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Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance

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A fundamental question in hematopoietic stem cell (HSC) biology is how self-renewal is controlled. Here we show that the molecular regulation of two critical elements of self-renewal, inhibition of differentiation and induction of proliferation, can be uncoupled, and we identify Notch signaling as a key factor in inhibiting differentiation. Using transgenic Notch reporter mice, we found that Notch signaling was active in HSCs *in vivo* and downregulated as HSCs differentiated. Inhibition of Notch signaling led to accelerated differentiation of HSCs *in vitro* and depletion of HSCs *in vivo*. Finally, intact Notch signaling was required for Wnt-mediated maintenance of undifferentiated HSCs but not for survival or entry into the cell cycle *in vitro*. These data suggest that Notch signaling has a dominant function in inhibiting differentiation and provide a model for how HSCs may integrate multiple signals to maintain the stem cell state.

Hematopoietic stem cells (HSCs) are rare cells in the body that give rise to all the lineages of the blood. After HSCs were originally identified functionally[1], various methods have been used to isolate HSCs from the bone marrow[2–8], the primary site of hematopoiesis in adult life. A key feature of HSCs is their ability to self-renew as well as to differentiate into multiple lineages[9]. This ability is essential for HSCs to perpetuate themselves and to generate mature blood cells throughout the lifetime of an organism. Defining the signals that allow stem cells to maintain the balance between self-renewal and commitment is a critical issue in stem cell biology.

Extracellular signals such as Notch, Wnt and Hedgehog have been linked with the self-renewal and maintenance of HSCs and progenitors[10,11]. Notch proteins are highly conserved cell surface receptors that regulate development[12] and are mutant in a variety of cancers, including leukemia[13] and breast cancer[14,15]. Notch is a single-pass transmembrane receptor that is activated when its extracellular domain interacts with ligands of the Delta and Serrate families. Ligand-receptor interactions lead to cleavage and release of the intracellular fragment of Notch, which enters the nucleus and associates with the transcriptional repressor CBF-1 (also called CSL, for 'CBF-1 and RBP-Jκ in mammals, Suppressor of Hairless in *Drosophila* and Lag-1 in *C. elegans*')[12,15,16]. In association with the transcriptional coactivator mastermind-like 1 (MAML1)[17], the intracellular fragment of Notch binds to and converts CBF-1 to a transcriptional activator, thereby initiating expression of target genes.

Expression of constitutively active Notch1 in hematopoietic progenitors and stem cells allows the establishment of immortalized cell lines that retain the potential to generate both lymphoid and myeloid cells *in vitro* and in long-term mouse reconstitution assays[18]. Similarly, overexpression of activated Notch1 in recombination activating gene 1–deficient HSCs results in increased generation of HSCs *in vitro* and *in vivo*[19]. Finally, human bone marrow cell populations enriched for HSCs show increased engraftment *in vivo* when cultured in the presence of the Notch1 ligand Jagged 1 (ref. 20). Those findings indicate that Notch signaling can promote self-renewal of HSCs and progenitors.

Evidence also suggests that Wnt signaling accomplishes an important regulatory function in hematopoietic progenitors and stem cells during fetal and adult development[11]. Wnt proteins constitute a large family of secreted signaling molecules that are expressed in diverse tissues and influence multiple processes in vertebrate and invertebrate development[21]. In addition to the importance of the Wnt pathway in normal development, dysregulation of the Wnt pathway can have potent oncogenic effects in tissues such as colon, breast, prostate and skin[11,22–24]. Wnt proteins act by binding to the Frizzled family of seven-pass transmembrane proteins[25] as well as to proteins of the low-density lipoprotein receptor–related protein family, LRP5 and LRP6 (refs. 26–28). In the absence of a Wnt signal, β-catenin is associated with a large multiprotein complex (the 'destruction complex'), which includes the scaffold protein Axin and the serine-threonine kinase glycogen synthase kinase 3β (GSK3β). In this complex, β-catenin is phosphorylated and is thereby targeted for ubiquitination and degradation[21]. Axin is a key negative regulator of the pathway as it facilitates GSK3β-mediated phosphorylation and degradation of β-catenin. Binding of Wnt proteins to their receptors inhibits phosphorylation of β-catenin by GSK3β, resulting

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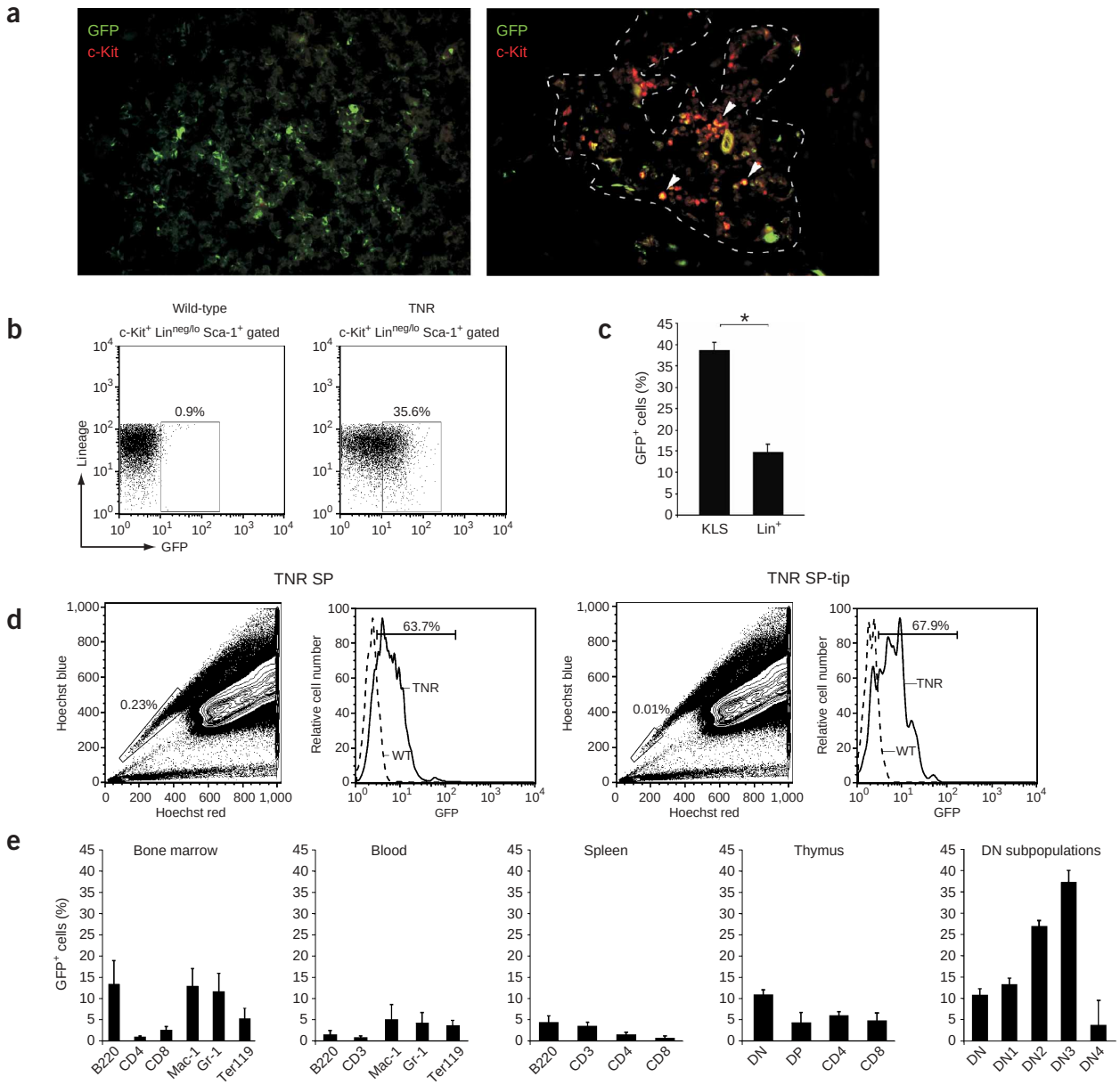

Figure 1 Notch signaling is highly active in HSCs and reduced in differentiated cells. (**a**) Bone sections from TNR mice were stained with antibodies to c-Kit and GFP. Localization of c-Kit$^+$ cells (red) undergoing active Notch signaling (GFP$^+$; green) in the bone marrow cavity (left) and the trabecular bone region (right). Arrowheads point to GFP$^+$c-Kit$^+$ cells. Dashed line indicates the bone marrow–bone boundary. (**b**) HSC marker and GFP expression in bone marrow cells from wild-type or TNR mice. Numbers indicate percentage of cells in outlined areas. Data are from one representative wild-type mouse (left) and TNR mouse (right) ($n > 20$ for each). (**c**) Average frequency of GFP$^+$ cells in KLS ($n = 33$) versus lineage-committed ($n = 16$) populations from TNR mice. The GFP background of wild-type cells was subtracted for each experiment before averaging. Data represent the average $\pm$ s.e.m. *, $P < 0.0001$. (**d**) Bone marrow cells of TNR and wild-type mice, loaded with Hoechst 33342. Left, side population (SP) region; right, side population cells with the highest dye efflux (SP-tip). Dotted lines, wild-type GFP expression; solid lines, TNR GFP expression. Percentages in dot plots indicate percent cells in outlined areas; numbers above bracketed lines indicate percentage of GFP$^+$ cells. Results are representative of two independent experiments. (**e**) Cells from bone marrow, blood, spleen and thymus of TNR mice analyzed for GFP and lineage-specific markers. Data represent the average $\pm$ s.e.m. percentage of GFP$^+$ cells ($n = 5$ for bone marrow and blood, $n = 4$ for spleen and thymus, and $n = 3$ for double-negative (DN) subpopulations). Background GFP expression was subtracted for each value.

in stabilization and accumulation of β-catenin in the cytosol[29]. β-catenin then translocates to the nucleus, where it binds to members of the LEF-TCF family of transcription factors. LEF-TCF proteins are normally associated with the transcriptional repressor Groucho[30,31]. Binding of β-catenin relieves this repression and allows LEF-TCF factors to induce expression of the appropriate target genes[32].

Exposure of mouse and human hematopoietic progenitors to conditioned media containing Wnt proteins results in an increase in immature colony formation *in vitro*[33,34]. In addition, purified Wnt proteins and viruses with genes encoding activated β-catenin enhance self-renewal of murine HSCs *in vitro* and HSC reconstitution *in vivo*[35,36]. The Wnt pathway is also required for HSC maintenance,

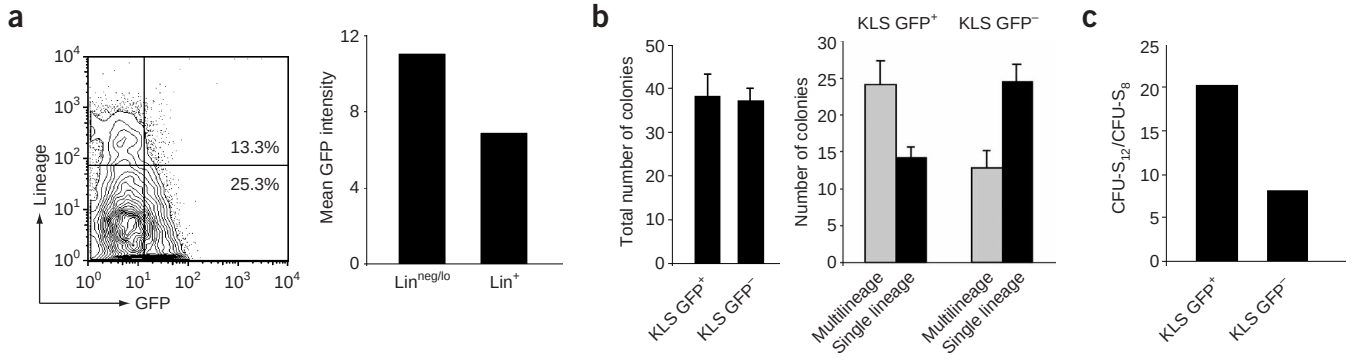

Figure 2 Notch signaling is downregulated during differentiation and defines a more primitive subset within the HSC population. (**a**) Left, expression of lineage markers and GFP of KLS GFP$^+$ cells cultured in the presence of SLF and IL-3 for 3 d. Percentages indicate the frequency of GFP$^+$ cells for Lin$^{neg/lo}$ and Lin$^+$ populations. Right, mean GFP fluorescence intensity of GFP$^+$Lin$^{neg/lo}$ or GFP$^+$Lin$^+$ populations. Results are representative of three experiments. (**b**) KLS GFP$^+$ and KLS GFP$^-$ cells were sorted at a density of one cell per well into 96-well plates and were cultured in methylcellulose for analysis of *in vitro* colony-forming ability. Colonies were assigned scores for the total number of colonies (left) and the presence of single or multiple lineages (right). Data represent average ± s.e.m. of eight independent experiments. (**c**) 'CFU spleen' capacity of KLS GFP$^+$ and KLS GFP$^-$ cells assessed as a ratio of day-12 colonies to day-8 colonies (CFU-S_{12}/CFU-S_8) after transfer into lethally irradiated syngeneic recipients. Data are representative of five independent experiments using 5–20 recipients per condition.

as expression of the Wnt inhibitor Axin leads to inhibition of HSC proliferation and viability *in vitro* and reduced reconstitution *in vivo*[35,36]. The effects of Wnt signaling on HSCs in mice have been recapitulated in a nonobese diabetic–severe combined immunodeficient xenotransplant model, in which delivery of Wnt5A conditioned medium *in vivo* results in increased reconstitution by human HSCs[37].

These studies suggest that the Notch and Wnt pathways are important regulators of HSC function. But whether these signals influence distinct elements of self-renewal (such as proliferation or inhibition of differentiation) and how these signals are integrated with one another remains less clear. Here we examine which aspects of self-renewal are controlled by Notch signaling and the relative functions of Notch and Wnt signaling in maintaining the stem cell state.

RESULTS

Notch signaling in HSCs

To determine whether Notch signaling is active in HSCs *in vivo*, we used a transgenic Notch reporter (TNR) mouse that has a transgene composed of a CBF-1 response element with four CBF-1-binding sites and a minimal SV40 promoter followed by an enhanced green fluorescent protein (GFP) sequence (K.Y. and N.G., data not shown, and **Supplementary Fig. 1** online). HSC-containing populations are preferentially localized to the trabecular bone regions referred to as the 'HSC niche'[38,39]. To determine whether Notch signaling occurs in the hematopoietic niche, we analyzed bone sections for expression of HSC markers and Notch reporter activity. Consistent with published studies[38], we found that trabecular bone was highly enriched in c-Kit$^+$ cells (**Fig. 1a**, right, and **Supplementary Fig. 2** online) in contrast to the bone marrow cavity (**Fig. 1a**, left). A substantial fraction of the c-Kit$^+$ cells were also responding to Notch signals (**Fig. 1a**, right). Whereas some c-Kit$^+$GFP$^+$ cells lay along the periphery of the bone (marked with dashed gray line), others were also present adjacent to large cells in the interior regions of the marrow. The data provide a unique *in vivo* analysis of active signaling in HSCs and progenitor cells in their native microenvironment.

Expression of c-Kit marks a population that includes both hematopoietic stem and progenitor cells. To test whether Notch signaling was active in a fraction more highly enriched for HSCs, we examined reporter activity specifically in the fraction within the c-Kit$^+$ population positive for expression of the surface marker Sca-1 and with negative to low expression of lineage markers (Lin$^{neg/lo}$; called KLS cells here). We found that 38% of KLS cells were GFP$^+$ (**Fig. 1b,c**). In contrast, only 14% of all lineage-committed cells (that is cells that expressed markers of committed lineages such as myeloid, erythroid, B and T cells) were positive for Notch reporter activity (**Fig. 1c**). Additionally, 36% of the Lin^{lo} fraction of bone marrow cells (which contains progenitor cells) was GFP$^+$, and 16–34% of common lymphoid progenitors and common myeloid progenitors were GFP$^+$ (data not shown). We were unable to use Thy-1.1 as a marker to identify c-Kit$^+$Thy-1.1$^+$Lin$^{neg/lo}$Sca-1$^+$ cells (KTLS cells) from TNR mice, as they do not express Thy-1.1. Thus, we isolated stem cell–enriched populations based on the dye efflux properties of HSCs. We analyzed Notch reporter activity in the bone marrow 'side population–tip' cells, which are highly 'enriched' for long-term repopulating activity[40]. On average, 59% of the total side population and 68% of side population–tip cells were positive for Notch reporter activity (**Fig. 1d**).

Although Notch signaling was highly active in HSCs, it was substantially less active in fully differentiated cells of the peripheral lymphoid organs (blood and spleen; **Fig. 1e** and **Supplementary Fig. 3** online). These data suggest that most mature hematopoietic cells do not actively use CBF-1-mediated Notch signaling. In addition, we analyzed reporter activity in the thymus, as involvement of this pathway has been established in thymic development and some Notch reporter activity would be expected. We found reporter activity in CD4$^-$CD8$^-$ double-negative cells, which comprise the most immature thymocytes (**Fig. 1e**), and at a lower frequency in CD4$^+$CD8$^+$ double-positive cells and CD4$^+$ or CD8$^+$ single-positive cells (**Fig. 1e**). Subfractionation of the double-negative compartment showed that the Notch reporter was more highly active in the DN1–DN3 populations and activity was reduced substantially in the DN4 population (**Fig. 1e** and **Supplementary Fig. 4** online).

Notch signaling and HSC function

Our observations in the bone marrow and peripheral blood suggested that Notch reporter activity is downregulated as HSCs differentiate. To test this idea directly, we isolated KLS GFP$^+$ cells and forced them to

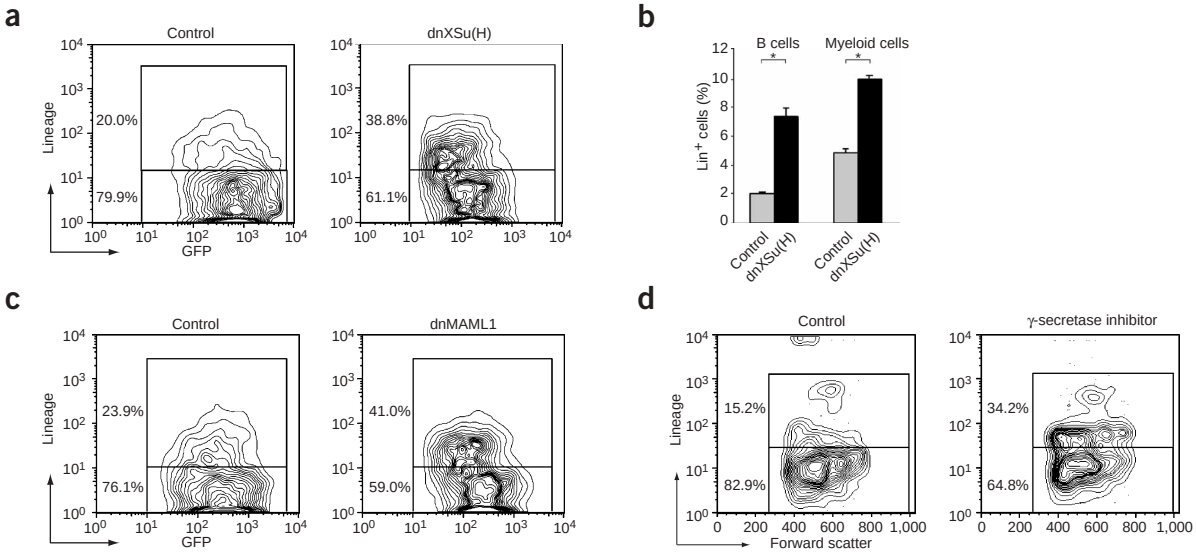

Figure 3 Inhibition of Notch signaling in HSCs leads to accelerated differentiation. Freshly purified wild-type KTLS cells were infected with vector control retroviruses (Control) or with retroviruses producing dnXSu(H) (**a**,**b**) or dominant negative MAML1 (dnMAML; **c**). After 48 h of infection, cells were analyzed by flow cytometry for expression of GFP and lineage markers. Percentages indicate the frequency of GFP$^+$Lin$^+$ (top box) and GFP$^+$Lin$^-$ (bottom box) cells. Results are representative of three to ten independent experiments. (**b**) Expression of the lineage markers B220, Mac-1 and Gr-1 on dnXSu(H)- or vector control–infected cells. Data represent average ± s.e.m. ($n = 3$). *, $P < 0.02$. (**d**) Purified wild-type KTLS cells were cultured in the presence of 5.0 μM γ-secretase inhibitor II or vehicle control along with SLF (10 ng/ml). After 72 h in culture, cells were analyzed for lineage marker expression. Percentages indicate the frequency of Lin$^+$ (top box) and Lin$^-$ (bottom box) cells. Results are representative of three independent experiments.

differentiate *in vitro* in response to interleukin 3 (IL-3) and Steel factor (SLF). After 3 d of treatment, KLS GFP$^+$ cells differentiated to Lin$^+$ cells (**Fig. 2a**, left), which mainly included cells of the myeloid and B lineages (**Supplementary Fig. 5** online). The frequency of GFP$^+$ cells was lower in the Lin$^+$ fraction than in the Lin$^{neg/lo}$ fraction (13% versus 25%; **Fig. 2a**, left). Moreover, GFP fluorescence intensity was lower in the Lin$^+$ fraction (**Fig. 2a**, right), suggesting not only that fewer differentiated cells were signaling to Notch but also that the level of signaling in each cell was reduced. Consistent with this observation, we found that HSCs also downregulated *Notch1*, *Maml1* and the Notch pathway element *Dtx3* after 24 h of treatment with IL-3 and SLF (data not shown).

As only a subset of KLS cells express the Notch reporter, we tested whether Notch signaling functionally divides the KLS population and 'marks' a more undifferentiated state in this population. We first compared the colony-forming ability of KLS GFP$^+$ and KLS GFP$^-$ cells when cultured in methylcellulose. The generation of single-lineage colonies is indicative of restriction of parent cells to one lineage; in contrast, the presence of multilineage colonies is indicative of more-immature parent cells with the capacity to differentiate into multiple lineages. Although KLS GFP$^-$ and KLS GFP$^+$ cells generated the same number of colonies (**Fig. 2b**, left), populations derived from KLS GFP$^+$ cells gave rise to fewer single-lineage colonies and a greater frequency of multilineage colonies (**Fig. 2b**, right). These data suggest that a higher fraction of multipotential cells reside in the KLS fraction that responds to Notch signals. We also tested our hypothesis *in vivo* using the colony-forming unit (CFU) spleen assay. We monitored lethally irradiated mice, transplanted with KLS GFP$^+$ and KLS GFP$^-$ cells, for the formation of colonies in the spleen. CFU spleen colonies that form after 8 days are derived mainly from more-differentiated precursor cells, whereas day-12 CFU spleen colonies are generated by more-primitive populations. Compared with KLS GFP$^-$ cells, KLS GFP$^+$ cells had a higher ratio of day-12 to day-8 colonies in the CFU spleen assay (**Fig. 2c**), indicating that the Notch signaling fraction of KLS cells contains more primitive cells. These data also show that the signaling status of highly primitive cells may differentially mark their functional state.

Notch signaling and HSC differentiation

That Notch signaling marked the most primitive, multilineage cells suggested that the pathway may be important for maintenance of the undifferentiated state. If this hypothesis were correct, we reasoned that inhibition of the Notch pathway should lead to accelerated differentiation. We thus inhibited Notch signaling in HSCs by engineering retroviruses that express a dominant negative mutant form of xenopus suppressor of hairless (dnXSu(H); the xenopus homolog of CBF-1)[41]. This mutant protein has been shown to be a 'pan' (CBF-1-dependent) Notch signaling inhibitor in both mammalian and nonmammalian cells[42]. Additionally, given the early embryonic death (by day 10.5) of CBF-1-deficient mice[43], dnXSu(H) is an effective tool for abrogating Notch signaling. We confirmed expression of dnXSu(H) in HSCs by RT-PCR analysis after infection and found it decreased expression of the Notch target gene *Hes1* (hairy and enhancer of split 1; **Supplementary Fig. 6** online). HSCs in which CBF-1-dependent Notch signaling was inhibited showed accelerated differentiation compared with that of control cells (39% versus 20% Lin$^+$; **Fig. 3a** and **Supplementary Fig. 7** online). The cells generated *in vitro* after inhibition of Notch signaling were predominantly of the myeloid lineages, although we also noted some increase in B220$^+$ cells (**Fig. 3b**). To determine whether other commonly used inhibitors of Notch signaling recapitulate the effects of dnXSu(H), we inhibited Notch signaling with a dominant negative form of MAML1 (dn(MAML1)), which has been shown to inhibit Notch signaling in several contexts[17]. After retroviral transduction, expression of dn(MAML1) in HSCs led to enhanced differentiation of HSCs (41% versus 24% Lin$^+$; **Fig. 3c**). In addition to using dnXSu(H)

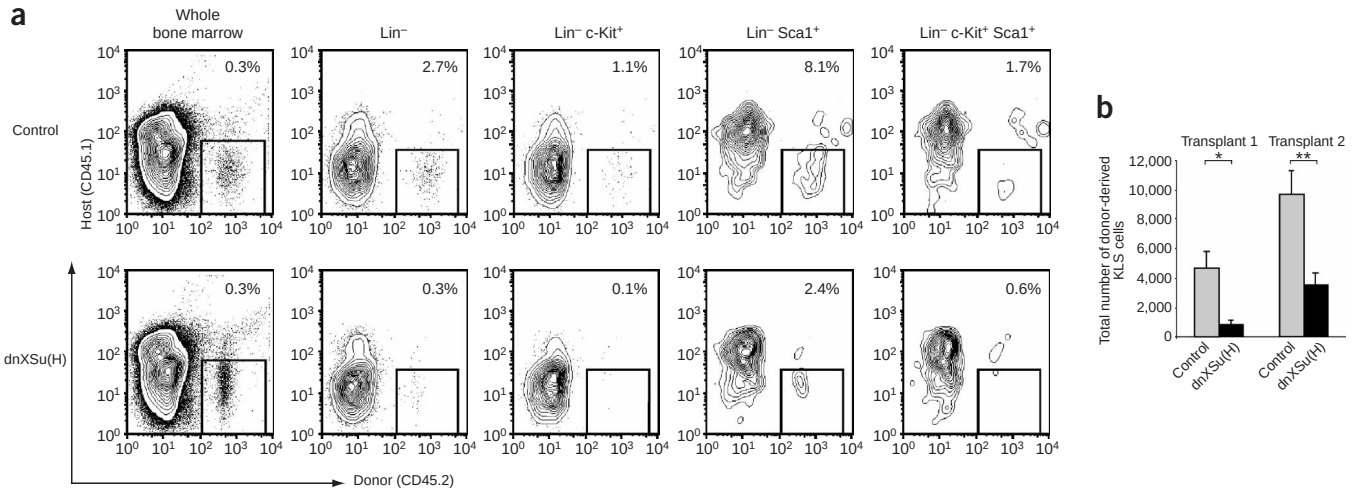

Figure 4 Inhibition of Notch signaling *in vivo* leads to depletion of the HSC population after long-term reconstitution. (**a**) Reconstitution of mice transplanted with control- or dnXSu(H)-infected HSCs (representative data). Percentages indicate the frequency of donor-derived cells (CD45.2$^+$) for each of the outlined populations. (**b**) Average donor-derived KLS cells in mice transplanted with dnXSu(H)-infected cells or control transplants. *, $P = 0.01$; **, $P = 0.05$. Data represent the absolute number of CD45.2$^+$ KLS cells $\pm$ s.e.m. in both transplant experiments; values were calculated by multiplying the observed KLS frequency by the estimated total number of bone marrow cells in host mice. Transplant 1 and 2 refer the first and second experiments, respectively, as described in Methods.

and dn(MAML1), we treated KTLS cells with γ-secretase inhibitor II. This inhibitor blocks Notch signaling by preventing cleavage of the intracellular fragment of Notch from the full Notch receptor, a crucial step in the Notch signaling cascade[10,39,44]. A higher fraction of HSCs incubated with γ-secretase inhibitor expressed lineage markers than did cells treated with vehicle control (34% versus 15% Lin$^+$; **Fig. 3d**). The similar effects of γ-secretase inhibitor II, dnXSu(H) and dn(MAML1) suggest that the accelerated differentiation observed was due to inhibition of Notch signaling and that it was unlikely to be due to nonspecific effects of any one inhibitor.

To determine whether an intact Notch signal is required to maintain the undifferentiated state of HSCs *in vivo*, we tested the ability of Notch-inhibited HSCs to reconstitute the hematopoietic system. For this, we infected wild-type CD45.2$^+$ KTLS cells with vector control or dnXSu(H) retroviruses and transplanted the cells into lethally irradiated CD45.1$^+$ recipient mice. Then we determined the frequency of donor-derived stem and progenitor cells in the bone marrow after long-term reconstitution (**Fig. 4**). We found a 65–80% reduction in donor-derived (CD45.2$^+$) KSLin$^-$ cells (true lineage-negative cells) in mice transplanted with dnXSu(H)-transduced cells compared with that of mice receiving vector control–transduced cells (**Fig. 4b**, **Table 1**, and **Supplementary Fig. 8** online). Additionally, at earlier time points, the peripheral reconstitution in mice transplanted with dnXSu(H)-infected cells was found to be higher (**Supplementary Fig. 8** online). These data collectively suggest that inhibition of Notch signaling leads to a higher rate of differentiation and thus inhibits HSC maintenance in context of the native bone marrow microenvironment.

Notch and Wnt signaling in HSCs

The data presented above suggest that Notch signaling is required for the prevention of lineage commitment and differentiation. As multiple signals are likely to be received by HSCs in context of the bone marrow niche, we were interested in determining whether Notch signaling has a dominant function relative to that of other signals in the maintenance of the undifferentiated state. We tested this specifically in context of Wnt signaling, which acts in synergy with SLF to promote HSC proliferation without substantial differentiation[35]. First, we determined whether both Wnt and Notch signal in the same cells within the native microenvironment. Thus, we crossed TNR mice with Wnt reporter mice (Tcf optimal promoter β-galactosidase (TOPGAL) mice)[45].

Table 1 Reconstitution efficiency of dnXSu(H)- or control-transduced HSCs

			Chimerism (%)	
Transplantation	Cell type	Frequency of reconstitution (%)	Average	Range
Control	Whole bone marrow	100 (4/4)	0.3	0.3–0.3
dnXSu(H)	Whole bone marrow	100 (5/5)	0.3	0.2–0.7
Control	Lin$^-$	100 (4/4)	1.6	0.1–0.3
dnXSu(H)	Lin$^-$	100 (5/5)	0.2	0.7–2.7
Control	Lin$^-$c-Kit$^+$	100 (4/4)	0.8	0.3–1.1
dnXSu(H)	Lin$^-$c-Kit$^+$	100 (5/5)	0.1	0.1–0.1
Control	Lin$^-$Sca-1$^+$	100 (4/4)	5.6	3.2–8.0
dnXSu(H)	Lin$^-$Sca-1$^+$	100 (5/5)	1.0	0.4–2.4
Control	Lin$^-$c-Kit$^+$Sca-1$^+$	100 (4/4)	1.0	0.6–1.7
dnXSu(H)	Lin$^-$c-Kit$^+$Sca-1$^+$	60 (3/5)	0.2	0.0–0.6

Chimerism of each recipient mouse as a percentage of the total population for each category (**Fig. 4c**). Results are from transplant experiment 1 (**Fig. 4c**). Ratios in parentheses indicate the number of transplanted mice used to derive chimerism data.

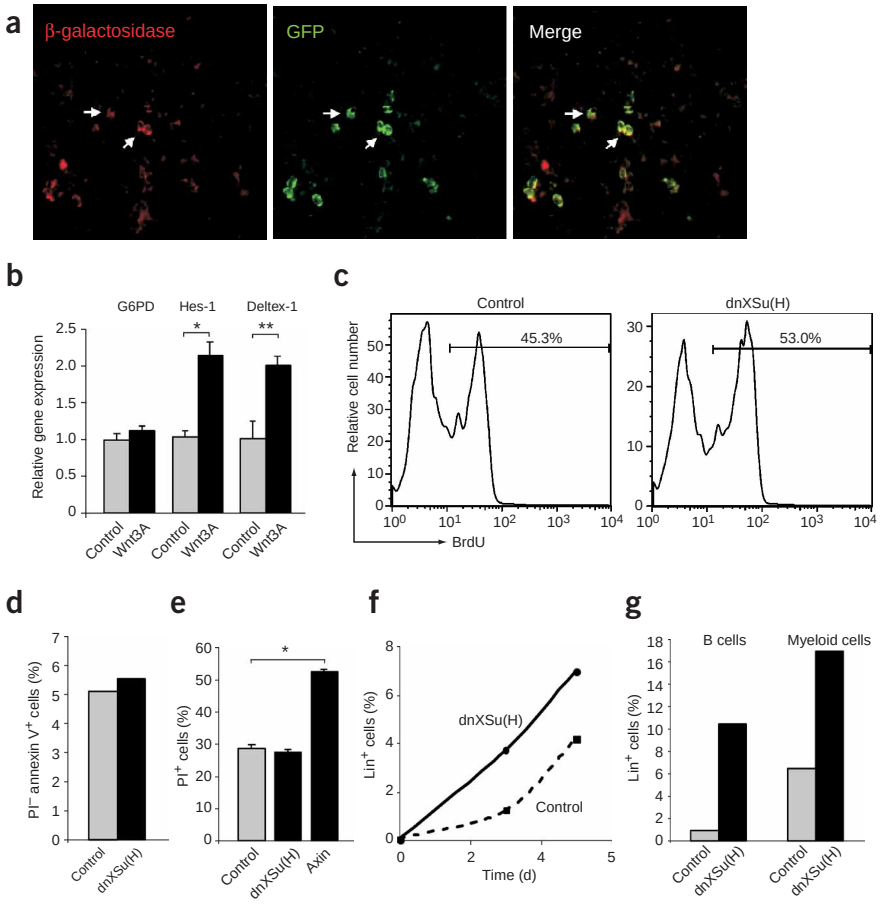

Figure 5 Inhibition of Notch signaling disrupts ability of Wnt signaling to maintain HSCs in an undifferentiated state. (**a**) Bone sections from TOPGAL-TNR double-transgenic mice stained with antibodies to β-galactosidase (red) and GFP (green). In double-positive cells (white arrows), Wnt and Notch pathways are simultaneously activated. (**b**) Wild-type KLS cells were stimulated for 12 h with either soluble Wnt3A or vehicle control in the absence of any other growth factors, and *G6pdx* (G6PD), *Hes1* (Hes-1) and *Dtx1* (Deltex-1) expression was analyzed by real-time PCR. Data represent relative gene expression ± s.e.m. averaged from three to four independent experiments. *, $P = 0.0002$; **, $P = 0.02$. (**c**,**d**) KTLS cells transduced with dnXSu(H) or control retrovirus were re-sorted after 48 h based on expression of GFP (GFP$^+$) and absence of lineage markers (Lin$^-$) and were cultured for 3 d in the presence of SLF and Wnt3A. They were then analyzed by flow cytometry for (**c**) 5-bromo-2′-deoxyuridine (BrdU) incorporation and (**d**) propidium iodide (PI) staining and Annexin V binding. Results are representative of three independent experiments. (**e**) c-Kit$^+$Lin$^{neg/lo}$ (KL) cells transduced with control, dnXSu(H) or Axin retrovirus were analyzed for propidium iodide incorporation after 48 h of infection ($n = 3$). The percentage of propidium iodide–positive cells ± s.e.m. was significantly higher in Axin-infected cells than in control cells (*, $P = 0.005$). (**f**,**g**) KTLS cells were transduced, re-sorted and cultured as described for **c**,**d**. The extent of differentiation was measured by (**f**) lineage marker expression over 5 d and (**g**) expression of B lineage (B220) and myeloid lineage (Mac-1 and Gr-1) markers after 3 d of culture.

TOPGAL transgenic mice, which have a minimal LEF-TCF promoter linked to the β-galactosidase gene, express β-galactosidase in response to Wnt signals. The trabecular bone area of the TOPGAL-TNR double-transgenic mice had a high frequency of cells that were double-positive for both β-galactosidase and GFP expression (85% of TOPGAL$^+$ cells and 65% of TNR$^+$ cells were double positive; **Fig. 5a**). This result indicated that both Wnt and Notch signals were active in the majority of cells in the HSC niche and that a large fraction of cells used both pathways simultaneously. In addition, stimulation of HSCs with Wnt3A upregulated expression of *Hes1* and *Dtx1*, downstream target genes of Notch signaling in HSCs (**Fig. 5b**), as well as Notch reporter activity in HSCs from TNR mice (data not shown). In comparison, of six Notch target genes tested, overexpression of the intracellular fragment of Notch itself upregulated only three target genes in HSCs: *Hes1*, *Dtx1* and *Hey1* (data not shown). These data suggest that Wnt contributes to the differential expression of known Notch targets in HSCs.

To test whether inhibition of Notch signaling affected the response of HSCs to an exogenous Wnt signal, we treated vector- or dnXSu(H)-transduced cells with purified Wnt3A and SLF[35]. In response to Wnt3A and SLF, HSCs expressing ectopic dnXSu(H) entered the cell cycle at a rate similar to that of vector control–infected cells (**Fig. 5c**). Additionally, blocking Notch signaling did not alter cell viability (**Fig. 5d**). This contrasts with the substantial reduction in cell viability noted when the Wnt pathway was blocked with Axin (**Fig. 5e**). However, when we monitored differentiation of these HSCs, we found that dnXSu(H) expression led to accelerated differentiation even in the presence of exogenous Wnt signals (**Fig. 5f**). Furthermore, as determined by expression of the lineage-specific markers Mac-1, Gr-1 and B220, differentiating HSCs committed preferentially to myeloid and B cell lineages (**Fig. 5g**). These data demonstrate that Wnt cannot maintain HSCs in an undifferentiated state when Notch signaling is inhibited. Thus, relative to soluble Wnt protein, Notch is a dominant signal required for inhibiting differentiation.

DISCUSSION

Here we have demonstrated that Notch signaling is used by HSCs in their native microenvironment and is downregulated as HSCs differentiate. Furthermore, inhibition of Notch signaling leads to accelerated differentiation of HSCs *in vitro* and depletion of HSCs *in vivo*. Finally, in the presence of Wnt3A and SLF, inhibition of Notch signaling diminishes the capacity of HSCs to maintain an undifferentiated state but allows normal proliferation and survival. These results suggest that Notch signaling is critical for the maintenance of an undifferentiated state by HSCs and may act as a 'gatekeeper' between self-renewal and commitment. These data also suggest that Notch signaling must be intact for Wnt proteins to enhance HSC renewal.

The Notch reporter mouse line TNR provides a new approach for *in vivo* analysis of active Notch signaling in HSCs. Using these mice, we have provided proof that HSCs signal through the Notch pathway *in vivo*. Specifically, c-Kit$^+$ cells actively signal through the Notch pathway in the trabecular bone marrow, a region described as the 'HSC niche'[38,39]. Analysis of freshly isolated cells from TNR mice

showed that active Notch signaling was predominant in populations most highly enriched for HSCs (that is, KLS and side population–tip populations). Several Notch receptors (Notch1–Notch4) and ligands (Delta-like 1, Delta-like 4 and Jagged 1) are expressed on both hematopoietic progenitors and stromal cells known to support hematopoiesis[20,39,46–48], indicating that Notch signaling can be triggered in HSCs either by neighboring hematopoietic cells or by cells of the microenvironment. Notch signaling seems to be characteristic of an immature state, as reporter activity was downregulated as HSCs differentiated *in vitro* and only a very small fraction of mature, lineage-committed cells showed reporter activity *in vivo*. TNR mice also allowed us to show that within the KLS population, the Notch-signaling cells represent a functionally distinct and more primitive subfraction. Finally, our loss-of-function studies indicated that disruption of Notch signaling leads to enhanced differentiation of HSCs *in vitro* and their depletion *in vivo*.

These data support a model in which HSC fate is critically dependent on Notch signaling. HSCs using the Notch pathway maintain an immature phenotype and the capacity for self-renewing divisions. When HSCs no longer receive Notch signals, their fate is altered: they become sensitive to commitment cues, lose their capacity for self-renewal and can adopt more differentiated fates. Inhibition of Notch signaling in HSCs may occur as a consequence of cells moving away from a niche containing Notch ligands, or it may result from intrinsic alterations in the HSCs themselves that reduce responsiveness to Notch signals. The regulation of Notch signaling in HSCs will be an avenue for future study.

Our observation that Notch signaling is active in HSCs and is important during the earliest stages of hematopoietic development is consistent with the results of other studies. For example, gain-of-function studies have shown that overexpression of the intracellular domain of Notch enhances HSC self-renewal[18,19]. Conversely, loss of Notch signaling impairs HSC development and function both *in vitro* and *in vivo*: γ-secretase inhibitor II reduces HSC frequency in the context of stromal cells[39] and Notch1 deficiency impairs the establishment and maintenance of hematopoietic stem cells[49,50]. Our finding that a critical function of Notch is to retard differentiation provides a mechanistic basis for how modulation of Notch signaling may control HSC numbers in these experiments. It also suggests that the function of Notch in the hematopoietic system is similar to its function in other systems[51,52]. The studies reported above as well as our results contrast with studies using interferon-γ-inducible Notch1-deficient mice, which have shown that inducible loss of Notch fails to influence hematopoiesis[53]. The reasons for the differences between these systems are unclear, but it is possible that interferon-γ-induced deletion sets up a substantially different context for the study of HSC development. Another way to address the importance of Notch signaling in HSCs would be to analyze CBF-1-deficient mice; unfortunately, those mice die by embryonic day 10.5, so analysis of the effect of CBF-1 loss on HSCs has not been possible[43].

Our findings also indicate that the ability of Notch signaling to inhibit differentiation is dominant relative to Wnt and SLF proteins. Thus, whereas the proliferation and survival of HSCs exposed to Wnt and SLF proteins seem unaffected when Notch signaling is impaired, their ability to remain undifferentiated is substantially altered. Our findings do not preclude the possibility that a stronger Wnt signal, such as activated β-catenin, may be able to overcome the consequences of loss of Notch signaling. However, it is likely that soluble Wnt better recapitulates physiological levels of Wnt signaling. The proliferative aspects of Wnt induced self-renewal may be driven by such genes as *Ccnd1*, *Ccnd2* and *Myc*, which have been shown to be expressed in response to Wnt3A in HSCs (A.W.D. and T.R., data not shown) and in other systems[54–56].

One possible interpretation of the finding that Notch signaling is required for the influence of Wnt on HSCs is that Wnt signaling exerts its influence by activating the Notch pathway. Our observation that Wnt3A upregulates Notch target genes is consistent with this possibility. The idea of a hierarchical relationship between Wnt and Notch signaling is also supported by studies in drosophila showing that mutations in *Notch* can produce phenotypes that mimic Wg (wingless) mutant phenotypes[57] and by studies in cell lines showing that Wnt1 can upregulate *Hes1* promoter activity[58]. In both cases, the effects have been suggested to be mediated by GSK3β, which can promote degradation of the intracellular fragment of Notch[58]. Thus, Wnt signaling, by repressing GSK3β, may lead not only to the accumulation of β-catenin and activation of Wnt target genes but also to the accumulation of the intracellular fragment of Notch and activation of Notch targets such as *Hes1*. It is also possible that Wnt and Notch represent parallel pathways in HSCs, with Wnt enhancing proliferation and survival and Notch preventing differentiation. If this is the case, the observed upregulation of *Hes1* and *Dtx1* in HSCs may reflect the possibility that Wnt and SLF selectively promote survival or growth of HSCs that signal in response to Notch.

The Wnt signaling pathway regulates stem cell fate in a variety of organs besides the hematopoietic system, specifically embryonic stem cells[59], neural stem cells[60,61], epidermal stem cells[62–64] and gut epithelial stem cells[65,66]. In most of those tissues, the function of Wnt relative to that of other signals remains unclear. Thus, our results here may also shed light on how Wnt integrates with other signals to influence stem and progenitor cell fate in tissues beyond the hematopoietic system. Finally, as it is becoming clear that signals are shared between stem cell renewal and cancer cell renewal[11,67], understanding how signals are functionally related to control stem cell renewal may also provide insight into how these signals coordinately drive oncogenic renewal.

METHODS

Mice. C57BL/Ka Thy-1.1 (CD45.2), CD1 and TNR mice were used at 6–10 weeks of age. Mice used as transplant recipients (CD45.1) were more than 10 weeks of age. Mice were bred and were provided with acidified water *ad libitum* in the animal care facility at Duke University Medical Center. All live animal experiments were according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Cell isolation and flow cytometry. HSCs were sorted from mouse bone marrow as described[68]. For analysis of lineage-positive cells from bone marrow, spleen and thymus, single-cell suspensions were prepared from the organs. Blood cells were obtained from 0.5 ml of blood collected by cardiac puncture and diluted in 0.5 ml of 10 mM EDTA in PBS. Then, 1 ml of 2% dextran was added to each sample and samples were depleted of red blood cells by sedimentation for 45 min at 37 °C. Red blood cells were lysed with 1× RBC Lysis Buffer (eBioscience) before staining.

HSCs were sorted and re-analyzed based on surface marker expression of c-Kit and Sca-1, low expression of Thy-1.1, and Lin^{lo} to Lin^{-} expression. The combination of the following antibodies defined the lineage markers: 145-2C11 (antibody to CD3ε (anti-CD3ε)), 53-7.3 (anti-CD5), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), RB6-8C5 Ly-6G (anti-Gr-1), M1/70 (anti-CD11b, Mac-1), Ter119 (anti-erythrocyte specific antigen) and 6B2 (anti-B220). Other antibodies used included clones 2B8 (anti-CD117, anti-c-Kit), D7 (anti-Ly-6A/E, anti-Sca-1) and HIS51 (anti-CD90.1, anti-Thy-1.1). All antibodies were purchased from Pharmingen or eBioscience. Analysis and cell sorting were done on a FACSVantage (Becton Dickinson) at the Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource.

Viral production and infection. dnXSu(H) and dominant negative MAML1 were cloned into the MSCV-IRES-GFP retrovirus expression vector.

MSCV-IRES-GFP alone was used as control vector. Virus was produced by triple transfection of 293T cells with mouse stem cell virus constructs along with gag-pol and vesicular stomatitis virus G glycoprotein constructs. Viral supernatant was collected for 2 d and was concentrated 100-fold by ultracentrifugation at 50,000*g*. For viral infection, 25,000–50,000 HSCs were cultured overnight in a 96-well U-bottomed plate in the presence of X-Vivo15, medium (Bio-Whittaker), 50 μM 2-mercaptoethanol, 2% FBS, 30 ng/ml of SLF and 30 ng/ml of Flt3 ligand. After 12–18 h, concentrated retroviral supernatant was added to the cells at a ratio of 1:5. Cells were then incubated at 32 °C for 12 h and were subsequently incubated at 37 °C for 36 h. Infected cells were then sorted based on their GFP expression for *in vitro* and *in vivo* assays. All cytokines were purchased from R&D systems.

Real-time PCR analysis. RNA from Wnt3A-stimulated or retrovirus-infected HSCs was isolated using RNAqueous-Micro (Ambion) and was converted to cDNA using Superscript II (Invitrogen). Concentrations of cDNA were measured with a fluorometer (Turner Designs) using RiboGreen reagent (Molecular Probes). Quantitative real-time PCR was done using an iCycler (BioRad) by mixing equal amounts of cDNAs, iQ SYBR Green Supermix (BioRad) and gene-specific primers (**Supplementary Methods** online).

***In vitro* HSC assays.** For *in vitro* differentiation, 2,000 KLS GFP$^+$ sorted cells were cultured in 96-well U-bottomed plates in medium containing X-Vivo15, 50 μM 2-mercaptoethanol, 10% FBS, 10 ng/ml of SLF and 300 ng/ml of IL-3. After a 3-day culture period, the cells were stained for lineage markers and were analyzed by flow cytometry for lineage and GFP expression.

For assessment of the colony-forming capacity of Notch signaling cells, KLS GFP$^+$ cells and KLS GFP$^-$ cells were sorted at a density of 1 cell per well in 96-well plates and were cultured in complete methylcellulose medium (Methocult GF M3434 from StemCell Technologies). Colonies were assigned scores after 7 d of culture and were identified based on apparent morphological criteria. HSCs that were either retrovirus infected or were treated with γ-secretase inhibitor II (Calbiochem) were cultured in 96-well U-bottomed plates in medium containing X-Vivo15, 50 μM 2-mercaptoethanol, 10% FBS, 10 ng/ml of SLF and Wnt3A (purified as described[36]; estimated working concentration, 100 ng/ml). Proliferation was monitored by culture of HSCs for 48 h, pulsing with 10 μM 5-bromo-2′-deoxyuridine (Sigma) for 12–18 h and staining of cells with anti-5-bromo-2′-deoxyuridine (BD Pharmingen). Cell death was determined by incubation with propidium iodide and Annexin V (BD Pharmingen) after 3 d in culture. The degree of differentiation of cultured cells was monitored at defined intervals by staining of HSCs for expression of lineage cell surface markers. All samples were analyzed by flow cytometry.

***In vivo* analysis of HSC function.** Virus-transduced HSCs (derived from mice expressing CD45.2) were injected retro-orbitally into groups of congenic recipient mice (expressing CD45.1) along with 300,000 bone marrow cells derived from an unirradiated recipient mouse. Two transplant experiments were done. In transplant experiment 1, 1,800 GFP$^+$ transduced cells were transplanted into recipient mice ($n = 4$ for control; $n = 5$ for dnXSu(H)). In transplant experiment 2, 2,000 GFP$^+$Lin$^-$ transduced cells were transplanted into recipient mice ($n = 5$ for control; $n = 6$ for dnXSu(H)). Host mice were lethally irradiated 4–24 h before transplantation with 9.5 Gy, accomplished with two doses of 4.75 Gy each using a 200-kV X-ray machine, and they were maintained on antibiotic-containing water (sulfamethoxazole and trimethoprim) after irradiation. Transplanted mice were killed 16 weeks after reconstitution and bone marrow was analyzed for determination of the frequency of the hematopoietic compartment derived from donor cells. Donor and host cells were distinguished by expression of CD45.1 (A20; eBioscience) and CD45.2 (104; eBioscience). The frequency of control donor–derived hematopoietic progenitor populations was consistent with the published range[69,70].

Immunofluorescence staining of bone sections. Freshly obtained bone specimens from TNR, TOPGAL-TNR and wild-type mice were decalcified, infiltrated with sucrose and embedded in optimum cutting temperature medium. Frozen sections were fixed in acetone and stored at −80 °C until staining. For immunostaining, sections were first blocked with 20% normal goat serum. For TNR bone analysis, samples were then incubated with c-Kit-specific biotinylated antibody (CalTag) and anti-GFP–fluorescein isothiocyanate conjugate (Molecular Probes), followed by incubation with streptavidin–Alexa 350 (Molecular Probes). For TOPGAL-TNR bone analysis, samples were then incubated with anti-GFP–fluorescein isothiocyanate conjugate as well as anti-β-galactosidase, followed by 7-amino-4-methylcoumarin-3-acetic acid secondary antibody. Samples were mounted using fluorescent mounting media and were viewed with appropriate filters.

Statistical analysis. Student's *t*-test was used to determine statistical significance. *P* values less than 0.05 were considered significant.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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