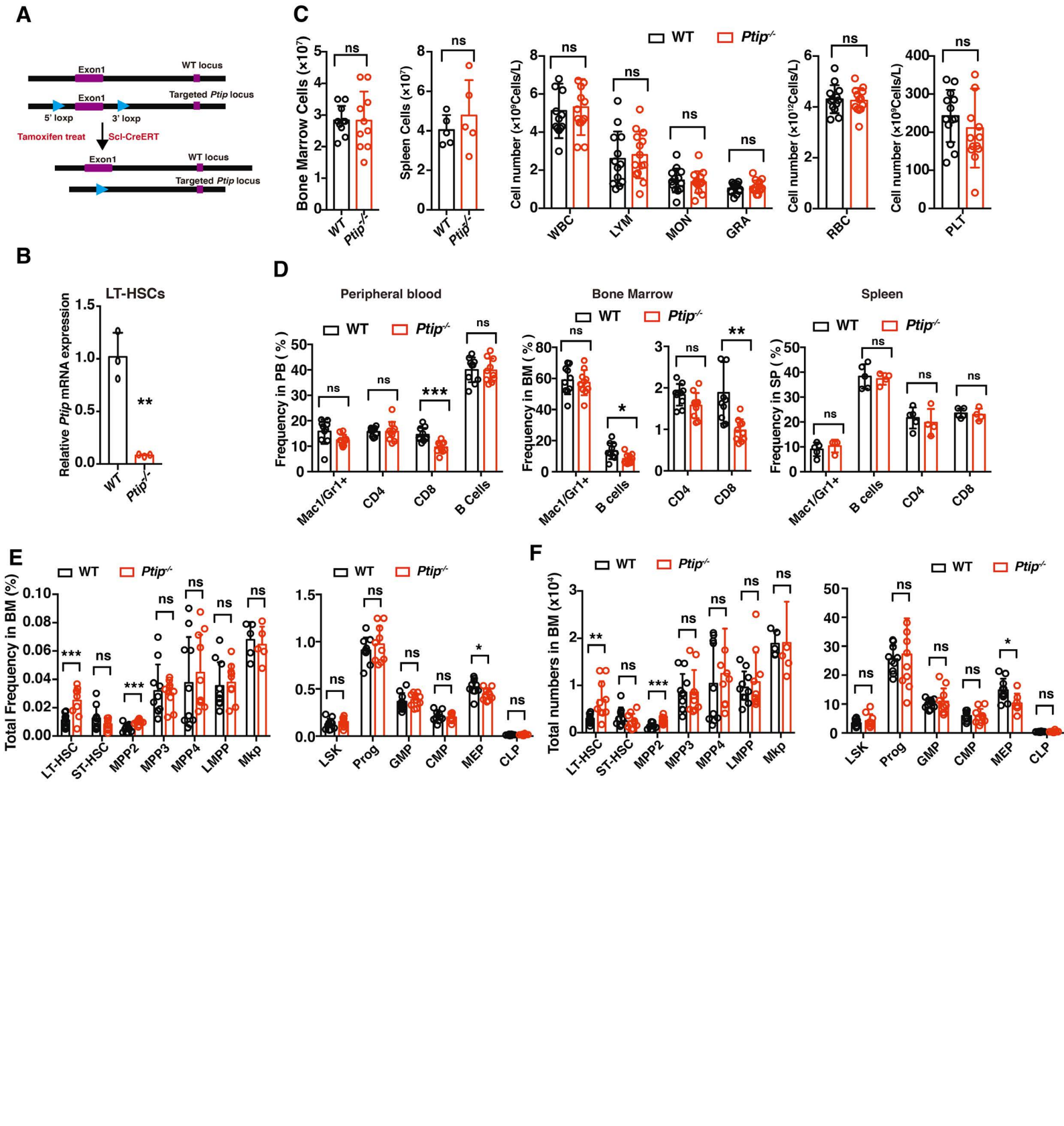
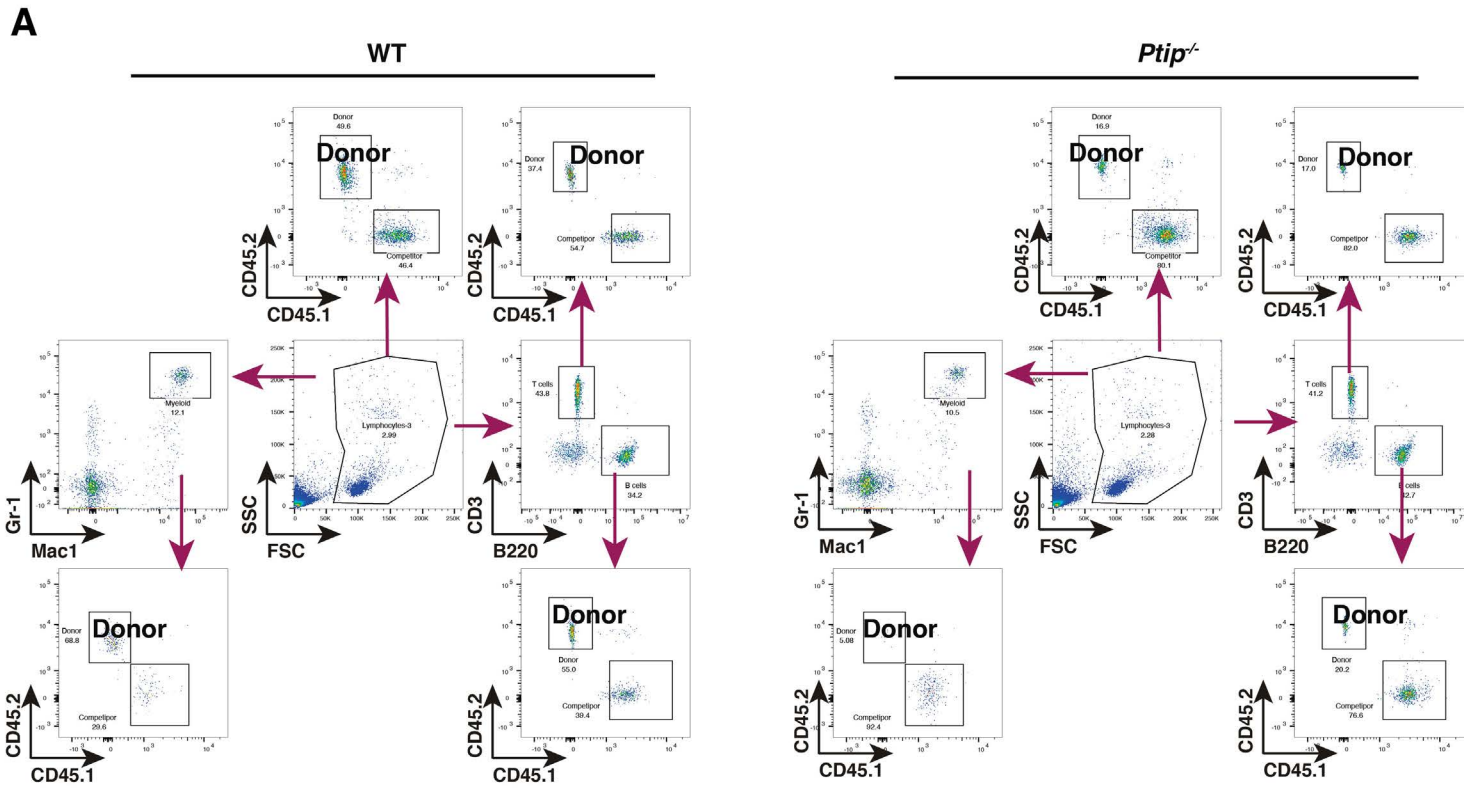
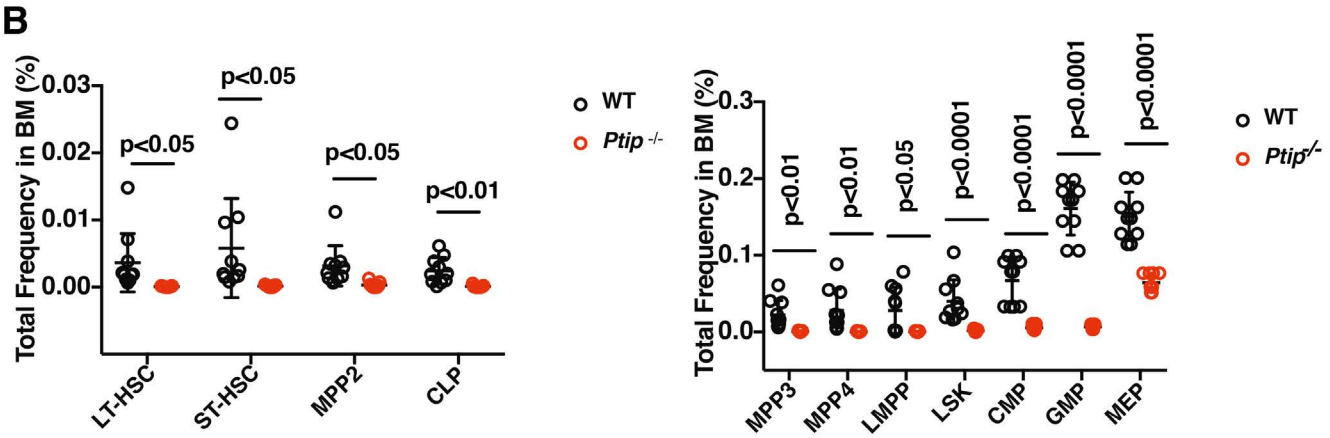
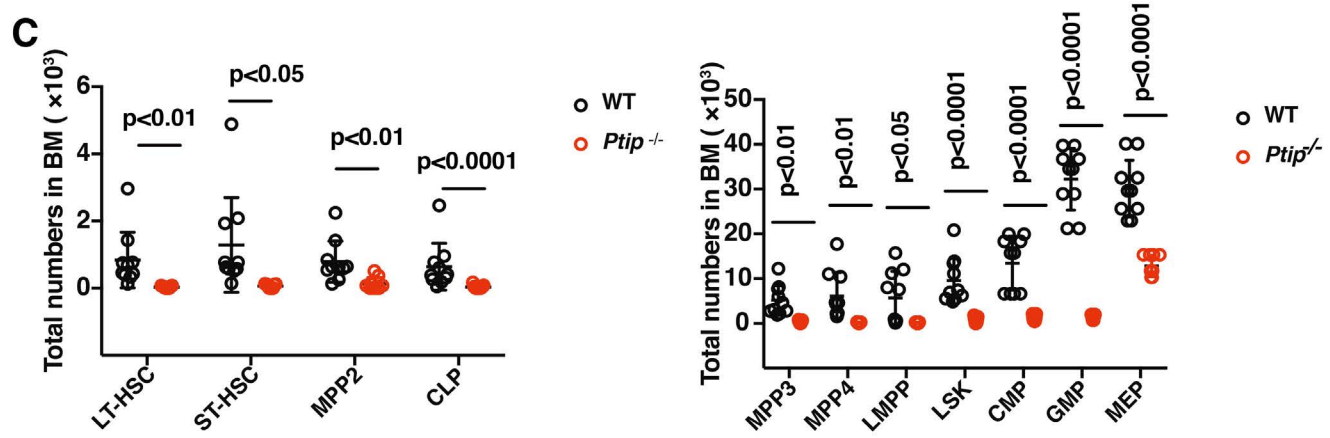

A
Exon1
WT locus
Exon1
Targeted Ptip locus
5' loxp
3' loxp
Tamoxifen treat
Scl-CreERT
Exon1
WT locus
Targeted Ptip locus
B
LT-HSCs
Relative Ptip mRNA expression
WT
Ptip-/-
C
WT
Ptip-/-
Bone Marrow Cells (×10^7)
Spleen Cells (×10^7)
Cell number (×10^9Cells/L)
WBC
LYM
MON
GRA
Cell number (×10^12Cells/L)
RBC
Cell number (×10^9Cells/L)
PLT
ns
D
Peripheral blood
Bone Marrow
Spleen
Frequency in PB (%)
Frequency in BM (%)
Frequency in SP (%)
Mac1/Gr1+
CD4
CD8
B Cells
B cells
E
Total Frequency in BM (%)
LT-HSC
ST-HSC
MPP2
MPP3
MPP4
LMPP
Mkp
LSK
Prog
GMP
CMP
MEP
CLP
F
Total numbers in BM (×10^4)

Figure S3


A
WT
Ptip-/-
Donor
Competitor
CD45.2
CD45.1
Gr-1
Mac1
SSC
FSC
CD3
B220
Myeloid
Lymphocytes-3
T cells
B cells


B
Total Frequency in BM (%)
WT
Ptip -/-
Ptip-/-
p<0.05
p<0.01
p<0.0001
LT-HSC
ST-HSC
MPP2
CLP
MPP3
MPP4
LMPP
LSK
CMP
GMP
MEP


C
Total numbers in BM (×10³)
WT
Ptip -/-
Ptip-/-
p<0.05
p<0.01
p<0.0001
LT-HSC
ST-HSC
MPP2
CLP
MPP3
MPP4
LMPP
LSK
CMP
GMP
MEP

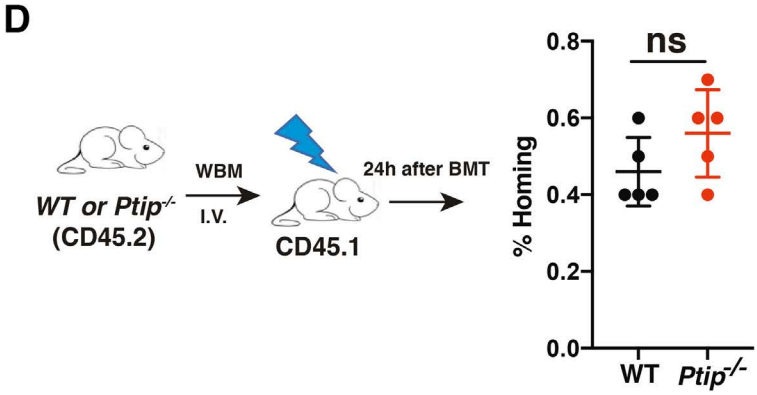
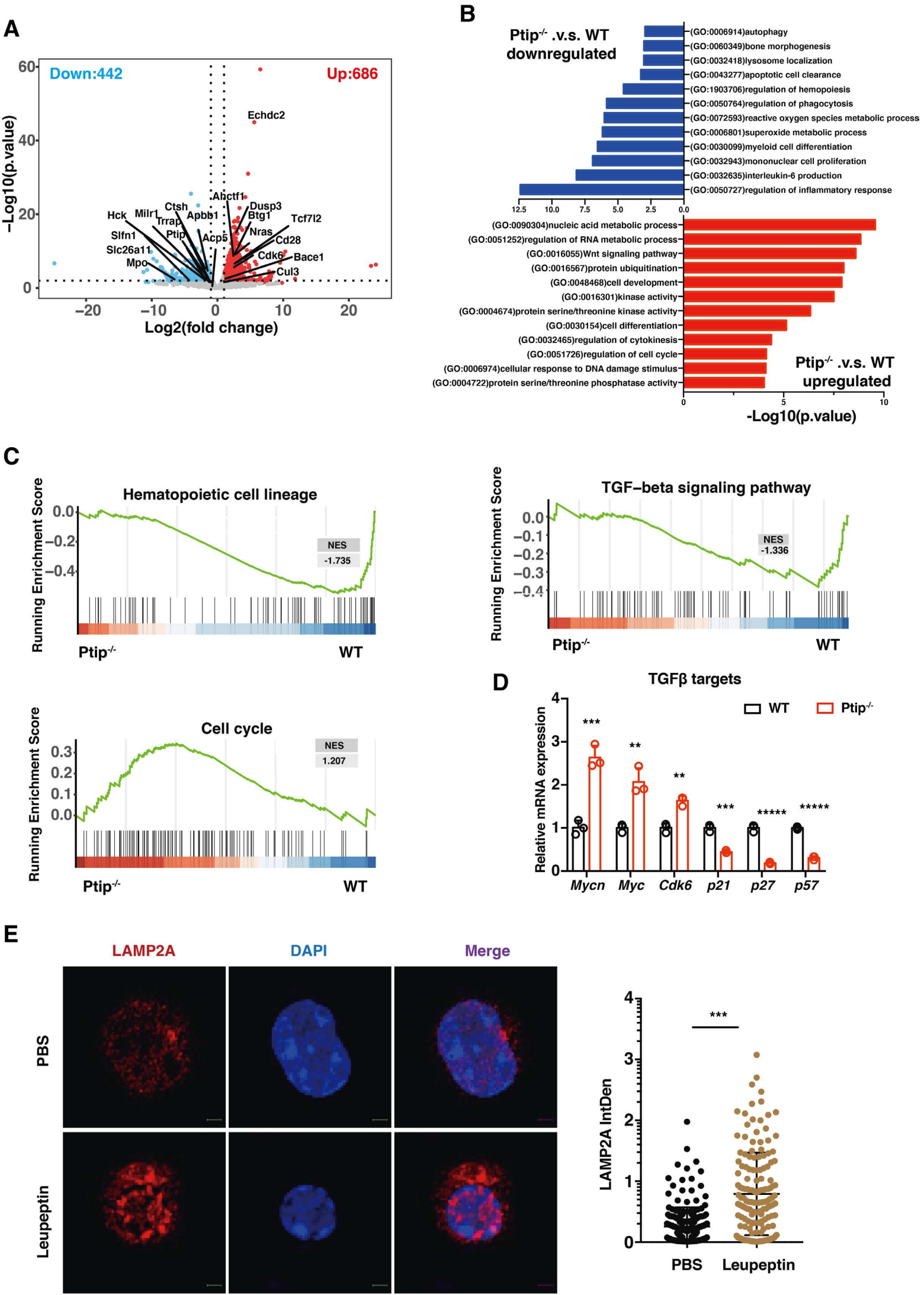

D
WT or Ptip-/-
(CD45.2)
WBM
I.V.
24h after BMT
CD45.1
% Homing
ns
WT
Ptip-/-

Figure S4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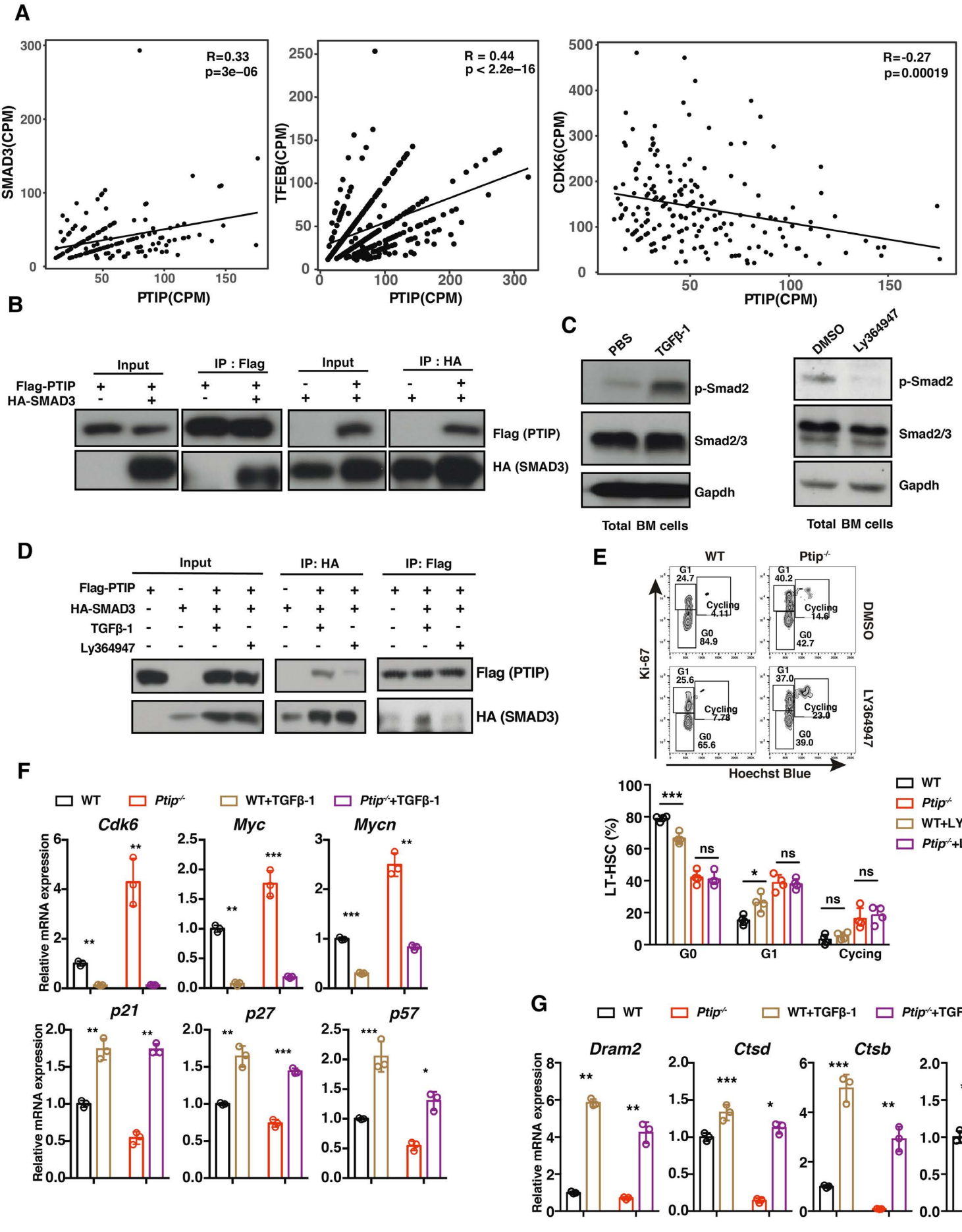
Down:442
Up:686
Echdc2
Ahctf1
Dusp3
Btg1
Tcf7l2
Nras
Cd28
Cdk6
Bace1
Cul3
Ctsh
Apbb1
Hck
Milr1
Trrap
Slfn1
Ptip
Acp5
Slc26a11
Mpo
−Log10(p.value)
Log2(fold change)
B
Ptip-/- .v.s. WT
downregulated
(GO:0006914)autophagy
(GO:0060349)bone morphogenesis
(GO:0032418)lysosome localization
(GO:0043277)apoptotic cell clearance
(GO:1903706)regulation of hemopoiesis
(GO:0050764)regulation of phagocytosis
(GO:0072593)reactive oxygen species metabolic process
(GO:0006801)superoxide metabolic process
(GO:0030099)myeloid cell differentiation
(GO:0032943)mononuclear cell proliferation
(GO:0032635)interleukin-6 production
(GO:0050727)regulation of inflammatory response
(GO:0090304)nucleic acid metabolic process
(GO:0051252)regulation of RNA metabolic process
(GO:0016055)Wnt signaling pathway
(GO:0016567)protein ubiquitination
(GO:0048468)cell development
(GO:0016301)kinase activity
(GO:0004674)protein serine/threonine kinase activity
(GO:0030154)cell differentiation
(GO:0032465)regulation of cytokinesis
(GO:0051726)regulation of cell cycle
(GO:0006974)cellular response to DNA damage stimulus
(GO:0004722)protein serine/threonine phosphatase activity
Ptip-/- .v.s. WT
upregulated
-Log10(p.value)
C
Hematopoietic cell lineage
Running Enrichment Score
NES
-1.735
Ptip-/-
WT
TGF−beta signaling pathway
NES
-1.336
Cell cycle
NES
1.207
D
TGFβ targets
WT
Ptip-/-
Relative mRNA expression
Mycn
Myc
Cdk6
p21
p27
p57
E
LAMP2A
DAPI
Merge
PBS
Leupeptin
LAMP2A IntDen
PBS
Leupeptin

Figure S5


A
R=0.33
p=3e−06
SMAD3(CPM)
PTIP(CPM)
R = 0.44
p < 2.2e−16
TFEB(CPM)
PTIP(CPM)
R=-0.27
p=0.00019
CDK6(CPM)
PTIP(CPM)
B
Input
IP : Flag
Input
IP : HA
Flag-PTIP
HA-SMAD3
Flag (PTIP)
HA (SMAD3)
C
PBS
TGFβ-1
DMSO
Ly364947
p-Smad2
Smad2/3
Gapdh
Total BM cells
Total BM cells
D
Input
IP: HA
IP: Flag
Flag-PTIP
HA-SMAD3
TGFβ-1
Ly364947
Flag (PTIP)
HA (SMAD3)
E
WT
Ptip-/-
G1 24.7
Cycling 4.11
G0 84.9
G1 40.2
Cycling 14.6
G0 42.7
G1 25.6
Cycling 7.78
G0 65.6
G1 37.0
Cycling 23.0
G0 39.0
DMSO
LY364947
Ki-67
Hoechst Blue
LT-HSC (%)
G0
G1
Cycing
WT
Ptip-/-
WT+LY364947
Ptip-/-+LY364947
F
WT
Ptip-/-
WT+TGFβ-1
Ptip-/-+TGFβ-1
Cdk6
Myc
Mycn
p21
p27
p57
Relative mRNA expression
G
WT
Ptip-/-
WT+TGFβ-1
Ptip-/-+TGFβ-1
Dram2
Ctsd
Ctsb
Gm2a
Relative mRNA expression