SUPPLEMENTARY APPENDIX

A Tie2-Notch1 signaling axis regulates regeneration of the endothelial bone marrow niche

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A Tie2-Notch1 signaling axis regulates regeneration of the endothelial bone marrow niche

By Lijian Shao et al.

SUPPLEMENTAL MATERIALS

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MATERIALS AND METHODS

Mice

Male C57BL/6J (or CD45.2) mice and B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (or CD45.1/2) mice were purchased from Jackson Lab (Bar Harbor, MA). Breeding pairs of Notch$^{+/\Delta TAD}$ mice were generated as in (Gerhardt et al. 2014) and Notch$^{+/-}$ mice were obtained from Jackson Lab (Bar Harbor, MA). *Notch1* conditional floxed mice were generated by crossing *Notch1*$^{f/f}$ (*generous gift from Dr. Jan Kitajewski)* to VE-Cadherin CreERT2 mice (Cancer Technology). RBPJ conditional floxed mice were generated by crossing RBPJ$^{f/f}$ to PDGFR β CreERT2 mice (*generous gift from Dr. Henar Cuervo).* All mice were backcrossed to C57BL/6J mice for more than 10 generations before being used in our study. Mice were housed at the University of Illinois at Chicago (UIC) AAALAC-certified animal facilities. They received food and water *ad libitum*. The Institutional Animal Care and Use Committees of UIC approved all experimental procedures used in this study.

5-FU treatment

5-Fluorouracil (5-FU) Sigma–Aldrich (St. Louis, MO, USA) was diluted in PBS and injected intraperitoneally to mice (150mg/kg). Male and female mice were monitored daily for 28 days following the first 5-FU injection. Mice that lost more than 20% of their body weight or exhibited signs of reduced mobility were euthanized before they reach the expected experimental end point according to our experimental plan.

Irradiation

Mice were exposed to a lethal (9.0Gy) dose of TBI in a Mark I ^{137}Ce γ-irradiator (JL Shepherd, Glendale, CA, USA) at a dose rate of 6.38Gy/min. Mice were irradiated on a rotating platform.

Tamoxifen treatment

To conditionally delete *Notch1* from endothelial cells, tamoxifen (Sigma) was dissolved in corn oil and given at a dose of 80 mg/kg to *Notch1*$^{f/f}$;VE-Cadherin Cre^{ERT2+} and Ve-Cadherin Cre^{ERT2-} mice via five consecutive IP injections, administered daily. Seven days after the last tamoxifen injection, tamoxifen-treated mice were treated with PBS(vehicle) or 5-FU treatment. To conditionally delete RBPJ from PDGFR β^+ cells, Tamoxifen was delivered through maternal milk by administering 250 μg/kg to the nursing mom at postnatal day 1, 2 and 3. Five-week old mice were then administered 5-FU treatment.

Peripheral blood counting

Blood was obtained through retroorbital bleeding and transferred to EDTA-coated tubes. Peripheral blood cell numbers were determined using a HemaVet analyzer (Drew Scientific, Dallas, TX) according to the manufacturer's instruction.

Preparation of BM mononuclear cells (BM-MNCs) and Thymus MNCs

Femur, tibia and thymus were harvested from mice immediately after euthanasia by 3-5% isoflurane and cervical dislocation. Bone marrow cells were

flushed into PBS containing 2% fetal bovine serum using a 22-gauge needle and syringe. Single cell suspensions from thymus were prepared by mincing and gently passing cells through 70 μm size of cell strainers. ACK lysing buffer (Invitrogen) was used to remove red blood cells to isolate mononuclear cells.

Flow cytometry

Bone marrow: BM-MNCs were isolated as described above***.*** Cells were preincubated with anti-CD16/32 antibody to block the Fcγ receptors. They were then incubated with anti-CD3e, anti-CD45R/B220, anti-Gr-1, anti-CD11b, and anti-Ter119 antibodies, and stained with appropriate antibody combinations as described in Supplemental Table 1, to analyze LSK cells ($Lin^-Sca1^+c\text{-}kit^+$ cells), HSCs ($CD150^+CD48^-$LSK cells) and CLP ($Lin^-Sca1^{low}c\text{-}kit^{low}CD135^+CD127^+$). The frequencies of LSK cells, HSCs and CLP were analyzed by Fortessa LSRII analyzer. Absolute cell numbers were calculated by multiplying the percentages of each population by of the total numbers of bone marrow cells from each mouse. DAPI was used to exclude dead cells. **Thymus:** Thymocyte suspensions were made as described above***.*** Thymocytes were preincubated with anti-CD16/32 antibody to block the Fcγ receptors and then incubated with anti-CD45R/B220, anti-Gr-1, anti-CD11b, and anti-Ter119 antibodies, and stained with appropriate antibody combinations as described in Supplemental Table 1, to analyze ETP cells ($Lin^-c\text{-}kit^+CD25^-CD44^+$) and DN3 cells ($Lin^-CD4^-CD8^-CD25^+CD44^-$). Frequencies of ETP and DN3 cells were analyzed with a Fortessa LSRII analyzer (BD Biosciences) and FlowJo (FlowJo, Ashland, OR)

software. Absolute cell numbers of each population from each mouse were calculated by percentages of each population multiply total numbers of thymocytes from each mouse. DAPI was used to exclude dead cells. **Peripheral blood:** Peripheral blood was collected via retroorbital bleeding and red blood cells were removed by ACK lysis buffer. Cells were preincubated with anti-CD16/32 antibody to block the Fcγ receptors and then stained with anti-CD45.2, anti-CD45.1, anti-CD45R/B220, anti-CD4, anti-CD8, anti-CD11b and anti-Gr-1 antibodies. Data analysis was performed using a Fortessa LSRII analyzer and FlowJo software. DAPI was used to exclude dead cells. Detailed antibody information is provided in the Supplemental Table 1.

Primary bone endothelial cells (pBECs) and osteoblasts:

Femora and tibiae were collected, and bone marrow cells were completely flushed out. Bones were cut into small pieces and digested with 0.1% collagenase type I and 15 μg /ml DNase I (Sigma–Aldrich, St. Louis, MO, USA). Digested bone cells were stained with anti-CD45, anti-Ter119, anti-CD31 antibodies. Osteoblasts and mesenchymal stem cells were identified by staining with anti-CD45, anti-Ter119, anti-CD31, anti-Sca-1, anti-CD51 antibodies. Data analysis was performed using a Fortessa LSRII analyzer and FlowJo software. DAPI was used to exclude dead cells.

Isolation of CD25$^+$ thymocytes and pBECs

Cell suspensions from thymus and digested bones was incubated with CD25 antibody- and CD31 antibody-labelled dynabeads (Invitrogen) for 30 min

at 4℃, respectively. $CD25^+$ thymocytes and bone $CD31^+$ cells (pBECs) were isolated and used for the following western blot and RT-PCR.

Retrovirus production

Retrovirus was produced after transient transfection of human embryonic kidney (HEK) 293T cells with PMIGR1 or PMIGR1-ICNΔTAD along with the packaging plasmids pCL-Eco using FuGEN6 (Promega) according to manufacturer's protocol. The supernatants containing viral particles were collected 48 h after transfection and filtered through 0.45 μm filter.

Bone-marrow derived endothelial cell culture (cBEC)

Bone-marrow derived endothelial cells (cBECs) were derived from C57BL/6 mouse and purchased from cell biologicals Inc (cat#, C57-6221) and cultured in endothelial cell medium. cBECs were treated with GSI (1 μM, Sigma), Tie2 kinase inhibitor (1 μM, Abcam), Ang1 (300 ng/ml, Peprotech) or 5-FU (100 μM, Sigma) as noted in Figure legends. cBECs were infected with retroviral particles containing PMIGR1 or PMIGR1-Notch1 ICNΔTAD under centrifugation (900xg) at 32℃ for one hour. GFP^+ cells were sorted out and expanded in endothelial cell medium. Expanded GFP^+ cells were named cBEC-PMIGR1 and cBEC-ICNΔTAD, respectively. To test 5-FU-induced apoptosis in endothelial cells, both cBEC-PMIGR1 and cBEC-ICNΔTAD cells were treated with 5-FU (100 μM) or DMSO for 24 hours. Cells were stained with Annexin V-APC and DAPI. Cellular apoptosis was measured by flow cytometry 24 and 48

hours after 5-FU treatment. Total cell number were counted and the expression of apoptotic related genes were analyzed by RT-PCR.

HSC transplantation

Sorted HSCs (350 cells) from 5-week old wild type and Notch$^{+/\Delta TAD}$ mice (CD45.2) were retro-orbitally transplanted with congenic splenocyte support cells ($4x10^5$) into 9.0 Gy irradiated congenic recipients (CD45.1/2). For secondary transplants, $1x10^6$ bone marrow cells from primary transplant recipients were transplanted into lethal dose irradiated secondary recipient mice (CD45.1/2). All cells were washed with cold PBS prior to injection. Mice were maintained on antibiotic water for 1 week before transplantation and 2 weeks post transplantation. To analyze engraftment ability of transplanted HSCs, peripheral blood was collected via retroorbital bleeding at 1, 2 and 3 months after transplantation.

Apoptotic assay.

Primary bone endothelial cells (pBECs) were isolated and incubated with anti-CD16/32 at 4^{o}C for 15 min to block the Fc-γ receptors and then stained with antibodies against CD45, Ter119 and CD31 in the dark. After annexinV staining with a kit from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions, apoptotic cells in CD45$^-$Ter119$^-$CD31$^+$ population were analyzed with a Fortessa LSRII analyzer.

Western blot analysis

Whole-cell lysates were prepared with RIPA buffer with protease inhibitors. Protein concentration was determined with the Bio-Rad protein assay dye reagent (Bio-Rad). Proteins were separated using SDS-PAGE and transferred to PVDF membranes. Antibodies used for Western blot were cleaved Notch1 (Val1744) antibody (Cell Signaling Technology, no. 2421), Notch1 (C37C7) antibody (Cell Signaling Technology, no. 3439), VE-Cadherin (Santa Cruz Biotechnology, no. SC-6458), Tie2 (Novus, no. NBP1-69753), phosphorylated Tie2 Y992 (Novus, no. AF2720), GAPDH (Sigma), Jag1 (Cell Signaling Technology, no. 2620), Dll4 (Novus, no. NB600-892) and secondary anti-rabbit-HRP or anti-goat-HRP or anti-mouse-HRP (Pierce). Blots were visualized with SuperSignal west femto chemilumenscence substrate (Thermo Scientific). The band intensities of ICN and ICNΔTAD in Notch1$^{+/\Delta TAD}$ endothelial cells were quantitated by ImageJ software.

Luciferase assay

U2OS cells were seeded 1x10^4 cells per well in 96-well plates, with 4 wells per condition. Four hours after seeding, the medium was changed, and cells were transfected with Fugene-6 and 25–50 ng of plasmid DNA per well, including the pGL3, pGL3-CSL4X, pcDNA3 (EV), pcDNA3-ICN, pcDNA3-ICNΔTAD, and internal transfection control pRL-TK. One day post-transfection, the medium was changed, and 48 h post-transfection, the cells were lysed, and firefly luciferase levels were measured in one step with Britelite plus (Perkin-Elmer). Renilla luciferase was measured with Stop-n-Glo reagent from

Promega. All readings were made using a Promega Glomax 96-microplate Luminometer at 1-sec intervals.

RT-qPCR

RNA was extracted using the Qiagen RNeasy minikit or microkit. cDNA was synthesized from RNA with the Super Script III kit (Invitrogen). Transcripts were amplified with SYBR Green PCR master mix (Applied Biosystems), and qPCR was performed on the ABI Prism 7900HT system (Applied Biosystems). GAPDH was used as a housekeeping gene. Primer sequences are provided in the Supplemental Table 2.

Chromatin immunoprecipitation assays (CHIP)

CHIP was performed as described previously (Yahiro-Ohtani et al. 2009). In brief, $2x10^6$ primary endothelial cells for each immunoprecipitation sample were fixed and immunoprecipitated with control antibody (rabbit IgG, Santa Cruz Biotechnologies) or anti-Notch1 antibody (Cell Signaling Technology, no. 2421). Local ChIP was performed using qPCR primers flanking RBPJ binding sites at Myc NDME (Yashiro-Ohtani et al. 2014), Hes1, Hey1, and Dtx1.GAPDH was used as an internal control as a non-Notch/RBPJ site. The DNA quantity recovered from each CHIP sample is shown as the relative value to the DNA input sample, which was not immunoprecipitated. The sequences of primers are provided in the Supplemental Materials (Supplemental Table S3).

Histology analysis and Immunohistochemistry

Tibia and femur were fixed overnight with 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 24 hours and decalcified in 14% EDTA for 10 days. Bones were then embedded in paraffin and 5-μm longitudinal sections were obtained. After de-paraffinization and rehydration, sections were processed to stain with hematoxylin and eosin Y. Pictures were taken at 40x magnification with an Olympus BX51 microscope.

For immunofluorescence staining on bone sections, sections were rehydrated in PBS and antigen retrieval was performed in the citrate solution at 95°C for 15 min. After washing with PBS, sections were blocked with 10% goat serum for 1 h at room temperature and then incubated with anti-mouse CD31 primary antibody (1:50, Dako) or anti-rat endomucin primary antibody (1:100, Santa Cruz Biotechnology) overnight at 4°C. Sections were washed three times with PBS and incubated with Texas Red or FITC conjugated secondary antibody for 1 h at room temperature. After three wash with PBS, DAPI was applied and sections were mounted with mounting media (Burlingame, CA, Vector). Stained sections were analyzed at 40x magnification with an Olympus BX51 fluorescence microscope.

For immunofluorescence staining on cultured bone marrow endothelial cells (cBECs), confluent cBECs were fixed with 4% paraformadehydrate (PFA) for 10 min at room temperature. Fixed cBECs were then permeabilized with 0.25% Triton-X-100 for 10 min at room temperature and blocked with 1% BSA for 15 min at room temperature. Primary antibody goat anti-mouse VE-Cadherin

and Alex 488 conjugated rabbit anti-goat secondary antibody were applied. DAPI was used to stain nuclei. Cells were analyzed at 40x magnification with an Olympus BX51 fluorescence microscope.

Bone microCT

Micro-Computed Tomography (microCT) images of femur bones from mice was performed on the X-CUBE (Molecubes NV., Gent, Belgium) by the Integrated Small Animal Imaging Research Resource (iSAIRR) at The University of Chicago. Spiral high-resolution CT acquisitions were performed with an x-ray source of 50-kVp and 460 μA. Volumetric CT images were reconstructed in a 1400 × 1400 × 800 format with voxel dimensions of 50 μm^3. Images were analyzed using VivoQuant 3.5 patch 2 (InviCRO, LLC, Boston, USA), and MATLAB 2017b (MathWorks, Natick, Massachusetts, USA). Cortical thickness was measured at the femur mid-diaphysis. Line profiles of the femoral endosteal and periosteal perimeters were obtained, and 80 thickness measurements were taken at evenly spaced interval.

Whole-mount imaging of the sternum vasculature

To label the vasculature we retro-orbitally injected each mouse with 5 μg of Alexa Fluor 647 anti-mouse CD31 (Biolegend) and 5μg of Alexa Fluor 647 anti-mouse CD144 (Biolegend) twenty minutes prior euthanasia. The sternum was collected and cleaned using a scalpel. Each sternum was cut into segments and divided into two halves by sectioning parallel to the frontal plane of the segment to expose the bone marrow. Each half-segment was fixed in 4%

paraformaldehyde (Sigma) in PBS for 60 minutes at 4ºC, washed three times in PBS and imaged immediately. Prior to imaging, each segment was glued to 35mm dishes and processed in a Nikon A1R Multiphoton Upright Confocal Microscope using a 10X, long-distance, water immersion objective, and NIS Elements AR software (version 4.50, Nikon). Z-stacks were acquired (2.3µm between slices) 20– 200µm, depending on the position of the bone. Each slice measured 1270µm in the x-axis and 1270µm in the y-axis and was 1024×1024 pixels. All images were acquired at room temperature. For Alexa Fluor 647, the wavelength of the excitation laser was 647 nm and emitted light detected between 660 to 720nm. Lmaris software (version 9.2, Bitplane) was used to generate 3D reconstructions and maximum projections from each image. Composite images of each sternum were assembled by stitching together the maximum projection images in Nikon NIS Elements AR software. Blood vessels numbers and length were counted manually in 3D reconstructions in order to be able to distinguish true vessels (with lumen) from vascular sheets (lacking lumen).

Statistical analysis.

The data were analyzed by analysis of variance (ANOVA). Differences among group means were analyzed by Student-Newman-Keuls multiple comparisons test after one- or two-way ANOVA. For experiments in which only single experimental and control groups were used, group differences were examined by unpaired Student's *t* test. Survival curves were constructed

using the Kaplan-Meier method and were compared using the Mantel-Cox test. Differences were considered significant at $p<0.05$. All analyses were done with GraphPad Prism from GraphPad Software.

Supplemental Tables

Table S1. List of antibodies used to stain various types of cells

Markers	Clone	Antibody isotype	Conjugate
CD45R/B220[1]	RA3-6B2	IgG_{2a}	PE
CD3[1]	17A2	IgG_1	PE
CD11b[1]	M1/70	IgG_{2b}	PE
Gr-1[1]	RB6-8C5	IgG_{2b}	PE
Ter-119[1]	Ter-119	IgG_{2b}	PE
CD45R/B220[1]	RA3-6B2	IgG_{2a}	FITC
CD45R/B220[1]	RA3-6B2	IgG_{2a}	APC
CD3e[1]	17A2	IgG_1	FITC
CD11b[1]	M1/70	IgG_{2b}	FITC
Gr-1[1]	RB6-8C5	IgG_{2b}	FITC
Ter-119[1]	Ter-119	IgG_{2b}	FITC
CD16/CD32[1]	93	IgG_{2a}	purified
CD45.2[1]	104	IgG_{2a}	FITC
CD45.2[1]	104	IgG_{2a}	Alexa Fluor 700
CD45.1[1]	A20	IgG_{2a}	PerCP-Cy5.5
Sca-11	D7	IgG_{2a}	PerCP-Cy5.5
c-kit[1]	2B8	IgG_{2b}	APC-CY7
c-kit[1]	2B8	IgG_{2b}	FITC

PDGFR-β [1]	APB5	IgG_{2a}	APC
CD150[1]	9D1	IgG_{2a}	PE-CY7
CD48[1]	HM481	IgG_{2a}	APC
CD48[1]	HM481	IgG_{2a}	Alexa Fluor 700
CD127[1]	A7R34	IgG_{2a}	APC
CD135[1]	A2F10	IgG_{2a}	PE
CD25[1]	3C7	IgG_{2b}	APC
CD44[1]	IM7	IgG_{2b}	PerCP-Cy5.5
CD4[1]	GK1.5	IgG_{2b}	PE-CY7
CD8a[1]	53-6.7	IgG_{2a}	APC-CY7
CD31[2]	390	IgG_{2a}	PE-CY7
CD31[1]	MEC13.3	IgG_{2a}	Alexa Fluor 647
CD144[1]	BV13	IgG_1	Alexa Fluor 647
CD51[1]	RMN-7	IgG_1	PE
Annexin V[1]	-	-	FITC

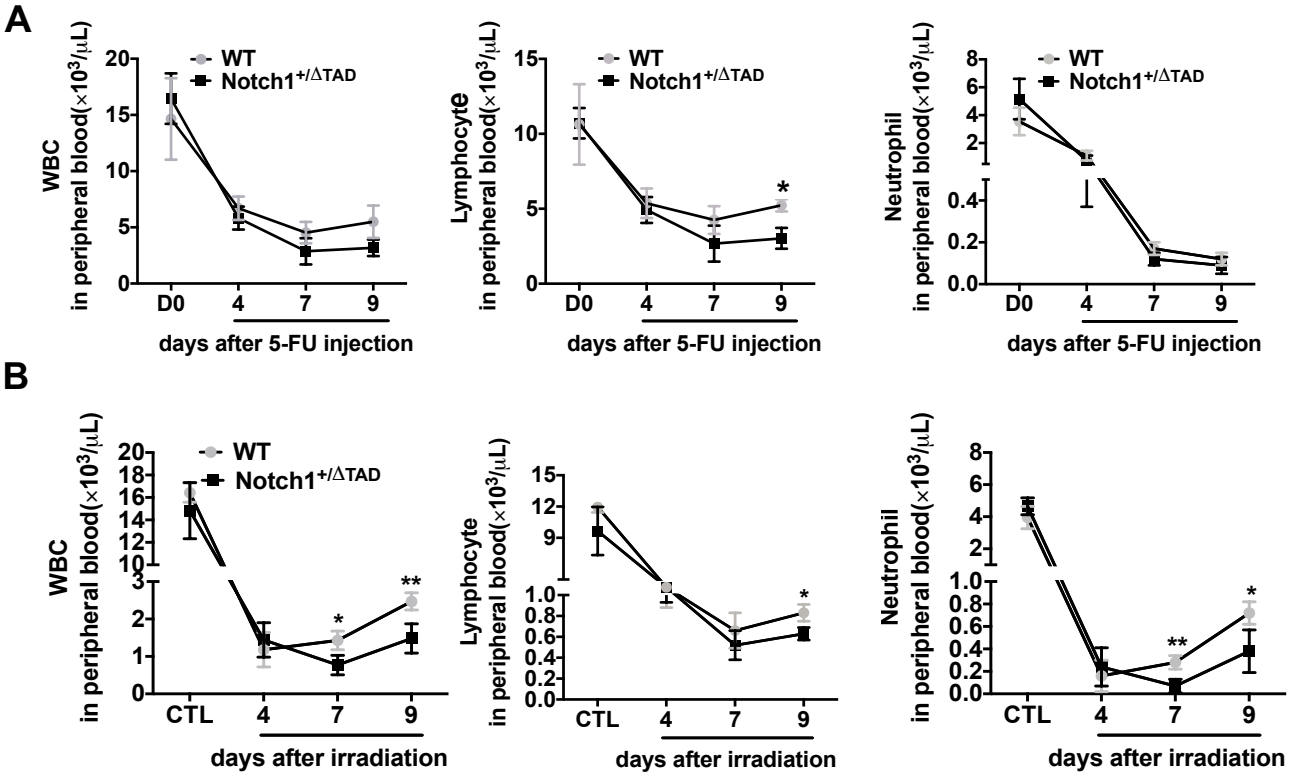
Footnotes: [1] Biolegend, San Diego, CA; [2]eBioscience, San Jose, CA.

Table S2. Sequences of the primers used for RT-qPCR

Genes	Forward sequences	Reverse sequences
Notch1	*5'-CTGTCCTCTGCCATATACAGGAGC-3'*	*5'-ACCTCGCAGGTTTGACCTTGCCAG-3'*
Tie2	*5'-GGGTTGCAGTGCAATGAAGCATGCC-3'*	*5'-TCATCCTTGGCCTGCCTTCTTTCTC-3'*
Jag1	*5'-GGCCCCACGTGTTCCACAAACATC-3'*	*5'-TTGCATCTAACTGACAAGTCTTGC-3'*
Dll4	*5'-AGACGACCTGCGGCCAGAGACTTC-3'*	*5'-CGGTAAGAGTAGCTCAGTCTGGTG-3'*
Hes1	*5'-GAAAGATAGCTCCCGGCATT-3'*	*5'-GTCACCTCGTTCATGCACTC-3'*
EphB2	*5'-ATTGCCATCGTATGTAACAGACGG-3'*	*5'-TCTTCATGCCTGGGGTCATGTGTC-3'*
Myc	*5'-CTCGCCCAAATCCTGTACCTCGTC-3'*	*5'-TCTTCTTGCTCTTCTTCAGAGTCG-3'*
Hey1	*5'-GGTACCCAGTGCCTTTGAGA-3'*	*5'-ACCCCAAACTCCGATAGTCC-3'*
HeyL	*5'-CACTGCCTTTGAGAAACAGGGCTC-3'*	*5'-GCTCGGGCATCAAAGAACCCTGTG-3'*
Dtx1	*5'-GTACTCCAATGGCAACAAGGATGG-3'*	*5'-ACCAGGCAGCGAGTGCGGGATGAG-3'*
Angiopoietin 1	*5'-CAGTGACTTTCATCAATGAAGGAC-3'*	*5'-CCAGGATCCAAACTGTTCTCACAC-3'*
Angiopoietin 2	*5'-GATGTCATCACCCAACTCCAAGAG-3'*	*5'-CATGTCACAGTAGGCCTTGATCTC-3'*
Socs3	*5'-CACGGAACCCTCGTCCGAAGTTCC -3'*	*5'-GGGTGGCCACGTTGGAGGAGAGAG-3'*
CD31	*5'-CGAGGTGAAGGTGCATGGCGTATC-3'*	*5'-TCCTTCGCTTGACAAACTTTGTCC-3'*
Bcl2	5'-ATAACGGAGGCTGGGATGCCTTTG-3'	5'-GTATGCACCCAGAGTGATGCAGGC-3'
Mcl-1	*5'-ACCAAGAAAGCTTCATCGAACCATTAGCAG-3'*	*5'-GGAAGAACTCCACAAACCCATCCCAGCCTC-3'*
Puma	*5'-GTACGAGCGGCGGAGACAAG-3'*	*5'-GCACCTAGTTGGGCTCCATTTCTG-3'*
Bax	*5'-TGGAGCTGCAGAGGATGATTGCTGAC-3'*	*5'-TTGCTAGCAAAGTAGAAGAGGGCAAC-3'*
Stat3	*5'-TGGCTGAGAAGCTCCTAGGGCCTG-3'*	*5'-AAACCCATGATGTACCCTTCATTC-3'*
Stat5	*5'-CCCCATTGGAATGATGGGGCTATC-3'*	*5'-TCAGATTCCAGAGGTTTCGGTCCG-3'*
GAPDH	*5'-TAAACTCAGGAGAGTGTTTCCTCG-3'*	*5'-ACTGCAAATGGCAGCCCTGGTGAC-3'*

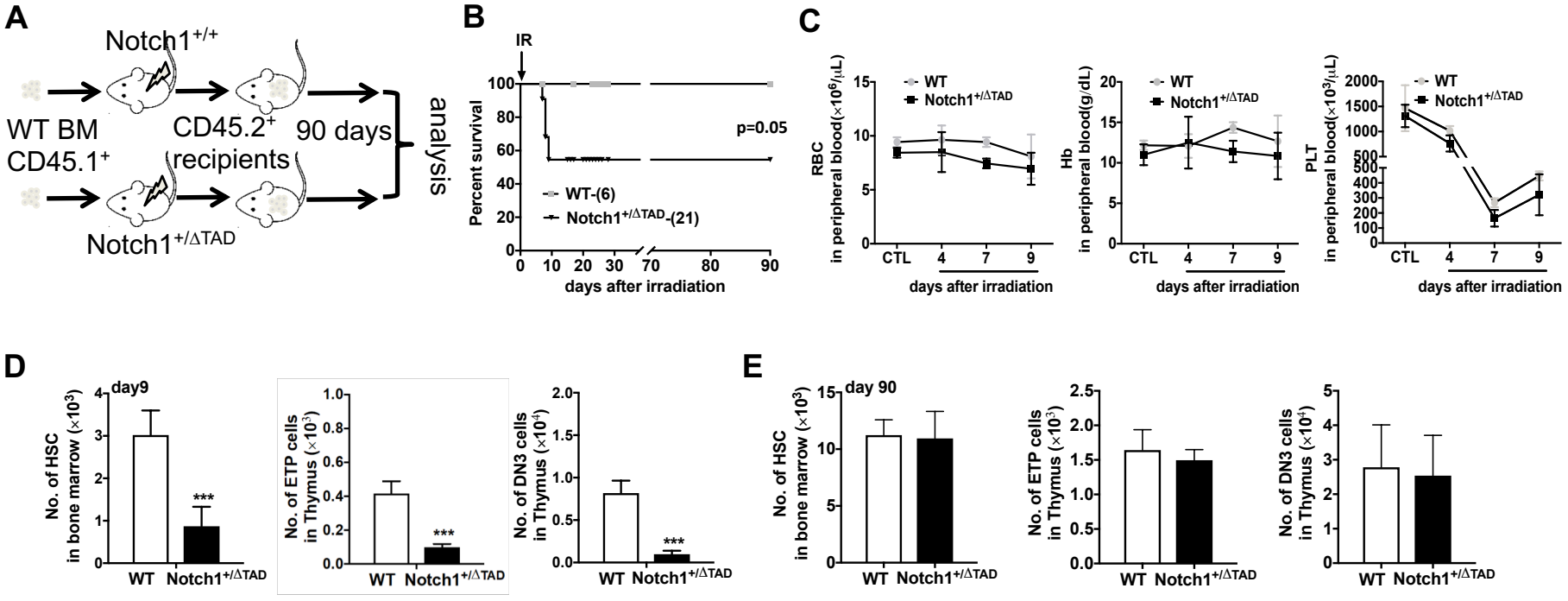
Table S3. CHIP primer sequences

Genes	Forward sequences	Reverse sequences
Myc	*5'-CACGGGACCTGAAAGGTTCT-3'*	*5-GGGTTAGGGCACAGGTGAGA-3'*
Hes1	*5'-CGTGTCTCTTCCTCCCATTG-3'*	*5'-CCAGGACCAAGGAGAGAGGT-3'*
Hey1	*5'-CACTATCTGAGGAGCTGTAGTCTG-3'*	*5'-AGCAGCCAGCCTCGGCCTCCGCTC-3'*
Dtx1	*5'-GGAAAAGGACTCTTGAGATCTTGG-3'*	*5'-TACCAAGCAGAGCCTCCAGTGAAG-3'*
GAPDH	*5'-AAACACTCTCCTGAGTTTAC-3'*	5'-*ATTTCCTCTCCTGTGTTCTC*-3'



Supplemental Figure S1. Frequency of peripheral blood cells in WT and Notch1$^{+/\Delta TAD}$ mice after 5-FU and irradiation.

(A) Peripheral blood counts of white blood cell (WBC), lymphocyte and neutrophil from WT and Notch1$^{+/\Delta TAD}$ mice (n=5-9 mice/group) were performed before (D0) and at days 4, 7 and 9 after 5-FU injection. (B) Peripheral blood counts of white blood cell (WBC), lymphocyte and neutrophil from WT and Notch1$^{+/\Delta TAD}$ mice were performed before (CTL) and at days 4, 7 and 9 after total body γ-irradiation (n=6 mice/group).



Supplemental Figure S2. Notch1$^{+/\Delta TAD}$ recipients show defective regeneration after irradiation damage.

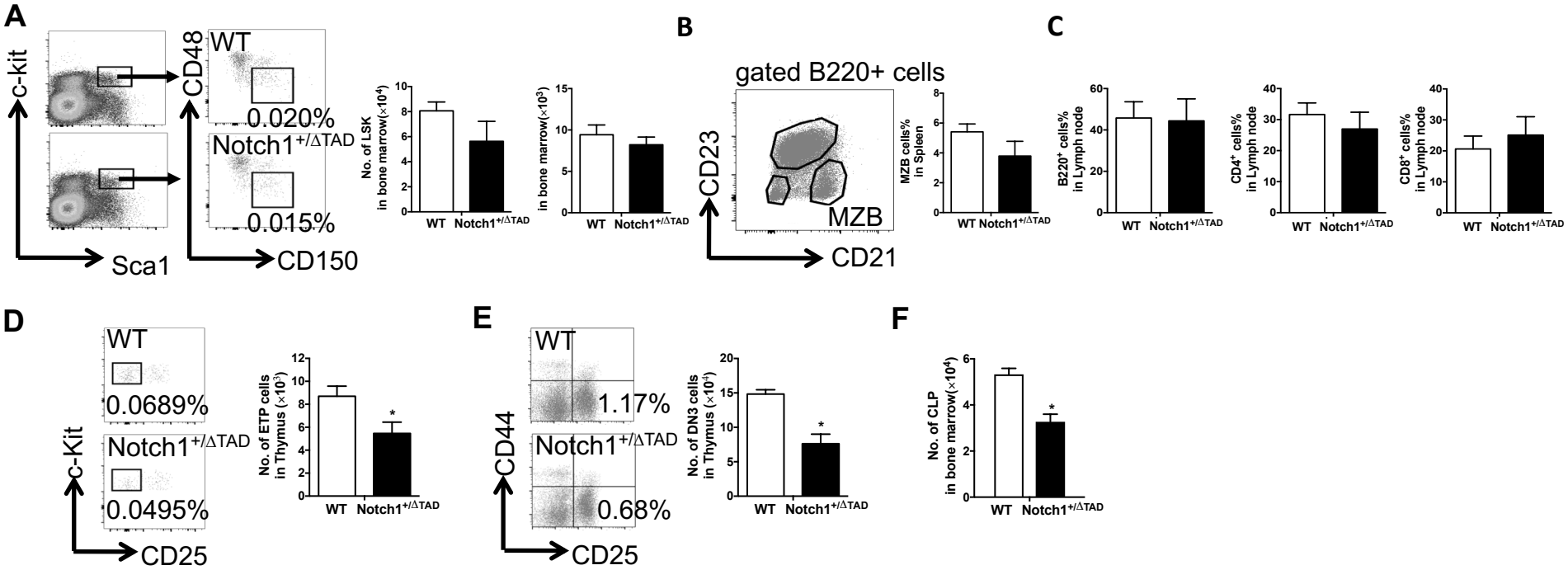
(A) Experimental design of gamma irradiation treatment. 2×10^6 bone marrow cells (BMCs) from WT CD45.1$^+$ mice were injected into 9.0 Gy irradiated Notch1$^{+/+}$ (WT) and Notch1$^{+/\Delta TAD}$ recipients.

(B) Kaplan-Meyer plot of WT and Notch1$^{+/\Delta TAD}$ recipients after irradiation. Transplanted mice were daily monitored until terminal analysis at day 90 (WT, n=6; Notch1$^{+/\Delta TAD}$, n=21). Significance (p=0.05) was determined by Mantel-Cox test.

(C) Numbers of erythrocytes (RBC) and platelet (PLT) and concentration of hemoglobin (Hb) from WT and Notch1$^{+/\Delta TAD}$ recipient mice were determined before (CTL) and at days 4, 7 and 9 after irradiation.

(D) Absolute numbers of donor-derived HSCs from the BM of morbid recipients at day 9 after transplantation/irradiation (left panel). Absolute numbers of ETP and DN3 donor-derived thymocytes in WT and Notch1$^{+/\Delta TAD}$ recipients were analyzed at day 9 after transplantation/irradiation (center and right panel).

(E) Absolute numbers of donor-derived HSCs from the BM of recipients were analyzed at day 90 after transplantation/irradiation (left panel). Absolute numbers of ETP and DN3 donor-derived thymocytes in WT and Notch1$^{+/\Delta TAD}$ recipient were analyzed 90 days after transplantation/irradiation (center and right panel). Absolute number of different populations from each mouse are expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure S3. Loss of one allele of Notch1 TAD adversely affects developing T cells.

(A) Representative gating strategy of flow cytometric analysis for LSK cells (Lin-Sca1$^+$c-kit$^+$ cells) and HSCs (Lin$^-$Sca1$^+$c-kit$^+$CD150$^+$CD48$^-$ cells) in BM shown from WT and Notch1$^{+/\Delta TAD}$ littermates. Total numbers (right panel) of LSK cells and HSCs from each mouse are presented as mean ± SD (n=5).

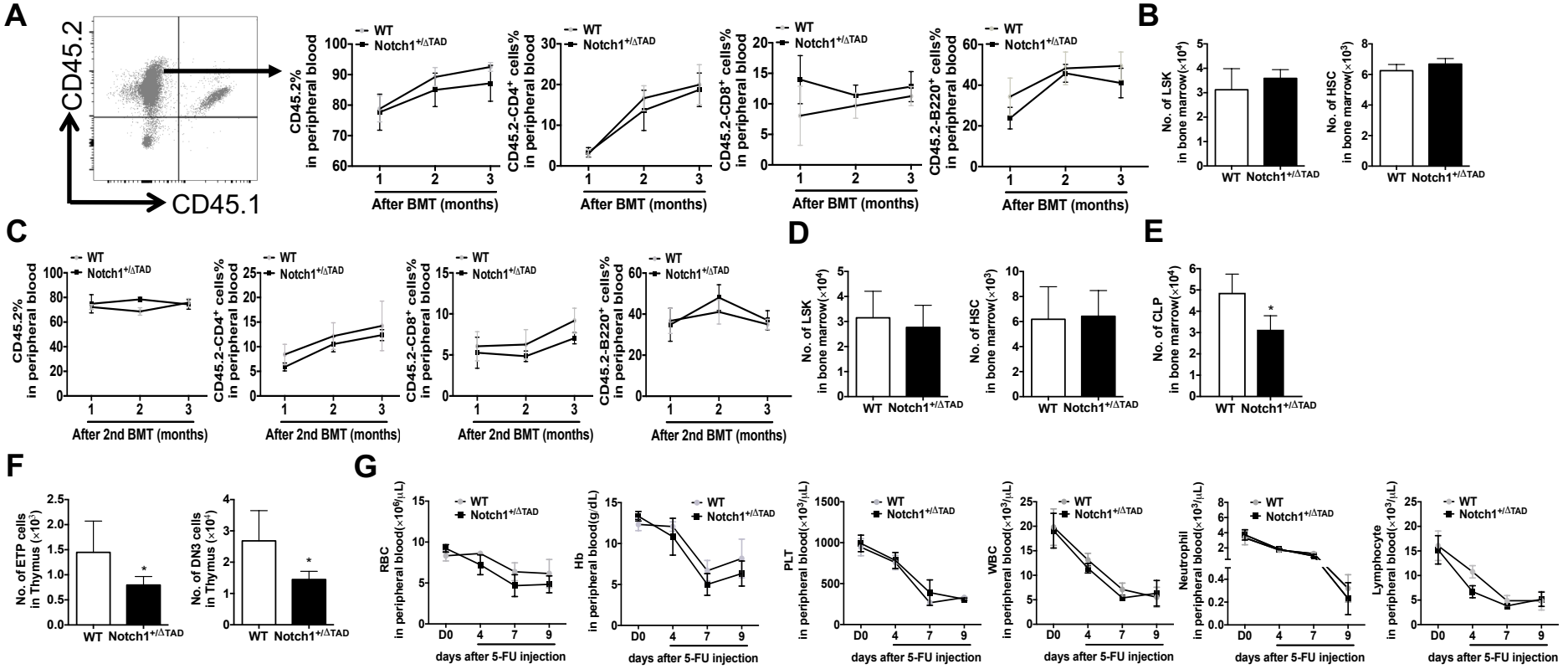
(B) MZB population analysis. MZB gating strategy was indicated in the left panel. Percentages of MZB cells in each mouse are presented as mean±SD.

(C) Analysis of CD4 and CD8 cells in lymph node from wild type and Notch1$^{+/\Delta TAD}$ mice. Lymph nodes were taken from 5-week old wild type and Notch1$^{+/\Delta TAD}$ mice. Lymph node cells were stained with B220, CD4 and CD8 antibodies. Percentages of B220^{+} cells, CD4^{+} cells, and CD8^{+} cells in each mouse are presented as mean±SD.

(D) Representative gating strategy of flow cytometric analysis for thymic ETP (Lin^{-}c-kit^{+}CD25^{-}CD44^{+} cells) from WT and Notch1$^{+/\Delta TAD}$ littermates. Absolute numbers (right panel) of ETP from each genotype are presented as mean ± SD (n=5).

(E) Representative gating strategy of flow cytometric analysis for thymic DN3 (Lin^{-}CD4^{-}CD8^{-}CD44^{-}CD25^{+}cells) from WT and Notch1$^{+/\Delta TAD}$ mice. Absolute numbers (right panel) of DN3 from each genotype are presented as mean ± SD (n=5). The statistical significance for the difference between WT and Notch1$^{+/\Delta TAD}$ datasets is indicated by asterisks. *$p<0.05$.

(F) CLP was identified by Lin^{-}Sca1^{low}c-kit^{low}CD135^{+}CD127^{+} cells in the BM from WT and Notch1$^{+/\Delta TAD}$ mice. Absolute CLP numbers from each genotype are presented as mean ± SD (n=5).



Supplemental Figure S4. HSCs derived from WT and Notch1$^{+/\Delta TAD}$ littermates show similar reconstitution ability

(A) Representative gating strategy for flow cytometric analysis of CD45.2 donor cell engraftment 3 months after BMT (left panel). The percentages of CD4^{+} cells, CD8^{+} cells and B220^{+} cells from donor-derived WT or Notch1$^{+/\Delta TAD}$ HSCs in the peripheral blood of the recipients are presented as means ± SD (n=8).

(B) Absolute numbers of donor-derived bone marrow LSK cells ($CD45.2^+Lin^-c\text{-}Kit^+Sca1^+$) and HSCs ($CD45.2^+Lin^-c\text{-}Kit^+Sca1^+CD150^+CD48^-$) at 3 months after transplantation.

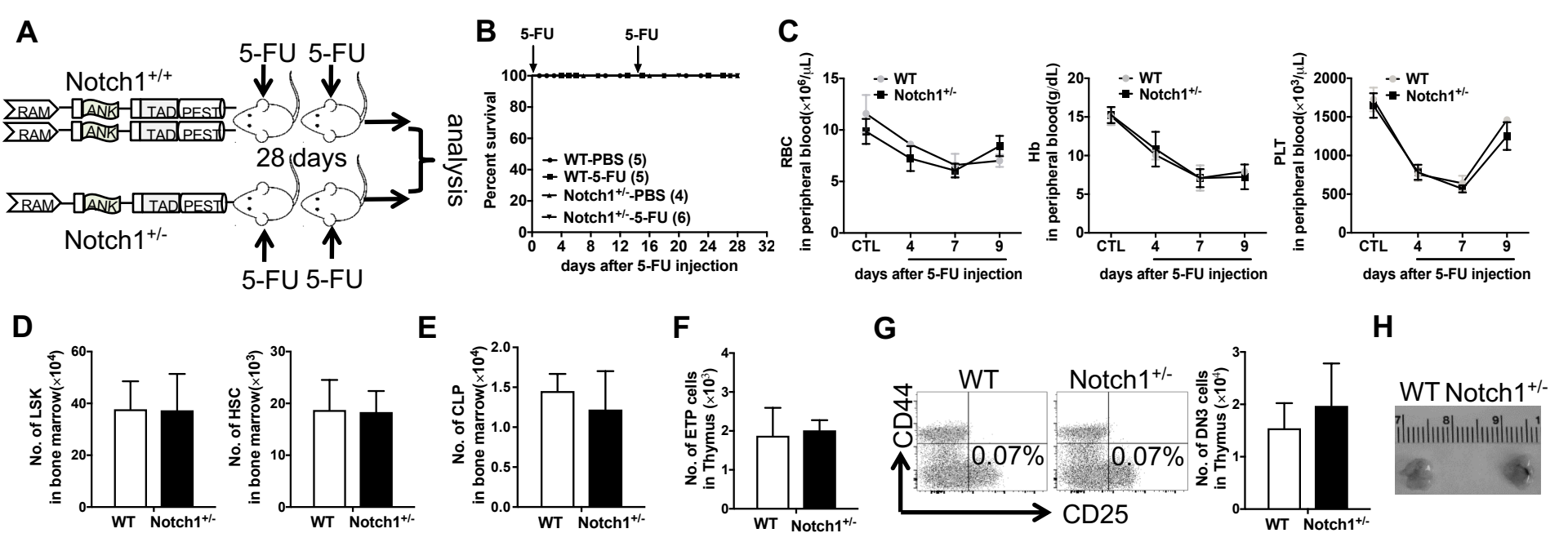
(C) The percentages of total donor-derived (CD45.2) hematopoietic cells in the peripheral blood of the secondary recipients 3 months after BMT are presented as means ± SD (left panel, n=5). The percentages of $CD4^+$ cells, $CD8^+$ cells and $B220^+$ cells from donor-derived hematopoietic cells in the peripheral blood of the secondary recipients are presented as means ± SD (n=5).

(D) Absolute numbers of donor-derived BM LSK cells and HSCs in secondary recipients 3 months after secondary transplantation.

(E) Absolute numbers of CLP ($CD45.2^+Lin^-c\text{-}Kit^{low}Sca1^{low}CD135^+CD127^+$) in BM of donor-derived populations 3 months after secondary transplantation expressed as mean±SD.

(F) Absolute numbers of ETP and DN3 cells in thymus of secondary recipient mice 3 months after secondary transplantation. ETP and DN3 were defined by $CD45.2^+Lin^-c\text{-}kit^+CD25^+CD144^+$ (left panel) and $CD45.2^+Lin^-CD4^-CD8^-CD44^-CD25^+$ (right panel), respectively. Absolute numbers of ETP and DN3 from each genotype are presented as mean± SD.

(G) Peripheral blood counts of erythrocyte (RBC), hemoglobin (Hb), platelet (PLT), white blood cell (WBC), neutrophil and lymphocyte from WT and $Notch1^{+/\Delta TAD}$ HSC reconstituted recipient mice were conducted before (D0) and at days 4, 7 and 9 after 5-FU injection. *$p<0.05$.



Supplemental Figure S5. Notch1$^{+/-}$ mice suffer no adverse effects post-chemotherapy.

(A) Experimental design of 5-FU treatment on Notch1$^{+/-}$ mice. 5-FU (150 mg/kg) was IP injected into WT and Notch1$^{+/-}$ 5-week old mice at day 1 and day 14.

(B) Kaplan-Meyer plot of WT and Notch1$^{+/-}$ mice after 5-FU treatment. Mice were daily monitored until day 28 (n=4-6 mice/group).

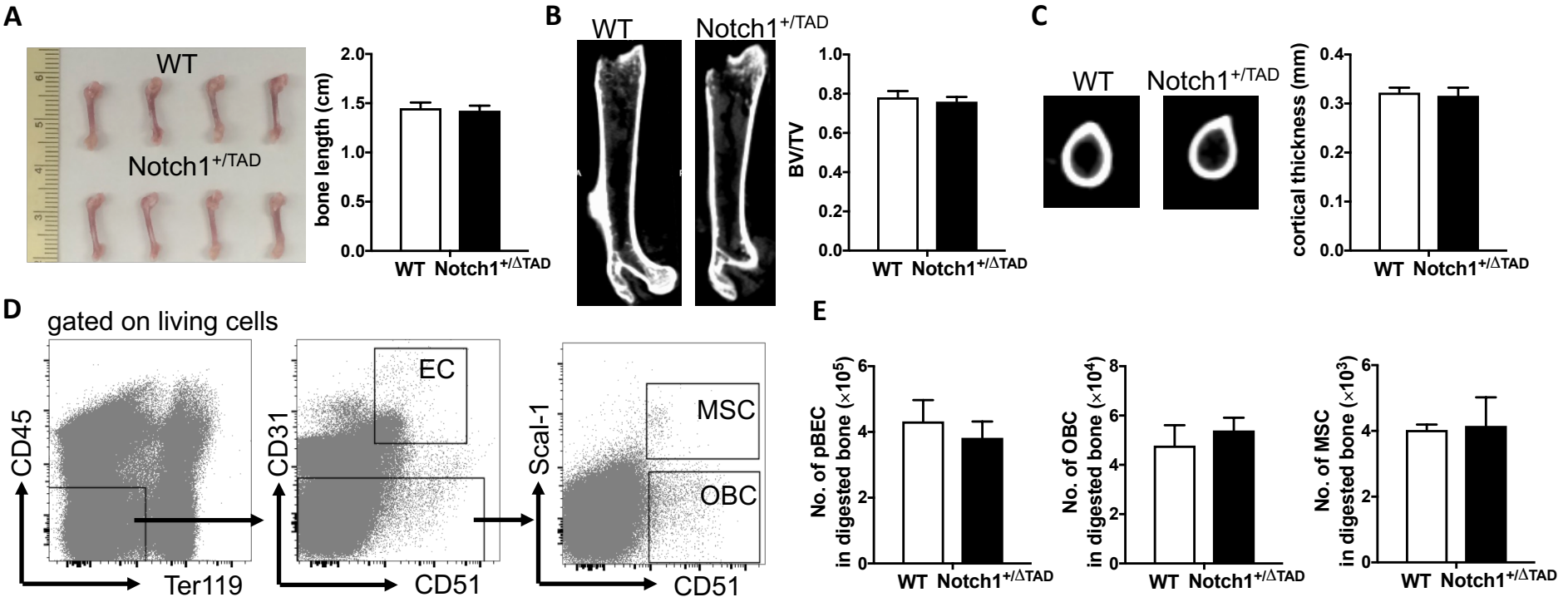
(C) Peripheral blood counts of erythrocyte (RBC), hemoglobin (Hb) and platelet (PLT) from WT and Notch1$^{+/-}$ littermates were performed before (CTL) and at days 4, 7 and 9 after 5-FU treatment.

(D) Absolute numbers of LSK (left panel) and HSCs (right panel) from each genotype are presented as mean±SD at day 9 after 5-FU treatment.

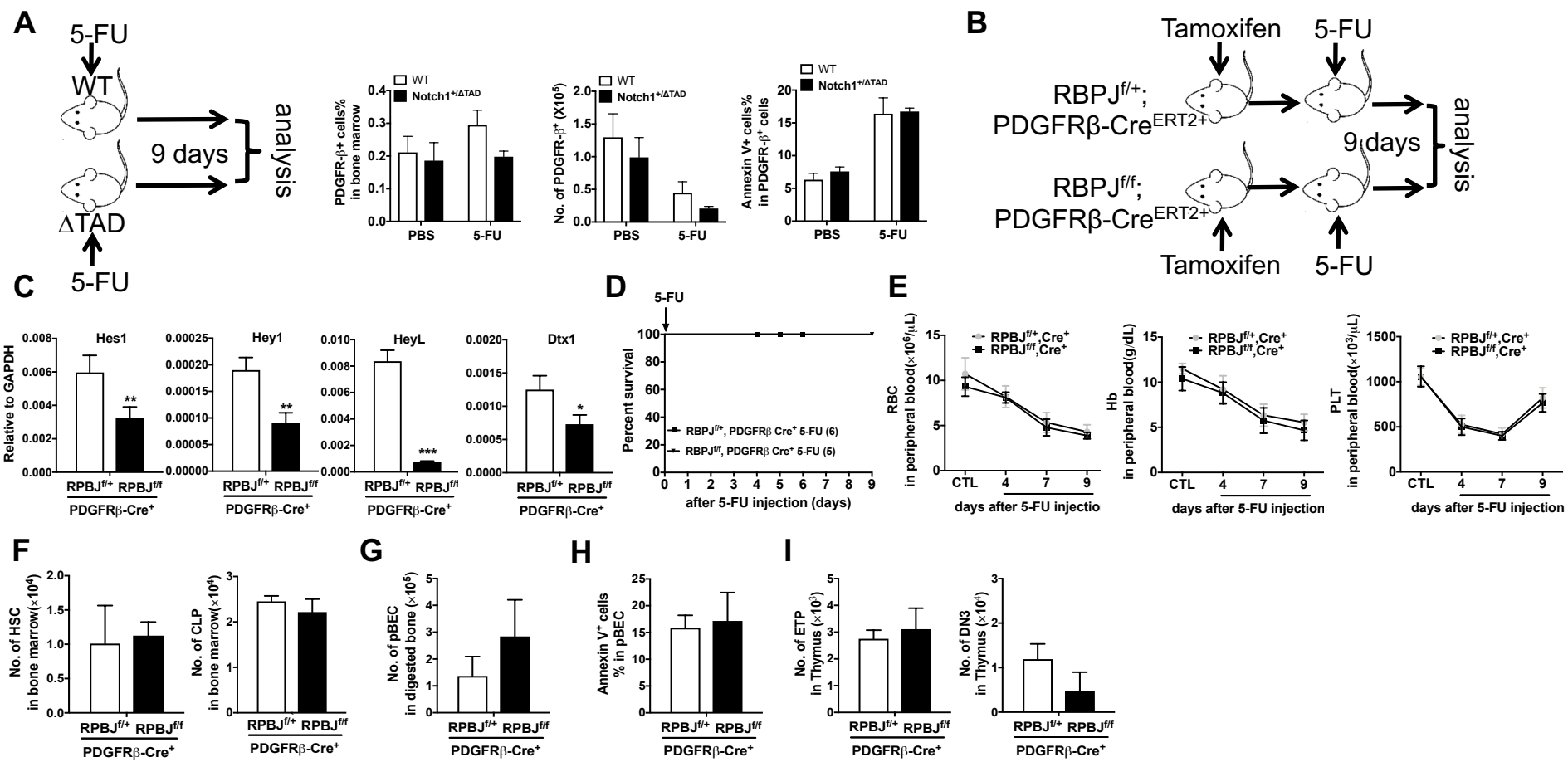
(E) Absolute numbers of CLP in BM from WT and Notch$^{+/-}$ littermates were analyzed at day 14 after 5-FU injection.

(F and G) Absolute numbers of ETP (F) and DN3 (G) in thymus from WT and Notch1$^{+/-}$ littermates were analyzed at day 9 after 5-FU treatment and presented as mean±SD.

(H) Representative images of the thymus from WT and Notch1$^{+/-}$ littermates at day 9 after 5-FU treatment.



Supplemental Figure S6. Loss of one allele of TAD does not affect bone development. (A-C) Bone phenotype. Femur bones were collected from 6-week old WT and Notch1$^{+/\Delta TAD}$ mice (n=4). The femur length was measured and represented as mean±SD (A). Total volume (TV) and bone volume (BV) were measured by microCT. Bone volume fraction (BV/TV) was presented (B). Cortical thickness was presented in C. (D-E) Bone progenitor phenotype. Femur and tibia were collected from 6-week old WT and Notch1$^{+/\Delta TAD}$ mice and digested with collagenase I as described in materials and methods. Gating strategy is indicated in D after cells from digested bones were stained with CD45, TER119, CD31 and CD51 antibodies. Numbers of EC, OBC and MSC from each mouse were presented as mean±SD (E, n=4).



Supplemental Figure S7. Loss of Notch signaling in PDGFRβ+ cells has no effect on chemotherapeutic recovery.

(A) WT and Notch1$^{+/\Delta TAD}$ mice were treated with 5-FU (left panel). Percentages and absolute numbers of CD45$^-$PDGFRβ$^+$ cells in BM were analyzed. Percentages of pro-apoptotic cells in CD45$^-$PDGFRβ$^+$ population were examined by Annexin V staining (n=4).

(B) Experimental design of 5-FU injection on RBPJ$^{f/f}$-PDGFRβCre^{ERT2+} mice. Tamoxifen was delivered through maternal milk by administering 250 μg/kg to the nursing mon at postnatal 1, 2 and 3. 5-FU (150 mg/kg) was intraperitoneally injected into RBPJ$^{f/f}$-PDGFRβCre^{ERT2+} or RBPJ$^{f/+}$-PDGFRβCre^{ERT2+} mice pretreated with tamoxifen at 5 weeks of age. Analysis were performed at day 9 after 5-FU injection.

(C) Expression of Hes1, Hey1, Heyl and Dtx1 was measured by RT-qPCR in FACS sorted CD45^{-}Ter119^{-}PDGFRβ^{+} cells from the BM of RBPJ$^{f/+}$-PDGFRβCre^{ERT2+} or RBPJ$^{f/f}$-PDGFRβ Cre^{ERT2+} mice.

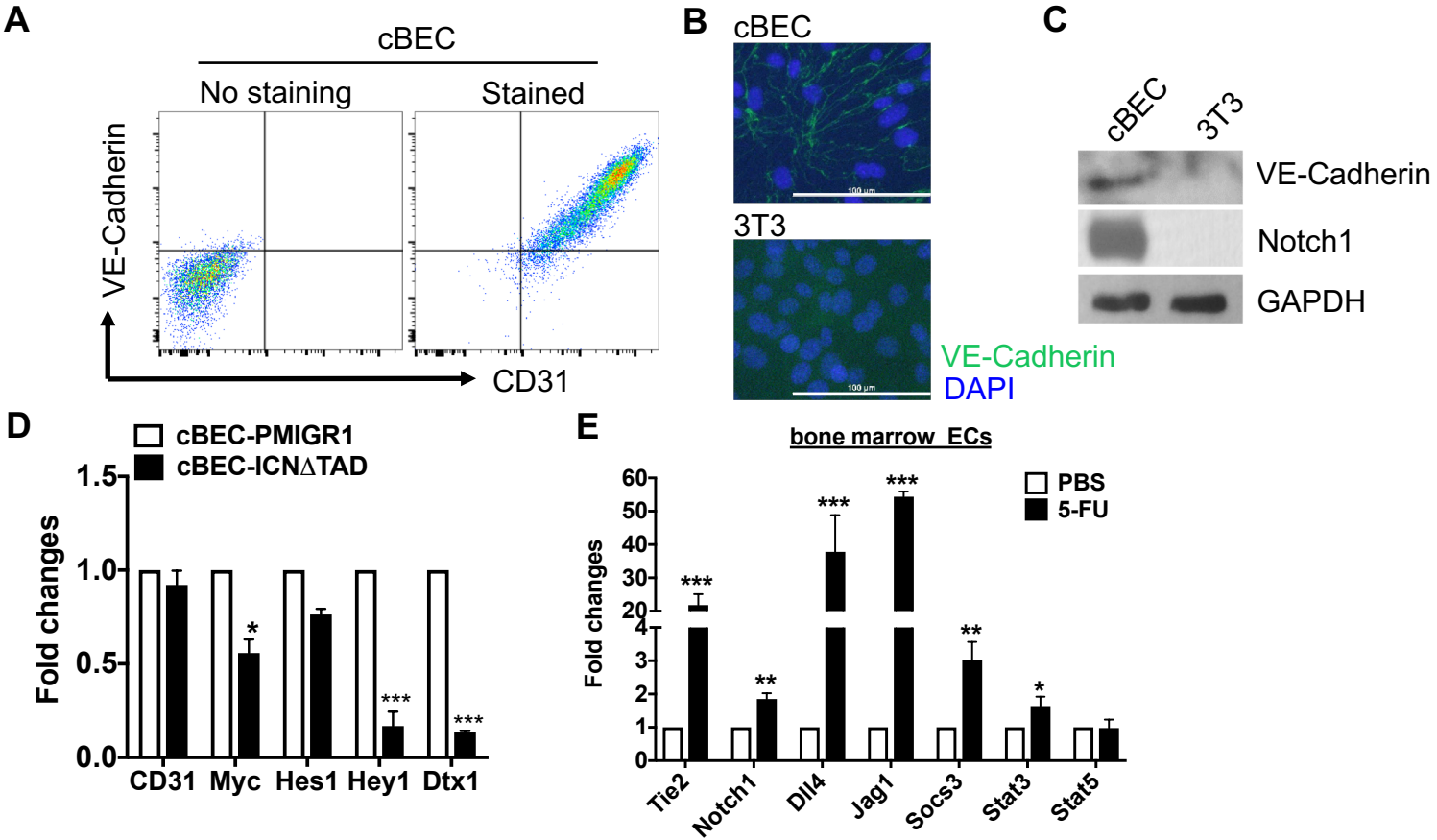
(D) Kaplan-Meyer plot of RBPJ$^{f/+}$-PDGFRβ Cre^{ERT2+} and RBPJ$^{f/f}$-PDGFRβ Cre^{ERT2+} mice after tamoxifen and 5-FU injection. Mice were daily monitored until day 9 after 5-FU injection (n=5-6 mice/group).

(E) Numbers of erythrocyte (RBC), hemoglobin (Hb) and platelet (PLT) in the peripheral blood from RBPJ$^{f/+}$-PDGFRβ Cre^{ERT2+} and RBPJ$^{f/f}$-PDGFRβ Cre^{ERT2+} mice were counted before (CLT) and at days 4, 7 and 9 after 5-FU injection.

(F) Absolute numbers of HSCs and CLP cells in the BM were analyzed at day 9 after 5-FU injection and expressed as mean± SD.

(G) Absolute numbers of pBEC were analyzed at day 9 after 5-FU injection. (H) pBECs from RBPJ$^{f/+}$-PDGFRβ Cre^{ERT2+} and RBPJ$^{f/f}$-PDGFRβ Cre^{ERT2+} mice were analyzed by flow cytometry for Annexin V staining at day 9 after 5-FU injection. Percentages of Annexin V^{+} cells are presented as mean± SD.

(I) Absolute numbers of ETP and DN3 in thymus from RBPJ$^{f/+}$-PDGFRβ Cre^{ERT2+} and RBPJ$^{f/f}$-PDGFRβ Cre^{ERT2+} mice at day 9 after 5-FU injection. Thymus cells were isolated from each genotype at day 9 after 5-FU injection. Numbers of ETP and DN3 from each mouse are expressed as mean± SD. *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure S8. Notch1 ICNΔTAD interferes with WT ICN1 in bone marrow endothelial cells.

(A and B) Cultured bone endothelial cells (cBEC) were stained for VE-Cadherin and CD31 and analyzed by flow cytometry (A). Confluent cBECs were fixed in 4%PFA and immunostained for VE-Cadherin (green). DAPI was used to stain nuclei (B). 3T3 cells were used for a negative control for VE-Cadherin staining (n=3). Scale bar=100 μm.

(C) Whole cell lysate from cBECs and 3T3 cells was extracted with RIPA buffer and analyzed by western blot for VE-Cadherin and Notch1 Val1744 expression (n=3). GAPDH was used as a loading control.

(D) The expression of indicated genes was determined by RT-qPCR in cBEC-PMIGR1 and cBEC-ICNΔTAD cells (n=3). Values are normalized to PMIGR1 transduced cells. GAPDH was used as an internal expression control. *$p<0.05$, ***$p<0.001$.

(E) Expression of indicated genes was determined by RT-qPCR in bone marrow ECs treated with PBS and 5-FU (PBS, n=5; 5-FU, n=15). GAPDH was used as an internal expression control.