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

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### ABSTRACT

Loss-of-function studies have determined that Notch signaling is essential for hematopoietic and endothelial development. By deleting a single allele of the Notch1 transcriptional activation domain we generated viable, post-natal mice exhibiting hypomorphic Notch signaling. These heterozygous mice, which lack only one copy of the transcriptional activation domain, appear normal and have no endothelial or hematopoietic phenotype, apart from an inherent, cell-autonomous defect in T-cell lineage development. Following chemotherapy, these hypomorphs exhibited severe pancytopenia, weight loss and morbidity. This phenotype was confirmed in an endothelial-specific, loss-of-function Notch1 model system. Ang1, secreted by hematopoietic progenitors after damage, activated endothelial Tie2 signaling, which in turn enhanced expression of Notch ligands and potentiated Notch1 receptor activation. In our heterozygous, hypomorphic model system, the mutant protein that lacks the Notch1 transcriptional activation domain accumulated in endothelial cells and interfered with optimal activity of the wildtype Notch1 transcriptional complex. Failure of the hypomorphic mutant to efficiently drive transcription of key gene targets such as *Hes1* and *Myc* prolonged apoptosis and limited regeneration of the bone marrow niche. Thus, basal Notch1 signaling is sufficient for niche development, but robust Notch activity is required for regeneration of the bone marrow endothelial niche and hematopoietic recovery.

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### Introduction

Chemotherapy and radiotherapy are widely used in the treatment of hematopoietic malignancies but broad cytotoxicity is an undesirable feature of these treatments.<sup>1</sup> These therapies damage multiple tissues including the bone marrow (BM) microvasculature.<sup>2-4</sup> The regeneration of the endothelial BM vascular niche is crucial for successful reconstitution of hematopoietic cells.<sup>5,6</sup> The interplay between the vascular and hematopoietic systems has multiple physiological and therapeutic implications. Endothelial cell (EC)-secreted growth factors such as vascular endothelial growth factor (VEGF)-A, enhance self-renewal and survival of hematopoietic stem cells (HSC) and mediate recovery of hematopoiesis.<sup>5,7,8</sup> Angiopoietin-1 (Ang1) signaling<sup>9,10</sup> via the activation of tyrosine kinase Tie2 has been proposed as the key endocrine mechanism mediating endothelial recovery and regeneration.<sup>2,11</sup> It is unknown whether paracrine signaling plays a role in the regeneration and reassembly of the BM endothelium.

Notch receptors are evolutionarily conserved transmembrane glycoproteins. Upon paracrine activation by neighboring cells through ligand interactions and proteolytic cleavage, they activate a transcriptional apparatus.<sup>12</sup> *Notch1* and *Notch4*

are highly expressed in the endothelium during embryonic development and control EC specification<sup>13</sup> and Notch1/Dll4 in coordination with VEGF-A/VEGFR2 signaling regulates sprouting angiogenesis.<sup>14,15</sup> The function of Notch signaling in the adult vasculature is less understood. Studies showed that Notch1 signaling in the adult endothelium regulates expression of inflammatory genes.<sup>16</sup> Notch1 is also known to be activated by blood flow and shear stress forces, which contribute to vascular homeostasis.<sup>17</sup> Important, unresolved questions are whether Notch activation has a role in post-injury endothelial regeneration and whether it promotes the recovery of hematopoiesis.

The intracellular domains of Notch receptors have distinct roles. The RAM domain has a high affinity for binding to RBPJ, while the Ankyrin repeat (ANK) domains interact with a Mastermind-like (MAML) protein factor and recruit other co-activators. The PEST domain localized at the C-terminal facilitates Notch degradation.<sup>18</sup> In between the ANK and PEST domains there is a transcriptional activation domain (TAD), which is capable of autonomous transcriptional activity and directly binds co-activators PCAF and GNC5.<sup>19,20</sup> The TAD is a region of significant divergence among the four mammalian Notch receptors.<sup>20,21</sup> These differences among the Notch receptor TAD may be important in the tissue-specific variability of Notch signaling.

We previously developed a transgenic knock-in model system which deleted the TAD of Notch1.<sup>22</sup> This model system was used to study the role of Notch1 TAD function during fetal development. The loss of TAD in both *Notch1* alleles ( $\Delta TAD/\Delta TAD$ ), allowed mice to develop to late gestation when they succumbed to multiple Notch-dependent cardiovascular anomalies.<sup>22-24</sup> While definitive HSC emerged from the aorta-gonad-mesonephros region, they failed to expand in the fetal liver of  $\Delta TAD/\Delta TAD$  embryos. Furthermore, when transplanted into irradiated adult recipients,  $\Delta TAD/\Delta TAD$  HSC underperformed in primary transplants and failed to reconstitute the hematopoietic system efficiently in secondary transplants.<sup>22</sup> In contrast, mice heterozygous for one allele of *Notch1* $\Delta TAD$  (*Notch1*<sup>+/ $\Delta TAD$</sup> ) survive to adulthood and exhibit no overt hematopoietic phenotype.

In the present study, we made use of hypomorphic Notch signaling in the *Notch1*<sup>+/ $\Delta TAD$</sup>  model to address whether the Notch pathway is crucial for the recovery of the adult BM niche and regeneration of hematopoietic cells after injury. We observed that high levels of Notch signaling were dispensable for the development of the endothelial niche and high Notch activity was not required during adult BM endothelial homeostasis. In the hematopoietic system, *Notch1*<sup>+/ $\Delta TAD$</sup>  only displayed cell-autonomous defects in the development of the T-cell lineage. However, following myelosuppressive injury, robust Notch signaling was critical for recovery of the BM endothelial niche and thereby the regeneration of HSC. Notch signaling was stimulated by a burst of Tie2-dependent activation, which induced expression of Notch1 ligands. Interestingly, expression of Notch1 $\Delta TAD$  protein in *Notch1*<sup>+/ $\Delta TAD$</sup>  EC decreased expression of Notch target genes and led to severe apoptosis. This phenotype could not be rescued by enhanced activation of Tie2 signaling. Our results suggest a crucial role for TAD-regulated Notch activity in mediating EC survival and promoting recovery of hematopoiesis following chemotherapeutic stress.

## Methods

### Animals

The following strains of mice were used in our studies under the guidelines and protocols approved by the Institutional Animal Care and Use Committees of University of Illinois at Chicago: C57BL/6J (or CD45.2), B6.SJL-*Ptprc*<sup>o</sup>*Pep3*<sup>o</sup>/BoyJ (or CD45.1), *Notch*<sup>+/ $\Delta TAD$</sup> , *Notch*<sup>+/+</sup>, *Notch1*<sup>fl</sup>, *RBPJ*<sup>fl</sup>, *PDGFR $\beta$ -Cre*<sup>ERT2</sup> and *VE-Cadherin Cre*<sup>ERT2</sup> mice. More details about the mice can be found in the *Online Supplementary Methods*.

### 5-Fluorouracil treatment and irradiation

5-Fluorouracil (5-FU, 150 mg/kg) was peritoneally injected into mice. For irradiation treatment, mice were exposed, on a rotating platform, to a lethal dose (9.0 Gy) of total body irradiation in a Mark I <sup>137</sup>Ce  $\gamma$ -irradiator (JL Shepherd, Glendale, CA, USA) at a dose rate of 6.38 Gy/min. More details about chemotherapy and radiotherapy can be found in the *Online Supplementary Methods*.

### Analysis of bone marrow and thymic mononuclear cells

Mononuclear cells from BM and thymus were prepared as described in the *Online Supplementary Methods*. The frequencies of LSK cells (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup> cells), HSC (CD150<sup>+</sup>CD48<sup>-</sup>LSK cells), common lymphoid progenitors (CLP: Lin<sup>-</sup>Sca1<sup>low</sup>c-kit<sup>low</sup>CD135<sup>+</sup>CD127<sup>+</sup>), thymic early T-cell precursors (ETP: Lin<sup>-</sup>c-kit<sup>+</sup>CD25<sup>+</sup>CD44<sup>-</sup>) and double-negative 3 cells (DN3 cells: Lin<sup>-</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup>CD44<sup>-</sup>) were analyzed with a flow cytometer, Fortessa LSRII, after immunostaining with appropriate antibodies as described in the *Online Supplementary Methods*. All antibodies used are listed in *Online Supplementary Table S1*.

### Isolation of CD25<sup>+</sup> thymocytes and primary bone endothelial cells

Cell suspensions from the thymus or digested bones were incubated with dynabeads labeled with CD25 antibody and CD31 antibody (Invitrogen), respectively, for 30 min at 4°C. CD25<sup>+</sup> thymocytes and bone CD31<sup>+</sup> cells (pBEC) were isolated and used for western blot and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) analysis.

### Bone marrow-derived endothelial cell culture

Cultured bone marrow-derived endothelial cells (cBEC) were purchased from Cell Biologicals Inc (cat#, C57-6221) and cultured in EC medium. cBEC were treated with a  $\gamma$ -secretase inhibitor (1  $\mu$ M, Sigma), a Tie2 kinase inhibitor (1  $\mu$ M, Abcam), Ang1 (300 ng/mL, Peprotech) or 5-FU (100  $\mu$ M, Sigma). More detailed information can be found in the *Online Supplementary Methods*.

### Western blot analysis

Expression of cleaved/active Notch1 (Val1744), Notch1, VE-Cadherin, Tie2, and phosphorylated Tie2-Y992 was measured by western blot. The procedure is detailed in the *Online Supplementary Methods*.

### Quantitative reverse transcriptase polymerase chain reaction

Expression of various genes was measured by RT-qPCR and calculated using the comparative C<sub>T</sub> method. The sequences of all primers used in the RT-qPCR assay are listed in *Online Supplementary Table S2*.

### Chromatin immunoprecipitation assays

RBPJ binding sites at *Myc NDME*, *Hes1*, *Hey1* and *Dtx1* locus

were analyzed by chromatin immunoprecipitation assay. The procedure is described in detail in the *Online Supplement*. The sequences of primers are provided in *Online Supplementary Table S3*.

### Analysis of hematopoietic stem cell transplantation, apoptosis, luciferase activity, bone micro-computed tomography, sternum vasculature whole-mount imaging and immunostaining

These assays and methods are described in the *Online Supplementary Methods*.

### Statistical analysis

Details of the statistical analysis of the data are provided in the *Online Supplementary Methods*.

## Results

### Myelosuppression by 5-fluorouracil or $\gamma$ -irradiation causes pancytopenia in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice

Myelosuppression by 5-FU is achieved by incorporating an analog of uracil into RNA or DNA of proliferating cells.<sup>25</sup> The result of 5-FU treatment during hematopoiesis is apoptosis of proliferating progenitors, followed by activation of HSC and reconstitution of the hematopoietic system.<sup>26</sup> To test for a possible hematopoietic defect in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice, 5-week old wildtype (WT) and *Notch1*<sup>+/ $\Delta$ TAD</sup> littermates were treated with two doses of 5-FU, 14 days apart. Terminal hematopoietic analysis was performed 28 days after the first injection (Figure 1A). The condition of the *Notch1*<sup>+/ $\Delta$ TAD</sup> mice deteriorated rapidly after treatment. Analysis of peripheral blood from *Notch1*<sup>+/ $\Delta$ TAD</sup> mice revealed a sharp decrease in red blood cell and platelet counts, as well as hemoglobin concentration (Figure 1D). White blood cell and lymphocyte counts were also reduced in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice (*Online Supplementary Figure S1A*). During the first 9 days after treatment, all *Notch1*<sup>+/ $\Delta$ TAD</sup> mice exhibited severe pancytopenia. For half of the *Notch1*<sup>+/ $\Delta$ TAD</sup> mice, the body-score condition index and body weight dropped below humane levels (body condition index = 2) (Figure 1B,C), and the animals were sacrificed.

We analyzed bone marrow LSK (Lin<sup>−</sup>Sca1<sup>+</sup>cKit<sup>+</sup>) progenitors and HSC (LSK<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup>) after 5-FU treatment. *Notch1*<sup>+/ $\Delta$ TAD</sup> mice showed significant decreases in the numbers of BM progenitors and HSC (Figure 1E) 9 days after treatment. The BM-residing common lymphoid progenitors (CLP), thymic early T-cell precursors (ETP) and CD4<sup>+</sup>CD8<sup>−</sup> double negative 3 (DN3) T-cell progenitors were also significantly reduced in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice after treatment (Figure 1F–H). Thymus size was reduced in 5-FU-treated *Notch1*<sup>+/ $\Delta$ TAD</sup> mice compared to controls (Figure 1I). These findings indicate that TAD-dependent Notch1 signaling plays a critical role in recovery of the hematopoietic system following myelosuppression.

To determine whether <sup>137</sup>Cs  $\gamma$  total body irradiation had a similar effect as 5-FU treatment, WT or *Notch1*<sup>+/ $\Delta$ TAD</sup> recipient mice (CD45.2<sup>+</sup>) were lethally irradiated and subsequently transplanted with 2.0x10<sup>6</sup> WT donor (CD45.1<sup>+</sup>) BM cells. Recipient mice were monitored for 90 days after irradiation/transplantation (*Online Supplementary Figure S2A*). We observed that the *Notch1*<sup>+/ $\Delta$ TAD</sup> mice suffered severe weight loss (*data not shown*) and 50% of them had low body-score condition leading to mortality 9 days following irradiation/transplantation (*Online Supplementary*

*Figure S2B*). Total body irradiation has a limited effect on enucleated erythroid cells<sup>1</sup> (*Online Supplementary Figure S2C*) but the numbers of white blood cells, neutrophils and lymphocytes were significantly lower in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice than in controls (*Online Supplementary Figure S1B*). At the 9-day time point, the numbers of donor-derived HSC, ETP, and DN3 cells in the *Notch1*<sup>+/ $\Delta$ TAD</sup> recipient mice were markedly reduced (*Online Supplementary Figure S2D*), suggesting that the *Notch1*<sup>+/ $\Delta$ TAD</sup> BM microenvironment plays a critical role during hematopoietic recovery. However, 90 days after transplantation, the numbers of the donor-derived HSC, ETP, and DN3 populations were comparable between recipients (*Online Supplementary Figure S2E*) suggesting that in the surviving *Notch1*<sup>+/ $\Delta$ TAD</sup> recipients, hematopoietic homeostasis was normalized. Thus, early hematopoietic recovery following  $\gamma$  irradiation also depends on Notch1-TAD signaling.

### Transplantation with *Notch1*<sup>+/ $\Delta$ TAD</sup> hematopoietic stem cells reveals a cell-autonomous defect in T-cell development

We next investigated whether there was a pre-existing hematopoietic defect in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice. Neither progenitors nor HSC were significantly affected in 6-week old *Notch1*<sup>+/ $\Delta$ TAD</sup> animals (*Online Supplementary Figure S3A*). No effect was seen in marginal zone B cells in the spleen or in B- and T-cell populations in the lymph nodes in *Notch1*<sup>+/ $\Delta$ TAD</sup> mice (*Online Supplementary Figure S3B, C*). The presence of a single *Notch1*- $\Delta$ TAD allele significantly reduced the frequency and number of thymic ETP and DN3 populations (*Online Supplementary Figure S3D, E*). This T-cell lineage defect could be traced to a reduction in the BM-residing CLP population (*Online Supplementary Figure S3F*). These findings show an inherent early role for Notch1-TAD signaling in the BM lymphoid progenitor population.

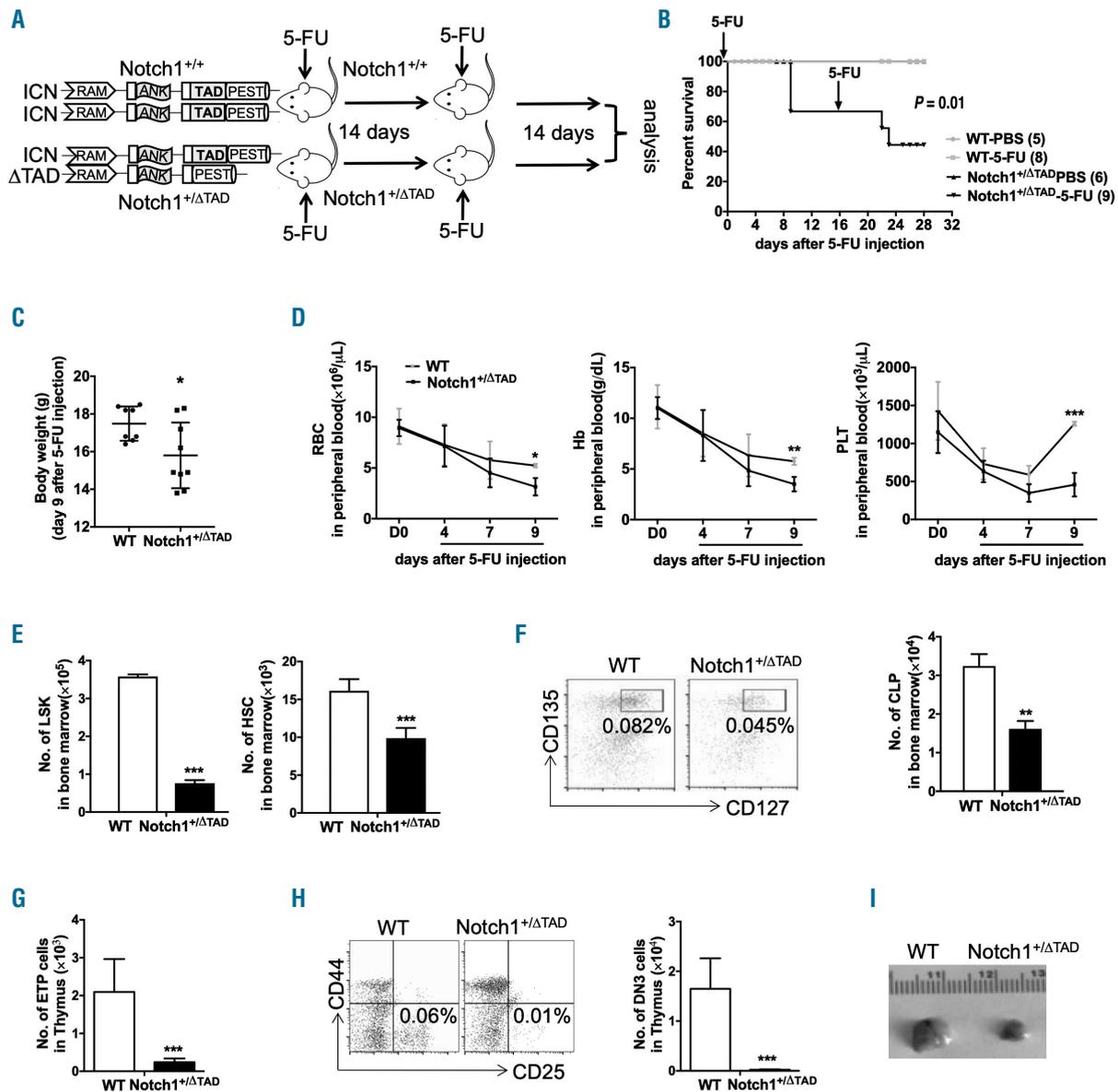
To investigate whether the effect of *Notch1*<sup>+/ $\Delta$ TAD</sup> on hematopoietic recovery is cell autonomous, 350 HSC from *Notch1*<sup>+/ $\Delta$ TAD</sup> or WT donors (CD45.2<sup>+</sup>) were transplanted into congenic (CD45.1<sup>+/2</sup>) recipients (Figure 2A). Reconstitution, defined by having 85–90% of blood cells in the recipients derived from *Notch1*<sup>+/ $\Delta$ TAD</sup> or WT donors, and multi-lineage potential was confirmed at 1, 2, and 3 months after transplantation (*Online Supplementary Figure S4A*). *Notch1*<sup>+/ $\Delta$ TAD</sup> HSC reconstituted recipients similarly to WT HSC. There were equal numbers of donor-derived LSK cells and HSC in recipient BM (*Online Supplementary Figure S4B*), indicating that *Notch1*<sup>+/ $\Delta$ TAD</sup> HSC were capable of homing and successfully engrafting into the WT recipient niche. In secondary transplants, mice receiving *Notch1*<sup>+/ $\Delta$ TAD</sup> BM cells had no defects in HSC reconstitution (*Online Supplementary Figure S4C, D*); however, the CLP, ETP, and DN3 populations were significantly reduced (*Online Supplementary Figure S4E, F*). This finding indicated an inherent T-cell defect resulting from loss of one *Notch1* TAD allele but also showed that HSC reconstitution was otherwise unaffected.

To test the effects of chemotherapy on the *Notch1*<sup>+/ $\Delta$ TAD</sup> hematopoietic system, we treated WT recipient mice reconstituted by *Notch1*<sup>+/ $\Delta$ TAD</sup> HSC with two rounds of 5-FU (Figure 2A). Recipients transplanted with *Notch1*<sup>+/ $\Delta$ TAD</sup> HSC exhibited no adverse effects when compared to those that received WT HSC (Figure 2B). Following treatment, comparable numbers of BM HSC were present in recipient mice (Figure 2C). Both sets of recipients had equivalent platelet, white blood cell, neutrophil, lymphocyte and red

blood cell counts as well as hemoglobin levels (*Online Supplementary Figure S4G*). 5-FU treatment continued to affect the recovery of the T-cell lineage populations originating from *Notch1<sup>+/-ΔTAD</sup>* donors (Figure 2D-F), which led to a 50% decrease in thymic mass (Figure 2G). Our findings showed that loss of a single *Notch1* TAD allele markedly impaired T-cell development in the reconstituted WT recipients, but had no other adverse effects on hematopoiesis.

### *Notch1* haploinsufficiency has no effect on hematopoietic recovery

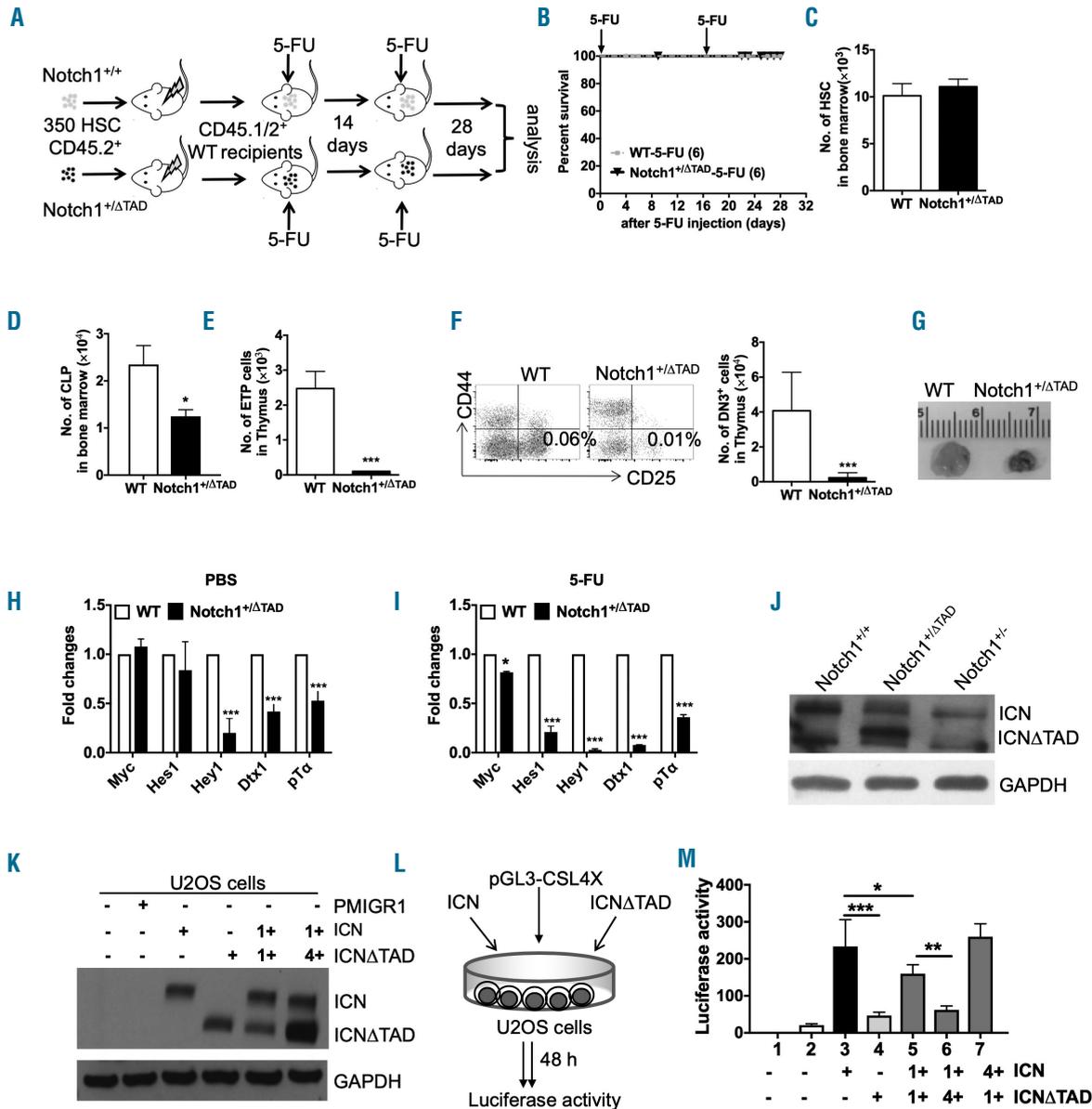
To test whether *Notch1* haploinsufficiency can account for pancytopenia, weight loss and morbidity after chemotherapy, we treated *Notch1<sup>+/-</sup>* mice with 5-FU (*Online Supplementary Figure S5A*). *Notch1<sup>+/-</sup>* mice exhibited no significant difference in body score index compared to WT mice and had no discernable hematopoietic phenotype (*Online Supplementary Figure S5B, C*). Furthermore, no dif-



**Figure 1. Limited recovery of *Notch1<sup>+/-ΔTAD</sup>* mice after chemotherapeutic stress.** (A) Experimental design of 5-fluorouracil (5-FU) injection. 5-FU (150 mg/kg) was injected intraperitoneally into *Notch1<sup>+/+</sup>* (WT) and *Notch1<sup>+/-ΔTAD</sup>* 5-week old mice on days 1 and 14. (B) Kaplan-Meier plot indicating survival of WT and *Notch1<sup>+/-ΔTAD</sup>* mice after 5-FU injection. Mice ( $n=5-9$ ) were monitored daily until terminal analysis at day 28 after the first 5-FU injection. The statistical significance ( $P=0.01$ ) between WT and *Notch1<sup>+/-ΔTAD</sup>* 5-FU-treated littermates was determined by the Mantel-Cox test. (C) Body weight of WT and *Notch1<sup>+/-ΔTAD</sup>* mice 9 days after 5-FU injection. (D) Red blood cell (RBC) and platelet (PLT) counts and hemoglobin (Hb) concentration were determined from peripheral blood of WT and *Notch1<sup>+/-ΔTAD</sup>* mice ( $n=5-9$  mice/group) before (D0) and at days 4, 7 and 9 after 5-FU injection. (E) Numbers of hematopoietic stem cells (HSC) and LinSca1<sup>+</sup>cKit<sup>+</sup> (LSK) progenitors in the bone marrow were analyzed 9 days after 5-FU injection. HSC were defined as Lin<sup>+</sup>cKit<sup>+</sup>Sca1<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells. The numbers of each population are expressed as mean  $\pm$  standard deviation (SD). (F) Cytometric analysis of common lymphoid progenitors (CLP) in bone marrow from WT and *Notch1<sup>+/-ΔTAD</sup>* mice at day 14 after 5-FU injection. CLP were defined as Lin<sup>+</sup>cKit<sup>low</sup>Sca1<sup>low</sup>CD135<sup>+</sup>CD127<sup>+</sup> (left panel). Absolute numbers of CLP from each mouse are expressed as mean  $\pm$  SD (right panel). (G, H) Cytometric analysis of early T-cell precursors (ETP) and double-negative 3 (DN3) cells in the thymus from WT and *Notch1<sup>+/-ΔTAD</sup>* mice at day 14 after 5-FU injection. ETP and DN3 populations were defined as Lin<sup>+</sup>cKit<sup>+</sup>CD25<sup>+</sup>CD144<sup>+</sup> (G) and Lin<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup> (H), respectively. Absolute numbers of ETP and DN3 cells from each mouse are expressed as mean  $\pm$  SD. (I) Representative images of the thymus from WT and *Notch1<sup>+/-ΔTAD</sup>* mice at day 14 after 5-FU injection. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ICN: intracellular domains of Notch; TAD: transcriptional activation domain; RAM: RBP-J-associated molecule domain; ANK: ankyrin repeats.

ferences were observed between *WT* and *Notch1*<sup>+/-</sup> littermates in hematopoietic progenitors and lineages in the BM and thymus (*Online Supplementary Figure S5D-H*). These findings show that a single allele of the *Notch1* receptor

was sufficient for recovery of the hematopoietic system and development of the T-cell lineage following myelosuppression. Thus, the phenotype observed in *Notch1*<sup>+ΔTAD</sup> mice was unique and depended on the function of the TAD.



**Figure 2. Transcriptional defect in T-cell development induced by *Notch1*<sup>+ΔTAD</sup>.** (A) Experimental design of 5-fluorouracil (5-FU) treatment of recipient mice reconstituted with hematopoietic stem cells (HSC) from *Notch1*<sup>+ΔTAD</sup> mice. Three hundred and fifty HSC from 5-week old wildtype (*WT*) or *Notch1*<sup>+ΔTAD</sup> donors were sorted and transplanted into irradiated CD45.1/2<sup>+</sup> *WT* recipients. After transplantation, 5-FU (150 mg/kg) was injected intraperitoneally into recipient mice on days 1 and 14. (B) Kaplan-Meier plot after 5-FU injection of recipients reconstituted by either *WT* or *Notch1*<sup>+ΔTAD</sup> HSC. Mice (n=6/group) were monitored daily until day 28 after the first 5-FU injection. Statistical significance was determined using the Mantel-Cox test. (C) Absolute numbers of donor-derived HSC in the bone marrow 14 days after the 5-FU injection. (D) Absolute numbers of donor-derived common lymphoid progenitors (CLP) in bone marrow at day 14 after the 5-FU injection are shown as mean ± standard deviation (SD). (E, F) Cytometric analysis and quantification of donor-derived early T-cell precursors (ETP) and double-negative 3 (DN3) cells from the thymus of reconstituted mice at day 14 after 5-FU injection. Absolute numbers are shown as mean ± SD. (G) Representative images of the thymus from recipient mice at day 14 after 5-FU injection. (H, I) Expression of the indicated genes in the DN3 population from *WT* or *Notch1*<sup>+ΔTAD</sup> mice (n=3). Thymic DN3 cells collected by flow-activated cell sorting at day 9 after injection of phosphate-buffered saline (PBS) (H) or 5-FU (I). Fold changes in the relative gene expression in *Notch1*<sup>+ΔTAD</sup> DN3 cells are shown normalized to *WT* expression and GAPDH. (J) Expression of Notch1-ICN and Notch1-ICNΔTAD in CD25<sup>+</sup> thymocytes (n=3). CD25<sup>+</sup> thymocytes from *WT*, *Notch1*<sup>+ΔTAD</sup> and *Notch1*<sup>-/-</sup> mice were isolated by anti-CD25 dynabead magnetic sorting and whole cells were lysed to measure protein expression of cleaved Notch1 ICN (val1744) by western blot. ICN is 100 kDa, ICNΔTAD is 75 kDa, and a non-specific band of ~65kDa is seen in all three lanes. (K) U2OS cells transfected with pMIGR1 control vector, pMIGR1-Notch1 ICN and pMIGR1-Notch1 ICNΔTAD. At 48 h, protein expression of Notch1 ICN and Notch1 ICNΔTAD was measured by western blot. (L) Experimental design of the luciferase assay. U2OS cells were transfected with reporters pRL-TK, pGL3-CSL4X, and with either pMIGR1 control vector or ICN or ICNΔTAD or both ICN and ICNΔTAD at indicated ratios (n=3). (M) Firefly luciferase activity was measured relative to renilla luciferase activity. Values are shown relative to the empty vector. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

### Transcriptional defect in *Notch1*<sup>+/ΔTAD</sup> impairs T-cell development

Notch function in T-cell development has been well documented,<sup>27,28</sup> so to determine how lack of a single TAD allele affects T cells, we measured target expression in sorted WT and *Notch1*<sup>+/ΔTAD</sup> DN3 cells before and after 5-FU treatment. During resting conditions, no significant change was observed in the expression of *Hes1* or *Myc*, but a decrease in the levels of *Hey1* and *Dtx1* was seen in *Notch1*<sup>+/ΔTAD</sup> DN3 cells (Figure 2H). However, the expression of *Hes1*, *Hey1*, and *Dtx1* was severely reduced after 5-FU treatment, and expression of pre-T-cell receptor alpha (*pTα*) and *Myc* was moderately reduced (Figure 2I), suggesting that Notch1ΔTAD strongly impairs target gene expression in developing thymocytes during regeneration.

To determine whether production of active Notch1 was equal among our different model systems, CD25<sup>+</sup> thymocytes were isolated from WT, *Notch1*<sup>+/−</sup>, and *Notch1*<sup>+/ΔTAD</sup> mice. The abundance of cleaved, full-length intracellular domains of Notch (ICN) was comparable between *Notch1*<sup>+/−</sup> and *Notch1*<sup>+/ΔTAD</sup> samples; however, there was an accumulation and a nearly 3-fold increase of the levels of cleaved ICN ΔTAD protein (Figure 2J).

Next, we employed an *in vitro* U2OS-based Notch luciferase reporter assay to assess the effects of accumulated ICN ΔTAD on transcription.<sup>29</sup> We chose U2OS cells, a human osteosarcoma cell line, because they express very low levels of endogenous Notch signaling and are highly transfectable. Constructs expressing Notch1 WT or ΔTAD mutant were transfected into U2OS cells at various ratios of ICN to ICNΔTAD (Figure 2K, L). ICNΔTAD had a lower capacity of transcriptional activation compared to ICN (Figure 2M, lanes 3 and 4). Dose-dependent transcriptional inhibition was observed when ICNΔTAD was co-transfected with ICN. The presence of a 1:4 ratio of ICN:ICNΔTAD caused a 4-fold decrease in luciferase activity (Figure 2M, lane 6). This TAD-dependent regimen was rescued by inverting the ratio of ICN to ICNΔTAD (Figure 2M, lane 7). Our findings uncovered a role for the ΔTAD-mediated suppression of WT Notch signaling which we termed TAD transcriptional interference. These findings showed that the presence of a Notch1ΔTAD protein impaired Notch target expression. Since Notch signaling is crucial for T-lineage development, we conclude that this transcriptional interference negatively affected *in vivo* development of thymocytes.

### Chemotherapy severely damages bone marrow microvasculature and induces endothelial cell apoptosis in *Notch1*<sup>+/ΔTAD</sup> mice

Our findings suggest that the cell-intrinsic hematopoietic defect in *Notch1*<sup>+/ΔTAD</sup> is limited to the T-cell lineage, which cannot account for the pancytopenia, morbidity, and the eventual mortality observed in the *Notch1*<sup>+/ΔTAD</sup> mice (Figures 1 and 2). Thus, our attention turned to the BM microenvironment. First, to elucidate whether loss of one copy of TAD in the *Notch1* locus affects bone and mesenchymal cells, we performed micro-computed tomography analysis of femoral bones. This assay indicated comparable bone length, bone volume fraction (BV/TV) and cortical thickness in WT and *Notch1*<sup>+/ΔTAD</sup> mice (Online Supplementary Figure S6A-C). Furthermore, CD51 and Sca1 were used to identify osteoblasts and mesenchymal stem/precursor cells.<sup>30</sup> Flow cytometry analysis showed that there were similar numbers of osteoblasts

and mesenchymal stem/precursor cells in digested bones from WT and *Notch1*<sup>+/ΔTAD</sup> mice (Online Supplementary Figure S6D, E). These data indicate that loss of one copy of *Notch1* TAD does not significantly affect bone development in *Notch1*<sup>+/ΔTAD</sup> mice.

To test whether the presence of one *Notch1*ΔTAD allele affects formation of the bone vasculature network, whole-mount sternal imaging was conducted. Imaging of the vasculature labeled by CD31 and VE-cadherin showed comparable vessel number and vessel length between WT and *Notch1*<sup>+/ΔTAD</sup> mice under resting conditions (Figure 3A). Next, we wanted to determine the effect of chemotherapy on the recovery and regeneration of BM EC and specifically the sinusoidal EC, which have been shown to be crucial components of the adult hematopoietic niche.<sup>31–33</sup> Primary bone CD31<sup>+</sup> EC (pBEC) were harvested from WT mice 3 days after 5-FU treatment. Expression of the *Notch1* receptor and several Notch canonical gene targets were upregulated after the chemotherapy (Figure 3B). Target gene upregulation corresponded with Notch1 receptor cleavage (Figure 3B, inset), showing an increase in Notch activation which occurred in pBEC as early as 3 days after 5-FU treatment.

To determine the condition of the BM EC niche at the time point when the *Notch1*ΔTAD-expressing heterozygous hypomorphs succumb to myelosuppressive treatment, pBEC in WT or *Notch1*<sup>+/ΔTAD</sup> littermates were analyzed 9 days after 5-FU treatment (Figure 3C). Annexin V staining showed that 20% of the pBEC in *Notch1*<sup>+/ΔTAD</sup> mice were apoptotic compared to <7% of WT pBEC (Figure 3D). This sorted pBEC population from digested bones, over 90% of which expressed CD31<sup>+</sup> and less than 5% expressed CD45<sup>+</sup> (Figure 3E, left panel), was further analyzed for expression of pro-apoptotic genes. A corresponding 2-fold increase in the expression of *Puma* and *Bax*<sup>34</sup> was observed in sorted *Notch1*<sup>+/ΔTAD</sup> pBEC (Figure 3E, right panel). Absolute numbers of *Notch1*<sup>+/ΔTAD</sup> pBEC were markedly decreased, reflecting apoptosis (Figure 3F).

To visualize the BM microenvironment,<sup>35</sup> sections of the tibia of WT and *Notch1*<sup>+/ΔTAD</sup> littermates were analyzed before and after chemotherapy. Under resting conditions, as suggested by the whole mount imaging of the sternum (Figure 3A), there was no difference in the abundance of hematopoietic cells in the BM, and similar CD31<sup>+</sup> and endomucin<sup>+</sup> sinusoidal endothelial structures were present in WT and *Notch1*<sup>+/ΔTAD</sup> littermates (Figure 3G–I, top panels). After 5-FU treatment, retention of hematopoietic cells was markedly reduced in *Notch1*<sup>+/ΔTAD</sup> BM (Figure 3G, bottom panel). Severe damage to the BM niche following 5-FU treatment was evident by the lack of CD31<sup>+</sup> cells and irregular endomucin<sup>+</sup> endothelial staining in the *Notch1*<sup>+/ΔTAD</sup> mice following this treatment (Figure 3H–I, bottom panel). Overall, our findings indicate that the hematopoietic defect observed in *Notch1*<sup>+/ΔTAD</sup> mice can be attributed to the failure of endothelial regeneration after chemotherapy.

### Endothelial cell-specific deletion of Notch1 receptor confirms the requirement for endothelial Notch signaling during niche recovery

To specify a tissue-specific role for Notch signaling in the recovery and regeneration of BM EC, *Notch1*<sup>fl/VE-cadherin-Cre<sup>ERT2</sup> mice were treated with tamoxifen 1 week prior to administration of 5-FU (Figure 4A). The tamoxifen regimen led to loss of Notch1 protein (Figure 4B) and a</sup>

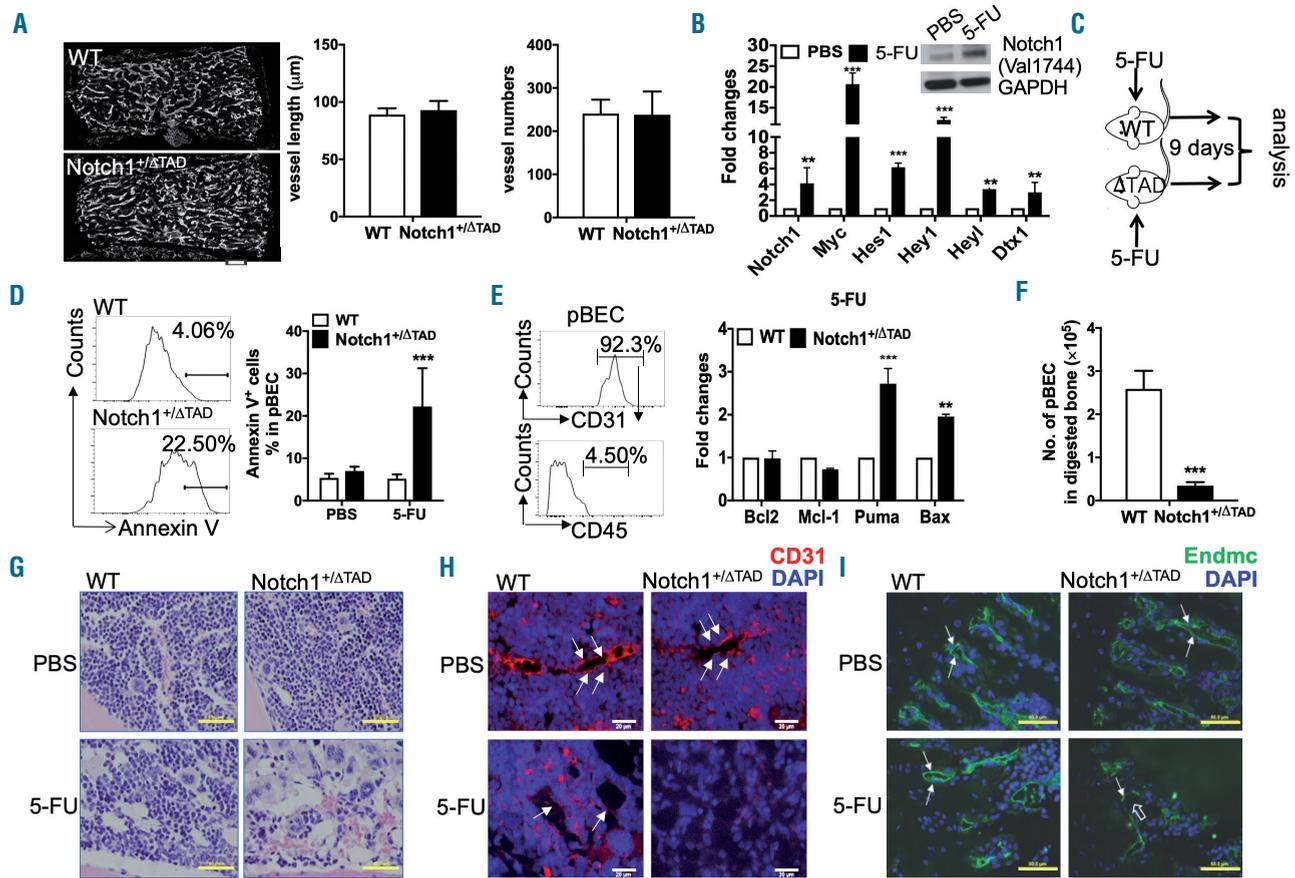
decrease in Notch target expression (Figure 4C) in pBEC. The condition of *Notch1<sup>fl/VE-cadherin-Cre<sup>ERT2+</sup></sup>* mice deteriorated (body condition index = 2) within a week after treatment, and five of 13 mice succumbed between days 7-9 after treatment (Figure 4D). The morbidity of *Notch1<sup>fl/VE-cadherin-Cre<sup>ERT2+</sup></sup>* mice was reflected in the low peripheral blood counts (Figure 4E) as well as decreases in the numbers of BM HSC, progenitors and CLP (Figure 4F, G). Consistent with increased endothelial apoptosis after treatment, the absolute number of pBEC in *Notch1<sup>fl/VE-cadherin-Cre<sup>ERT2+</sup></sup>* mice was significantly reduced (Figure 4H, I).

Besides the endothelium, perivascular cells have been implicated in the regulation of HSC function.<sup>36</sup> We observed a decrease in numbers of BM PDGFRβ<sup>+</sup> pericytes after chemotherapy in *Notch1<sup>+ΔTAD</sup>* mice. However, this effect was not due to increased apoptosis of the pericytes and may be attributed to a previously described loss of

BM EC (Online Supplementary Figure S7A). To determine whether Notch signaling in pericytes played a role in the recovery of the BM niche, *RBPJ<sup>fl</sup>-PDGFRβ-Cre<sup>ERT2+</sup>* and *RBPJ<sup>fl</sup>-PDGFRβ-Cre<sup>ERT2+</sup>* littermates were treated first with tamoxifen and then with 5-FU (Online Supplementary Figure S7B, C). We observed no significant hematopoietic differences between the two cohorts after 5-FU treatment (Online Supplementary Figure S7D-I). Overall these results show that robust Notch signaling in EC is essential for recovery of the BM niche following chemotherapeutic challenge.

### Loss of the transcriptional activation domain suppresses transcriptional activation of Notch1 targets in endothelial cells

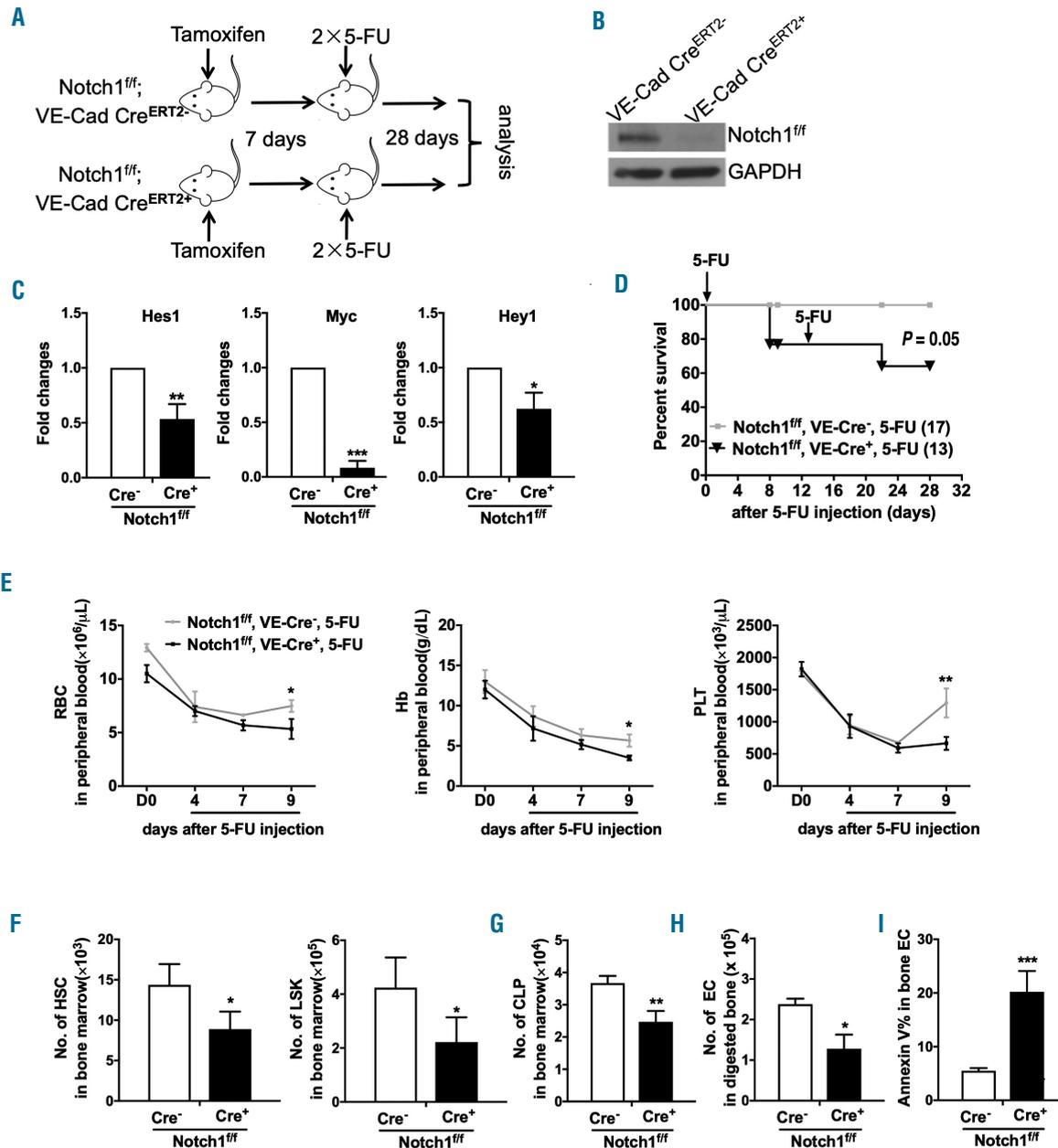
To determine whether the ΔTAD-dependent transcriptional interference observed in T cells also occurred EC



**Figure 3. Defective recovery of endothelial niche induced by *Notch1<sup>ΔTAD</sup>*.** (A) Whole mount imaging of the sternal vasculature after staining for CD31 and VE-cadherin was performed in WT and *Notch1<sup>ΔTAD</sup>* mice under resting conditions, including analysis of vessel length and vessel numbers (n=4). Scale bar=150 μm. (B) CD31<sup>+</sup> primary bone endothelial cells (pBEC) were isolated at day 3 after 5-fluorouracil (5-FU) injection and expression of the indicated genes was determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) normalized to expression of genes after injection of phosphate-buffered saline (PBS) (n=3). GAPDH was used as an internal expression control. Levels of active Notch1 protein in pBEC were measured by western blot at day 3 after 5-FU injection (insert). (C) Experimental design of 5-FU treatment for endothelial cell analysis in bone marrow (n=6). (D) pBEC harvested from long bones of WT and *Notch1<sup>ΔTAD</sup>* littermates gated for 4',6-diamidino-2-phenylindole (DAPI)CD45<sup>+</sup>TER119<sup>+</sup>CD31<sup>+</sup> and analyzed by flow cytometry for annexin V at day 9 after PBS or 5-FU injection (left panel). Percentages of annexin V<sup>+</sup> cells in CD31<sup>+</sup> endothelial cells (pBEC) are presented as mean±SD (right panel). (E) Isolated pBEC from digested bones were stained with DAPI/CD45/TER119/CD31<sup>+</sup>. The purity of CD31<sup>+</sup> cells as a percent of CD31<sup>+</sup>CD45<sup>+</sup> cells is demonstrated in the histogram analysis (left panel). The expression of indicated genes was determined by RT-qPCR in pBEC isolated from WT and *Notch1<sup>ΔTAD</sup>* mice at day 7 after 5-FU injection (right panel). Fold changes in the relative gene expression, normalized to WT samples with GAPDH used as an internal expression control, are shown as mean±SD. (F) Absolute numbers of pBEC (DAPI/CD45/TER119/CD31<sup>+</sup>) were analyzed at day 9 after 5-FU injection by flow cytometry. (G) Representative longitudinal bone sections from mice treated with PBS (upper panels) or 5-FU (lower panels) stained with hematoxylin and eosin-Y at day 9 after 5-FU treatment. Scale bar=50 μm. (H, I) Representative longitudinal bone section from mice treated with PBS (upper panels) or 5-FU (lower panel) fixed with formalin and stained for CD31 (H, red, scale bar=20 μm) or endomucin (Endmc) (I, green, scale bar=100 μm) at day 9 after 5-FU injection. DAPI was used to stain nuclei (blue). Filled white arrows point to vascular structures representative of the bone marrow endothelium. The open white arrow points to discontinuous vascular structures. \*\*P<0.01, \*\*\*P<0.001.

and whether it affected the recovery of EC in the BM niche, we tested the expression of Notch target genes in pBEC. Under basal conditions, expression of *Hes1*, *Hey1* and *Dtx1* was downregulated in *Notch1<sup>ΔTAD</sup>* pBEC, while expression of *Myc* and *EphB2* was unaffected (Figure 5A). However, 9 days after 5-FU treatment, expression of the

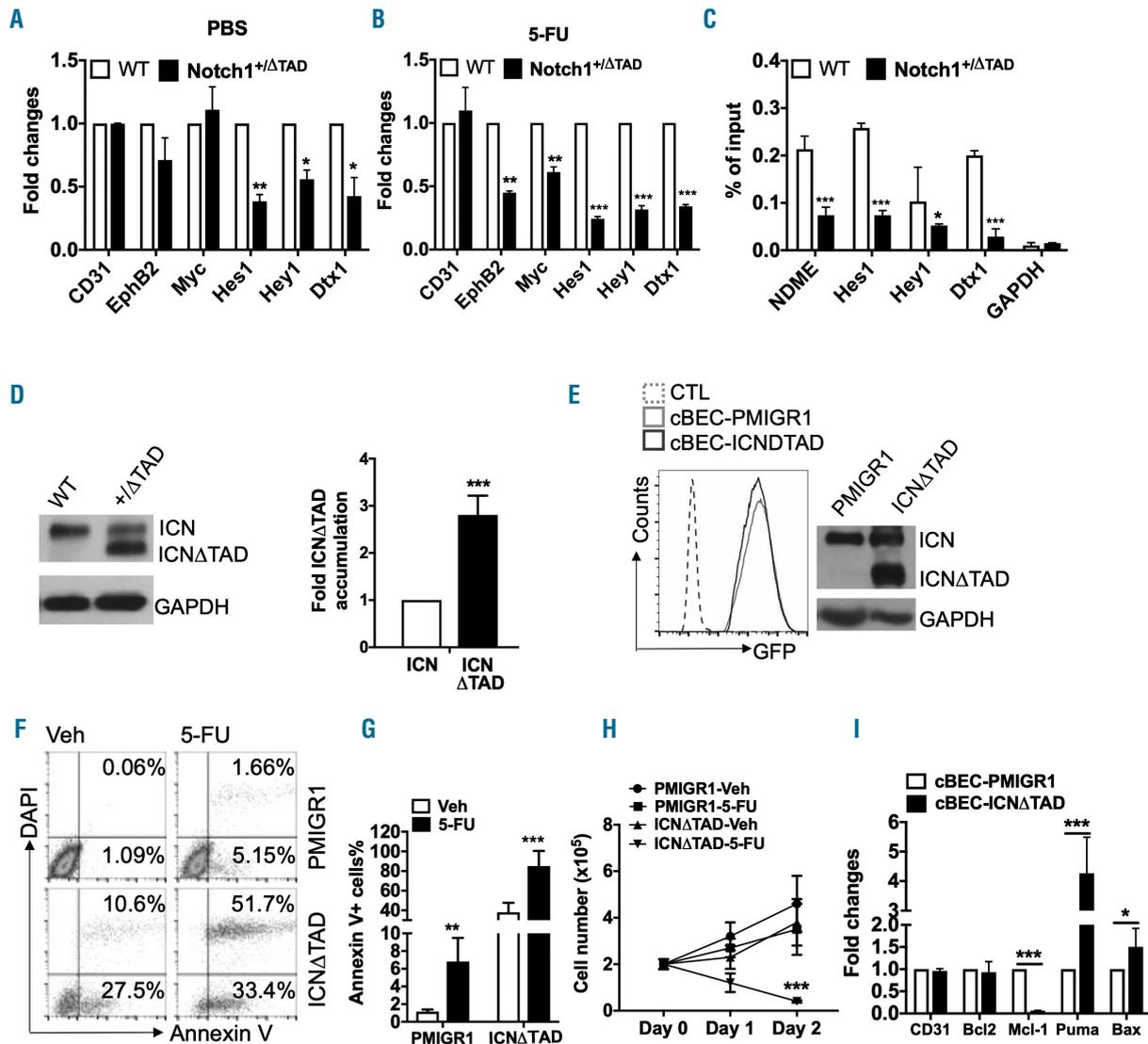
Notch target genes *EphB2*, *Myc*, *Hes1*, *Hey1* and *Dtx1* was markedly downregulated in *Notch1<sup>ΔTAD</sup>* pBEC (Figure 5B). To determine how Notch target expression was affected by the *Notch1ΔTAD* mutant, a chromatin immunoprecipitation assay was performed on purified pBEC from WT or *Notch1<sup>ΔTAD</sup>* littermates after 5-FU treatment. The chromatin



**Figure 4. Conditional deletion of *Notch1* in endothelial cells phenocopies *Notch1<sup>ΔTAD</sup>* mice after myelosuppression.** (A) Experimental design of 5-fluorouracil (5-FU) injection of *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* mice. Tamoxifen (80 mg/kg) was injected intraperitoneally (IP) into *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* mice for 5 consecutive days. One week later, 5-FU (150 mg/kg) was injected IP into *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* or *Cre<sup>ERT2</sup>* littermates on days 1 and 14. (B) Protein expression of cleaved Notch1 in CD31<sup>+</sup> primary bone endothelial cells (pBEC) from *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2</sup>* or *Cre<sup>ERT2+</sup>* littermates after tamoxifen treatment. GAPDH was used as a loading control. (C) Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) expression of *Hes1*, *Myc*, *Hey1* and *Dtx1* in pBEC harvested from *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* or *Cre<sup>ERT2</sup>* littermates after tamoxifen and 5-FU treatment. (D) Kaplan-Meier plot of *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* and *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2</sup>* littermates after tamoxifen and 5-FU treatment. Mice were monitored daily until day 28 after 5-FU injection (n=13-17 mice/group). Significance (P=0.05) was determined by the Mantel-Cox test. (E) Peripheral blood counts of red blood cells (RBC) and platelets (PLT), and concentration of hemoglobin (Hb) in *Notch1<sup>ff</sup>; VE-Cadherin Cre<sup>ERT2+</sup>* and *Cre<sup>ERT2</sup>* littermates were conducted before (D0) and at days 4, 7 and 9 after 5-FU injection. (F) Absolute numbers of hematopoietic stem cells (HSC) and LinSca1<sup>+</sup>c-kit<sup>+</sup> (LSK) cells in bone marrow were analyzed 9 days after the 5-FU injection and expressed as mean ± standard deviation (SD). (G) Absolute numbers of common lymphoid progenitors (CLP) in bone marrow were analyzed at day 9 after the 5-FU injection and expressed as mean ± SD. (H) Absolute numbers of pBEC were analyzed at day 9 after the 5-FU injection by flow cytometry. (I) Percentages of annexin V<sup>+</sup> Lin-Sca1<sup>+</sup>c-Kit<sup>+</sup> pBEC 9 days after 5-FU injection are presented as mean ± SD. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

immunoprecipitation assay showed reduction of Notch1 occupancy at the Notch-dependent Myc enhancer *NDME*<sup>37</sup> and at *Hes1*, *Hey1* and *Dtx1* promoters<sup>38</sup> in *Notch1*<sup>+ΔTAD</sup> pBEC (Figure 6C). In line with our findings in thymocytes (Figure 2J), we observed a 3-fold accumulation of Notch1 ICN ΔTAD protein in pBEC when compared to cleaved Notch1 WT protein (Figure 5D). These results indicate that ΔTAD interference occurred in pBEC and contributed to the decreased target gene expression in EC, thus limiting regeneration and recovery of the BM niche.

For further *in vitro* study of ΔTAD transcriptional interference, pBEC were sorted from the BM by expression of CD31<sup>+</sup> and VE-cadherin<sup>+</sup> (*Online Supplementary Figure S8A*). These cultured bone EC (cBEC) exhibited typical endothelial morphology, VE-cadherin<sup>+</sup> adherent junctions and active Notch signaling (*Online Supplementary Figure S8B, C*). To replicate the effects of ΔTAD interference, we transduced cBEC with a retroviral vector expressing GFP (PMIGR1) or ICNΔTAD (Figure 5E, left). Accumulation of ICNΔTAD protein in GFP<sup>+</sup> sorted cBEC



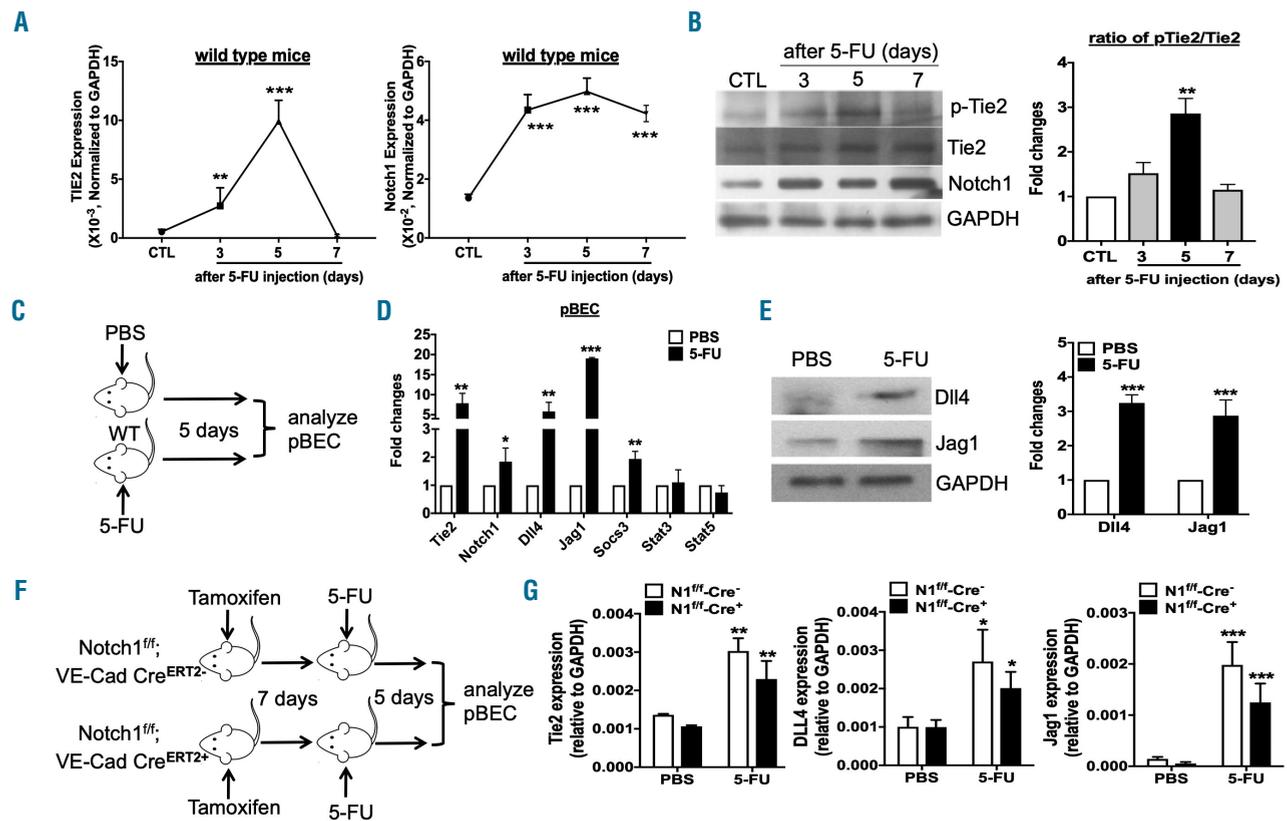
**Figure 5. Notch1 ICNΔTAD interference in bone marrow endothelial cells.** (A, B) The expression of the indicated genes was determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) in CD31<sup>+</sup> primary bone endothelial cells (pBEC) at day 9 after treatment of wildtype (WT) and *Notch1*<sup>+ΔTAD</sup> littermates (n=3) with phosphate-buffered saline (PBS) or 5-fluorouracil (5-FU). Values were normalized to expression of genes in WT littermates. *GAPDH* was used as an internal expression control. (C) Local chromatin immunoprecipitation (CHIP) assay to detect the occupancy of Notch1 complex on Rbpj DNA-binding sites of Notch-dependent Myc enhancer (*NDME*), *Hes1*, *Hey1*, *Dtx1* and *GAPDH* promoter elements. pBEC from WT and *Notch1*<sup>+ΔTAD</sup> mice were used for CHIP analysis at day 9 after 5-FU treatment (n=3). Values represent the mean of signal intensity relative to input DNA normalized to IgG. (D) pBEC from WT and *Notch1*<sup>+ΔTAD</sup> mice were harvested and used to measure protein levels of cleaved Notch1 and Notch1ΔTAD by western blot (left panel). The band intensities of Notch1 ICN and Notch1 ICNΔTAD in *Notch1*<sup>+ΔTAD</sup> pBEC were quantified by ImageJ software (n=3, right panel). (E) Flow cytometry analysis of cultured bone marrow-derived endothelial cells (cBEC) (untransduced control) or cBEC transduced with retrovirus expressing PMIGR1 or PMIGR1-ICNΔTAD (left panel). Western blot analysis for expression of Notch1 protein in transduced and sorted GFP<sup>+</sup> cBEC expressing PMIGR1 or PMIGR1-ICNΔTAD. *GAPDH* was used as a loading control (n=3, right panel). (F, G) cBEC transduced with PMIGR1 or PMIGR1-ICNΔTAD were treated with 5-FU or vehicle (Veh) for 24 h (n=3). Representative annexin V and 4',6-diamidino-2-phenylindole (DAPI) flow cytometry analysis (F) and quantified percentages of pro-apoptotic annexin V<sup>+</sup> cells (G). (H) Cell counts of cBEC-PMIGR1 and cBEC-ICNΔTAD after Veh or 5-FU treatment. Cell numbers were counted at day 1 and day 2 after a single 5-FU treatment (n=3). (I) RT-qPCR for the indicated genes in cBEC-PMIGR1 and cBEC-ICNΔTAD cells 24 h after 5-FU treatment. Values were normalized to PMIGR1 transduced cells. *GAPDH* was used as an internal expression control. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

was confirmed by western blot analysis (Figure 5E, right).

Increased ICN $\Delta$ TAD in cBEC markedly impaired the expression of *Hey1* and *Dtx1* and significantly downregulated the expression of *Myc* (Online Supplementary Figure S8D). To mimic chemotherapy *in vitro*, cBEC were treated with 100  $\mu$ M of 5-FU. After 24 h, the cells were analyzed for apoptosis by flow cytometry. We observed that 27% of cBEC-ICN $\Delta$ TAD cells were pro-apoptotic (annexin V<sup>+</sup>) under resting conditions; furthermore, the apoptotic cells (annexin V<sup>+</sup>/DAPI<sup>+</sup>) cells increased to 50% in 5-FU-treated cBEC-ICN $\Delta$ TAD (Figure 5F, G). Apoptosis of cBEC-ICN $\Delta$ TAD led to a rapid decrease in cell numbers 48 h after 5-FU treatment (Figure 5H). This finding was validated by a decrease of Mcl-1 expression and an increase of Puma and Bax expression in cBEC-ICN $\Delta$ TAD (Figure 5I). These findings corroborate the *in vivo* phenotype observed in freshly isolated BM EC and indicate the importance of Notch signaling in the recovery and survival of EC after chemotherapy.

### Both Notch1 and Tie2 signaling are activated during recovery of bone marrow endothelium

Tie2 signaling has been proposed to facilitate endothelial regeneration.<sup>2,39</sup> Thus, we compared the expression of the *Notch1* and *Tie2* receptors 3, 5, and 7 days after 5-FU treatment in the pBEC of WT mice. Tie2 expression was low in resting pBEC and rapidly increased by 10-fold at day 5 after 5-FU treatment (Figure 6A). This increase in Tie2 expression corresponded with a significant increase in the phosphorylation of Tie2 protein (p-Tie2) (Figure 6B, right panel). Expression of *Notch1* receptor was increased 4-fold after treatment, but *Notch1* receptor expression and activation remained high throughout until day 7 (Figure 6A, B). Previous work has suggested that activation of Tie2 signaling by Ang1 in human vascular EC results in increased induction of Notch ligand delta-like 4 (Dll4).<sup>40</sup> To test this *in vivo*, we treated WT mice with 5-FU (Figure 6C). Five days later, expression of *Dll4* and *Jag1*, determined by analysis of both mRNA (Figure 6D) and protein levels



**Figure 6. Both Notch1 and Tie2 signaling are activated during endothelial niche recovery after chemotherapy.** (A) Expression kinetics of Tie2 and Notch1 after chemotherapy. Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) expression of *Tie2* and *Notch1* in sorted CD31<sup>+</sup> primary bone endothelial cells (pBEC) from wildtype (WT) mice before (CTL) and at days 3, 5 and 7 after 5-fluorouracil (5-FU) treatment. *GAPDH* was used as an internal expression control at each time-point. (B) Representative western blot of protein level analysis for phosphorylated Tie2 (p-Tie2), total Tie2, cleaved Notch1 and GAPDH from sorted pBEC before (CTL) and at days 3, 5 and 7 after 5-FU treatment (left panel). The band intensities of Tie2 and p-Tie2 in pBEC were quantified by ImageJ software. The ratios of pTie2/Tie2 at days 3, 5 and 7 after 5-FU treatment were normalized to that of the control (CTL) group. The fold changes of pTie2/Tie2 ratio are presented (n=3, right panel). (C) Experimental design of 5-FU treatment for pBEC analysis (phosphate-buffered saline, PBS, n=6; 5-FU, n=15). Five- to six-week old mice were treated intraperitoneally (IP) with PBS or 5-FU. CD31<sup>+</sup>Ter119<sup>+</sup>CD45<sup>+</sup> cells (pBEC) were sorted from digested bones 5 days after 5-FU treatment. (D) RT-qPCR expression of indicated genes from pBEC treated as described in (C). Values are normalized to PBS-treated pBEC. *GAPDH* was used as an internal expression control. (E) Protein expression of Dll4 and Jag1 was measured in pBEC from mice treated with PBS or 5-FU. *GAPDH* was used as a loading control (left panel). The band intensities of Dll4 and Jag1 in pBEC were quantitated by ImageJ software (n=3, right panel). (F) Experimental design of 5-FU injection of *Notch1*<sup>fl/fl</sup>;*VE-CadherinCre*<sup>ERT2</sup> mice (left panel). *Notch1*<sup>fl/fl</sup>;*VE-CadherinCre*<sup>ERT2</sup> mice were injected IP with tamoxifen daily for 5 days. One week later, 5-FU was injected IP into *Notch1*<sup>fl/fl</sup>;*VE-CadherinCre*<sup>ERT2</sup> or *Cre*<sup>ERT2</sup> littermates. pBEC were analyzed at day 5 after 5-FU injection (n=3). (G) Expression of *Tie2*, *Dll4* and *Jag1* was measured by RT-qPCR in pBEC as described in (F). Absolute expression values were compared to those of PBS-injected mice of each genotype. *GAPDH* was used as an internal expression control.

(Figure 6E), was significantly increased in pBEC from both BM (*Online Supplementary Figure S8E*) and digested bone (Figure 6D, E). Notch ligand upregulation corresponded with the upregulation of *Tie2*, *Notch1* and the Tie2 signaling target *Socs3* (Figure 6D, E, *Online Supplementary Figure S8E*).

Our initial expression timeline (Figure 6A) indicated that *Notch1* expression is upregulated prior to *Tie2* upregulation. To determine whether the induction of *Tie2* and Notch ligands after chemotherapy depended on active Notch signaling, we employed the previously described *Notch1<sup>fl/fl</sup>VE-cadherin-Cre<sup>ERT2+</sup>* model system and analyzed freshly harvested pBEC from WT (*Cre*<sup>-</sup>) and *Notch1<sup>fl/fl</sup>VE-cadherin-Cre<sup>ERT2+</sup>* (*Cre*<sup>+</sup>) mice after tamoxifen and 5-FU treatment (Figure 6F). Our data indicate that 5-FU treatment significantly increased expression of *Tie2*, *Dll4* and *Jag1* in pBEC in WT and *Notch1<sup>fl/fl</sup>VE-cadherin-Cre<sup>ERT2+</sup>* mice. However, loss of endothelial *Notch1* expression did not significantly affect upregulation of *Tie2* after 5-FU treatment (Figure 6G, left panel). Furthermore, loss of Notch signaling did not significantly affect the expression of Notch ligands *Dll4* and *Jag1* (Figure 6G), upregulation of which coincided with the increase in *Tie2* expression and activity (Figure 6A, B). Taken together, we show that Tie2 signaling is independent of Notch1 signaling during BM endothelial regeneration and recovery.

### Notch1 functions downstream of Tie2 signaling during the recovery of bone marrow endothelium

Previous studies have shown that hematopoietic progenitors and mesenchymal cells are responsible for the secretion of Ang1 in the BM.<sup>10</sup> To test this, hematopoietic progenitor (Lin<sup>-</sup>Sca-1<sup>+</sup>) cells and bone osteoblasts (CD51<sup>+</sup>) were sorted from the BM 5 days after 5-FU treatment. Expression of *Ang1* was markedly increased in 5-FU-treated hematopoietic progenitors (up to 150-fold) and osteoblasts (up to 30-fold) when compared to the same untreated cell populations (Figure 7A). To further analyze any possible crosstalk between the Notch and Tie-2 signaling pathways, we supplemented cBEC culture media with Ang1, which caused an increase in the levels of p-Tie2 and cleaved Notch1 (Figure 7B). Ang1 stimulation also induced the expression of Notch ligands (*Dll4* and *Jag1*) and its targets, such as *Hes1*, *Hey1* and *Myc* (Figure 7C). To determine whether Tie2 stimulation by Ang1 was essential for Notch ligand upregulation, we treated cBEC with a Tie2 inhibitor. Our results show that increased expression of *Dll4* and *Jag1* by Ang1 stimulation was completely blocked by pre-treatment with the Tie2 kinase inhibitor (Figure 7D). Our findings support a role for the Tie2 signaling pathway in the induction of Notch signaling by enhancing expression of Notch ligands.

To determine whether Notch signaling also influenced *Tie2* expression, we inhibited Notch activity by addition of a  $\gamma$  secretase inhibitor for 48 h followed by a 3 h washout. Notch targets, inhibited by  $\gamma$  secretase inhibitors, are rapidly upregulated during washout. We observed that this was the case for *Hes1* in cBEC, but not for *Tie2* and *Ang2* (Figure 7E). Interestingly, *Myc* expression in cBEC was not entirely dependent on Notch activity; however, *Myc* levels markedly increased after the washout of the  $\gamma$  secretase inhibitor, indicating that in EC *Myc* is responsive to higher levels of Notch signaling (Figure 7E). These data showed that, in BM EC, Tie2 activation enhanced downstream Notch signaling while Notch activity had no effect on Tie2 signaling.

To test whether Notch functioned downstream of Tie2 activation, ICNΔTAD-cBEC cells were treated with 5-FU for 24 h and then the medium was supplemented with Ang1 (Figure 7F, left). Ang1 stimulated moderate growth of resting cBEC but had no effect on the ICNΔTAD-transduced cBEC. After 5-FU treatment, Ang1 significantly increased growth of control cBEC but did not rescue ICNΔTAD-cBEC (Figure 7F, right). Tie2 activation by Ang1 had the expected result of increasing *Socs3* expression.<sup>41</sup> However, Tie-2 activation had no effect on the Notch targets *Hes1* and *Hey1* in ICNΔTAD-expressing cells (Figure 7G). These results show that whereas Tie2 activation accelerates the recovery of the BM EC niche after chemotherapy by promoting Notch ligand expression, ultimately, the resolution of niche recovery depends on activation of robust Notch signaling.

## Discussion

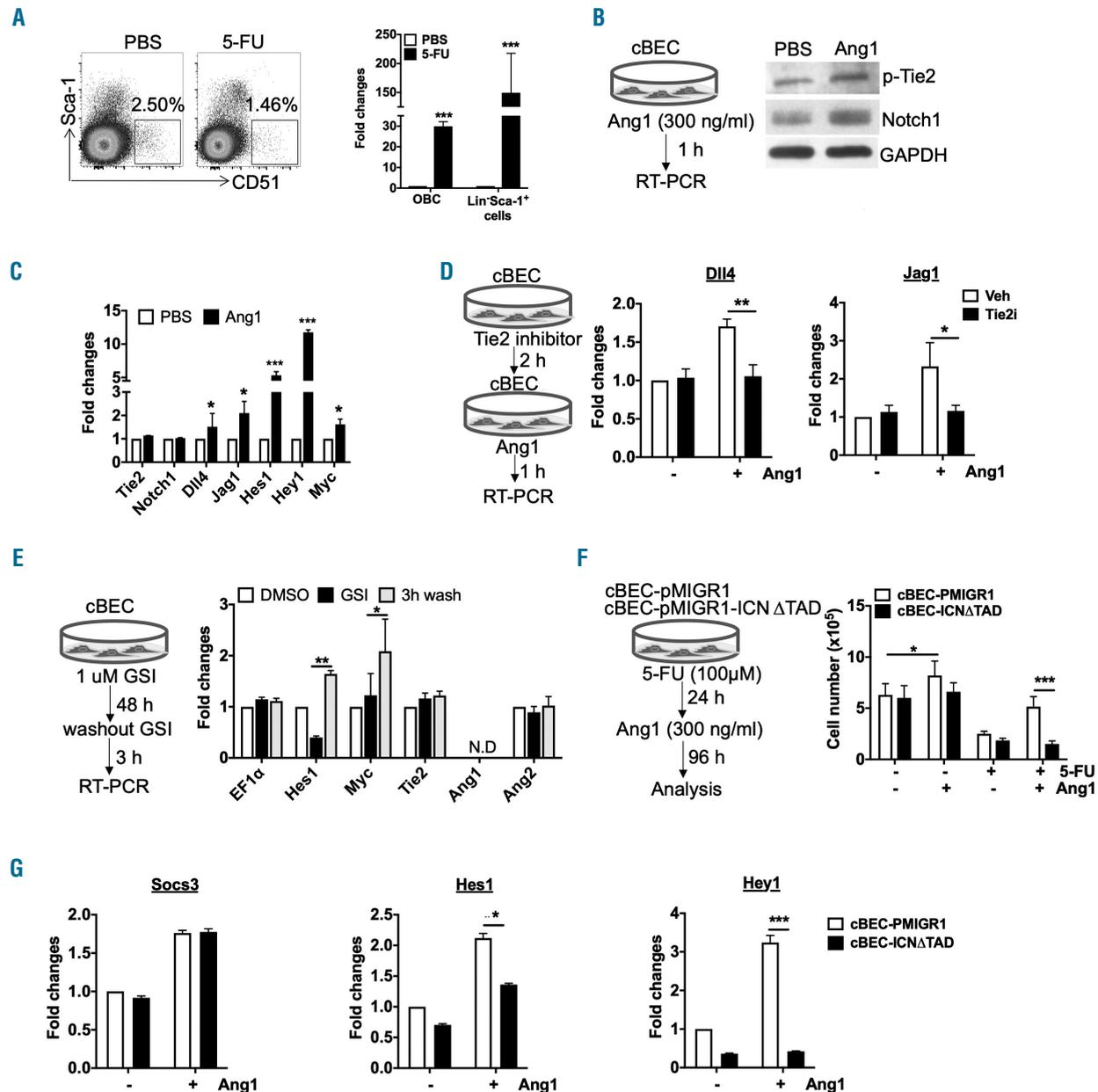
The Notch1 receptor is crucial for the emergence of the first definitive HSC from the embryonic hemangioblast<sup>22</sup> and expansion of fetal liver HSC.<sup>22</sup> The role of Notch signaling in adult BM HSC is not well understood and mired in controversy.<sup>43-46</sup> It has been suggested that Notch signaling is essential for HSC self-renewal, specifically with regard to Jagged1 activation of Notch1 signaling in HSC.<sup>46</sup> Even so, pan-inhibition of Notch signaling in HSC by deletion of the key transcriptional complex member and DNA-binding factor RBPJ, showed no adverse effects in maintenance and expansion of the stem and progenitor pool.<sup>47</sup> A separate recent study showed depletion of HSC numbers after complete ablation of Notch signaling in the endothelium, thus suggesting that the Notch1 receptor participates in the development of the EC niche.<sup>48</sup> An even more recent article indicated a possible alternative to Notch1 BM signaling by suggesting that Jagged2 triggers Notch2 signaling in HSC after myelosuppression.<sup>49</sup>

Regardless of its proposed involvement during hematopoietic and endothelial development, the specific role of Notch signaling, its mechanistic interplay with other known regenerative pathways and its temporal activation during and following myelosuppression are not known. In the present study, we investigated Notch signaling in adult hematopoietic and endothelial tissues by deleting the TAD from a single allele of *Notch1*. This mutation has the capacity to interfere with the activity of the WT Notch1 transcriptional complex and created a hypomorphic signaling environment. Our *Notch1<sup>+ΔTAD</sup>* heterozygous model system matured to adulthood and under homeostatic conditions exhibited no adverse effects in the bone and vasculature. We show that this heterozygous hypomorphic model system is useful for determining the requirement for robust Notch signaling activation in adult tissues. Using the *Notch1<sup>+ΔTAD</sup>* mice we determined that basal Notch1 signaling was sufficient for development of the endothelial hematopoietic niche; however, robust activation of Notch1 was required for the regeneration of the hematopoietic system.

We observed increased Notch1 receptor expression and cleavage in BM EC following treatment with the chemotherapeutic agent 5-FU. The WT BM microenvironment began to recover at ~9 days after exposure to 5-FU. However, the BM niche was persistently disrupted in *Notch1<sup>+ΔTAD</sup>* mice as evidenced by the presence of few CD31<sup>+</sup>

and endomucin<sup>+</sup> microvessels and a high frequency of apoptotic EC. Defective regeneration of EC in these mice caused gaps in sinusoidal vessels leading to severe hemorrhage.<sup>5,50</sup> The occupancy of the Notch transcriptional complex and expression of Notch target genes associated with survival and proliferation, such as *Hes1* and *Myc*,<sup>51,52</sup> was markedly decreased in *Notch1*<sup>+ΔTAD</sup> BM EC, indicating that

the regeneration of niche EC following chemotherapy was dependent on high levels of Notch signaling. Validation was obtained using conditional deletion of the Notch1 receptor in the VE-cadherin<sup>+</sup> EC. Similar to the *Notch1*<sup>+ΔTAD</sup>, this EC-specific, conditional, loss-of-function Notch1 model exhibited defective regeneration of BM EC highlighted by increased EC apoptosis and limited hematopoietic recovery.



**Figure 7. Notch1 functions downstream of Tie2 signaling to mediate endothelial niche recovery.** (A) Representative gating strategy for osteoblasts (Ter119-CD45<sup>+</sup> CD31<sup>+</sup> Sca1<sup>+</sup> CD51<sup>+</sup> cells) in digested bones from mice treated with phosphate-buffered saline (PBS) and 5-fluorouracil (5-FU) (PBS, n=5; 5-FU, n=15; left panel). Expression of *Ang1* was determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) in osteoblasts and Lin<sup>+</sup>Sca-1<sup>+</sup> cells treated with PBS and 5-FU (right panel). GAPDH was used as an internal expression control. (B) Schematic of *Ang1* treatment in serum-starved (16 h) cultured bone marrow-derived endothelial cells (cBEC) (left panel). Expression of p-Tie2, cleaved Notch1 and GAPDH protein levels in *Ang1*-treated cBEC was detected by western blot (right panel). (C) RT-qPCR expression of Notch target genes from cBEC treated as described in (B). Values are normalized to those in PBS-treated cBEC. GAPDH was used as an internal expression control. (D) Schematic of Tie2 inhibitor (Tie2i) and *Ang1* treatment in serum-starved (16 h) cBEC (left panel). Expression of *Dll4* and *Jag1* was determined by RT-qPCR in Tie2i and/or *Ang1*-treated cBEC. The vehicle for the Tie2 inhibitor was dimethylsulfoxide (DMSO). Vehicle for *Ang1* is PBS. (E) Schematic for treatment of cBEC with  $\gamma$  secretase inhibitors (GSI). RT-qPCR expression analysis of the indicated genes in cBEC treated with GSI/washout. Values were normalized to those of DMSO-treated cBEC. GAPDH was used as an internal expression control. (F) Schematic for treatment of control PMIGR1 or PMIGR1-ICN $\Delta$ TAD cBEC with 5-FU and *Ang1*. The indicated cBEC populations of cells were monitored for growth after 5-FU and *Ang1* treatment. (G) RT-qPCR expression of Tie2 target *Socs3*, and Notch targets *Hes1* and *Hey1* was examined after cell growth analysis of cBEC in (F). Values were normalized to those of PMIGR1 cBEC without *Ang1*-treatment. GAPDH was used as an internal expression control. Unless indicated differently, n=3 were used for experiments in this figure. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

While further work is required to determine whether specific subsets of BM EC are more susceptible to loss of Notch signaling during niche regeneration, our work suggests that a majority of EC, which express CD31, VE-cadherin and endomucin, require robust Notch1 signaling to recovery after myelosuppressive injury.

Mediators of BM niche recovery include VEGF-A, Tie2, and the Tie2 agonist Ang1.<sup>2,53,54</sup> Initiation of regenerative signaling is believed to hinge upon VEGF-A produced by osteoblasts and osteocytes<sup>55,56</sup> and Ang1 produced by osteoprogenitors and hematopoietic precursors.<sup>10,57</sup> We observed that there was a short pulse of Tie2 phosphorylation after chemotherapeutic damage while Notch signaling remained at elevated levels for at least 7 days after chemotherapy. Tie2 activation increased Notch receptor cleavage and the Tie2 agonist, Ang1 stimulated Notch signaling and promoted the expression of two canonical Notch ligands: *Dll4* and *Jag1*. However, Tie2 stimulation, after 5-FU treatment failed to rescue the survival of Notch-deficient EC which expressed the hypomorphic *Notch1 $\Delta$ TAD* allele. These results indicate that Notch signaling functions downstream of Tie2 and that Notch1 signaling is ultimately responsible for the regeneration of the BM endothelium.

The *Notch1<sup>+/ $\Delta$ TAD</sup>* mice exhibited no inherent HSC defect in the absence of BM injury, corroborating reports that indicate a lack of involvement of Notch signaling in adult HSC expansion.<sup>44,45,47</sup> However, chemotherapy severely impaired the generation of HSC in *Notch1<sup>+/ $\Delta$ TAD</sup>* mice suggesting that Notch signaling is required for HSC self-renewal. Transplantation of *Notch1<sup>+/ $\Delta$ TAD</sup>* HSC reconstituted irradiated WT hosts which showed no pancytopenic symptoms after chemotherapy, indicating that there is no cell-intrinsic defect in HSC generation due to the presence of the Notch1 $\Delta$ TAD protein. In fact, the reverse experiment, in which WT HSC were transplanted into *Notch1<sup>+/ $\Delta$ TAD</sup>* recipients had the same severe phenotype as the constitutive *Notch1<sup>+/ $\Delta$ TAD</sup>* or *Notch1<sup>fl/VE-cadherin-Cre<sup>ERT2+</sup></sup>* models, further supporting a specific role for Notch signaling in the regeneration of the BM EC niche. Thus, we attribute the loss of HSC and progenitor cells observed after 5-FU treatment to the destruction of the BM EC niche, a process which requires robust Notch activity for its recovery.

Previous work has established that successful T-cell development requires Notch signaling up until  $\beta$ -selection of  $\alpha\beta$  T cells in the thymus.<sup>27,58,59</sup> *Notch1<sup>+/ $\Delta$ TAD</sup>* mice exhibited a moderate decrease in developing T cells and had no defect in mature single-positive T cells (*data not shown*). This inherent defect was exacerbated by 5-FU treatment and extended to the BM-residing CLP population. These results suggest that the Notch-driven commitment of the T-cell lineage begins at an early stage in the BM. A recent study, in which genetic deletion of *Dll4* in the bone producing mesenchymal *Ocn<sup>+</sup>* cells decreased CLP numbers and limited the production of T cells in the thymus,<sup>60</sup> supports our observations. Thus, the present findings suggest an important role for Notch1 signaling in mediating the differentiation of early lymphoid hematopoietic progenitors and the existence of a specific Notch-signal-promoting niche in the BM for T-cell commitment prior to thymic migration.

In summary, we employed a novel strategy for physiological inhibition of Notch signaling in adult tissues using a hypomorphic *in vivo* model system. We demonstrated that high levels of Notch1 signaling are required for the regeneration of BM EC after myelosuppressive chemotherapy and radiotherapy. Optimal recuperation of the EC niche relies on several signaling pathways, but it is ultimately a Notch-dependent process that supports regeneration of the BM niche and hematopoietic recovery.

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