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The E3 ligase Itch is a negative regulator of the homeostasis and function of hematopoietic stem cells

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Although hematopoietic stem cells (HSCs) are the most thoroughly characterized type of adult stem cell, the intricate molecular machinery that regulates their self-renewal properties remains elusive. Here we showed that the E3 ubiquitin ligase Itch negatively regulated the development and functions of HSCs. *Itch*$^{-/-}$ mice had HSCs with enhanced frequency, competence and long-term repopulating activity. Itch-deficient HSCs showed accelerated proliferation rates and sustained progenitor properties, as well as more Notch1 signaling, due to more accumulation of activated Notch1. Knockdown of Notch1 in Itch-mutant HSCs resulted in reversion of the phenotype. Thus, we have identified Itch as a previously unknown negative regulator of HSC homeostasis and function.

Hematopoietic stem cells (HSCs) are a specialized subset of cells that give rise to the entire blood system throughout life[1–3]. Like any other stem cell, HSCs are able to self-renew and to differentiate into various lineages of the hematopoietic system. Self-renewal is a tightly controlled process through which stem cells divide and generate daughter stem cells with properties similar to those of the mother cell[4]. However, under certain conditions, HSCs differentiate into progenitor cells with less self-renewal properties. Since the discovery of stem cells, intense research aimed at understanding the genetic and molecular bases of self-renewal has identified candidates involved in HSC self-renewal. These include cell-intrinsic regulators, such as transcription factors, signal transducers, cell-cycle inhibitors and surface receptors, and cell-extrinsic regulators, such as the bone marrow niche and cytokines[2,4–6].

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AUTHOR CONTRIBUTIONS

C.R. conceived of, designed and did the study, analyzed and interpreted all data, and wrote the manuscript; L.M. provided the *Itch*$^{-/-}$ mice and corrected the manuscript; and R.A.F. provided advice and corrected the manuscript.

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The **Really Interesting New Gene** (RING) finger–type E3 ubiquitin ligase c-Cbl has been reported to have a role in the self-renewal of HSCs[7,8].

Ubiquitination is a post-translational modification of proteins by which polyubiquitin chains are added to lysine residues of target proteins[9,10]. The functional consequences of this modification include the targeting of proteins for proteasomal degradation, as well as other cellular functions, such as protein recycling, endocytic trafficking, DNA repair and transcriptional regulation[11–13]. Ubiquitination occurs through a three-step enzymatic cascade involving ubiquitin-activating (E1) enzymes, ubiquitin conjugating (E2) enzymes and ubiquitin ligase (E3) enzymes[14]. E3 ubiquitin ligases are considered crucial for the process, as they recognize, bind and recruit specific target proteins for ubiquitination. They are broadly classified into two main families based on their domains: the RING-finger domain, which promotes ubiquitination by simultaneously binding to the substrate and an E2 enzyme, and the **Homologous to E6-Associated Protein (E6AP) C-Terminus** (HECT) domain, that participates directly in catalysis by forming an obligate thiolester bond with ubiquitin during the ubiquitination reaction[12]. Because E3 ligases modify substrates at a specific time and place, comprehensive knowledge of their physiological roles is critical for understanding the events associated with post-translational modifications *in vivo*.

Itch is an E3 ubiquitin ligase that belongs to the HECT family[15]. It contains four amino-terminal domains containing two conserved tryptophan residues separated by 20–22 amino acids (the ‘WW’ domain), a C2 domain, and a HECT ligase domain. WW domains mediate protein-protein interactions, whereas the HECT domain recruits ubiquitin-loaded E2 ligases and transfers the ubiquitin to the substrate[10]. Biochemical studies, mainly *in vitro*, have identified more than 20 cellular targets of Itch proteins[15]. Mice deficient in Itch develop a skin-scratching phenotype (the ‘itchy’ phenotype) and severe immune dysregulation, including lymphadenopathy, splenomegaly and inflammation in the lungs and digestive tract[16]. On a C57BL/6 background, Itch-deficient mice die around ~6–8 months of age, most probably because of hypoxia associated with pulmonary chronic interstitial inflammation and alveolar proteinosis.

In correlation with the strong autoimmune disorder associated with Itch deficiency in mice, Itch has been shown to have important roles in T cells, such as controling AKT phosphorylation in thymic T cells[17], ubiquitylating JunB in Th2 cells[18], regulating the expression of Foxp3 in Tregs[19] and restricting the development of pathogenic αβ & γδ T cells[20]. However, the role of Itch in other cell types remains largely unknown. Here we investigate the involvement of Itch in hematopoiesis. We found that Itch negatively regulated the development and function of HSCs. Itch deficiency in HSCs was associated with more Notch1 signaling. Our data suggest that the ubiquitination events mediated by Itch are critical for the development and function of HSCs.

RESULTS

Itch restricts the HSC pool in the bone marrow

In an attempt to identify the importance of ubiquitination events and E3 ligases in HSC development, we studied the expression of three well-characterized E3 ligases, c-Cbl,

Cbl-B and Itch, in the lineage-negative (Lin$^-$: CD11b$^-$Gr-1$^-$220$^-$CD3ε$^-$Ter119$^-$) Sca-1$^+$c-Kit$^+$ (LSK) compartment of the bone marrow of C57BL/6 wild-type mice. Although real-time PCR analysis showed expression of all three candidate E3 ubiquitin ligases, Itch expression was much higher than that of c-Cbl and Cbl-B (Supplementary Fig. 1a). Direct comparison of LSK cells and CD4$^+$ T cells showed that *Itch* mRNA expression was higher in bone marrow LSK cells (Supplementary Fig. 1b). Further real-time PCR analysis of *Itch* mRNA expression in sorted long-term HSCs (LT-HSCs; CD150$^+$CD48$^-$ LSK cells), short-term HSCs (ST-HSCs; CD150$^+$CD48$^+$ LSK cells) and multipotent progenitors (MPPs; CD150$^-$CD48$^+$ LSK cells)[21,22] from the bone marrow of wild-type mice showed that *Itch* mRNA was detectable at all these stages of HSC development. However, the LT-HSCs had much more *Itch* mRNA (Fig. 1a).

To investigate whether Itch deficiency affects the bone marrow HSC compartment, we used previously characterized *Itch*$^{-/-}$mice[16]. Our analysis showed that Itch-deficient mice had a much greater frequency of LSK cells in the bone marrow (Fig. 1b). Further analysis of the LSK compartment indicated *Itch*$^{-/-}$ mice had much greater absolute numbers of CD34$^-$Flt3$^-$LSK, CD34$^+$Flt3$^-$LSK and CD34$^+$Flt3$^+$LSK cells (Fig. 1c), although the frequencies of these subsets were similar to those of wild-type mice (Fig. 1b). We obtained similar results by CD150- and CD48-based immunophenotyping of LT-HSCs, ST-HSCs and MPPs (Fig. 1d). Of note, wild-type and *Itch*$^{-/-}$ mice had a similar absolute number of total bone marrow cells (Supplementary Fig. 1c).

According to a published study, LSK cells can be further subcategorized on the basis of their expression of CD150, CD48, CD34 and Flt3 into the following five subsets: a most primitive HSC subset (CD34$^-$Flt3$^-$CD150$^+$CD48$^-$LSK), and the increasingly differentiated MPP1 subset (CD34$^+$Flt3$^-$CD150$^+$CD48$^-$LSK), MPP2 subset (CD34$^+$Flt3$^-$CD150$^+$CD48$^+$LSK), MPP3 subset (CD34$^+$Flt3$^-$CD150$^-$CD48$^+$LSK) and MPP4 subset (CD34$^+$Flt3$^+$CD150$^-$CD48$^+$LSK)[23]. Using that developmental scheme, we found that none of those subsets was perturbed in the absence of Itch (Fig. 1e); nevertheless, the absolute number of the most primitive HSCs (CD34$^-$Flt3$^-$CD150$^+$CD48$^-$LSK) and their downstream progenitors (MPP1, MPP2, MPP3 and MPP4) was greater in Itch-deficient bone marrow (Fig. 1f). Analysis of cells of the myeloid, erythroid and lymphoid lineages indicated that *Itch*$^{-/-}$ and wild-type mice had similar frequencies of these (Supplementary Fig. 2a–d). Consistent with that, the total number of myeloid- and lymphoid-restricted progenitors was similar in wild-type and *Itch*$^{-/-}$ mice (Supplementary Fig. 2e,f). Together these data suggest that the E3 ligase Itch restricts the size of the HSC pool in the bone marrow.

Augmented repopulation ability of *Itch*$^{-/-}$ HSCs

During development of the mouse embryo, hematopoiesis first originates from the mesodermal precursors and involves various organs, including the intraembryonic aorta-gonad-mesonephros region, the extraembrionic yolk sac, the placenta, the fetal liver and the thymus[24, 25]. Starting from embryonic day 12 (E12) through birth, the most prominent site of hematopoiesis is the fetal liver, where HSCs expand their populations and differentiate into myeloid, erythroid and lymphoid lineages before migrating into the bone marrow[25,26].

To investigate whether the augmented HSC pool is detectable at early stages of development in Itch-deficient mice, we collected fetal livers from embryos (at E15) and neonatal pups (at the second day after birth) and the HSC pool was evaluated. The frequency of HSCs was greater both on E15 (Fig. 2a–c) and at the second day after birth (Supplementary Fig. 3a,b). In line with that observation, *Itch* mRNA was expressed in LSK cells obtained from the fetal liver of wild-type embryos at E15 (Supplementary Fig. 3c).

Next we assessed whether the greater number of HSCs in Itch-deficient bone marrow was a cell-intrinsic phenomenon. We mixed LSK cells either from $Itch^{-/-}$ or wild-type animals (CD45.2) with bone marrow cells depleted of c-Kit$^+$ (to provide efficient short-term hematopoiesis after irradiation) from wild-type (CD45.1$^+$) mice and injected the mixture into lethally irradiated wild-type congenic (CD45.1$^+$) recipients. At 18 weeks after transplantation, the bone marrow of recipients that received $Itch^{-/-}$ LSK cells had more LSK cells (Fig. 2d and Supplementary Fig. 4a). Consistent with the results reported above, transfer of total bone marrow from $Itch^{-/-}$ (CD45.2$^+$) mice into lethally irradiated wild-type congenic recipients resulted in larger LSK pool (Fig. 2d and Supplementary Fig. 4b). Furthermore, we assessed the ability of Itch-deficient HSCs to repopulate the hematopoietic system under competitive settings. We mixed limiting dilutions of LSK cells derived either from $Itch^{-/-}$ or wild-type (CD45.2$^+$) mice with defined ratios of wild-type (CD45.1$^+$) LSK competitor cells and transplanted the mixtures into lethally irradiated wild-type (CD45.1$^+$) recipients as described before[7]. We obtained blood from recipient mice at regular intervals (4, 8, 12 and 24 weeks after transplantation) and calculated the proportion of hematopoiesis derived from donor (CD45.2$^+$) cells and competitor (CD45.1$^+$) cells. Notably, at all time points analyzed, hematopoiesis derived from donor (CD45.2$^+$) cells was greater in the recipients that received $Itch^{-/-}$ LSK cells than in those that received wild-type LSK cells (Fig. 2e). This was most probably due to the greater abundance LSK cells in the bone marrow (Fig. 2f), as the wild-type and $Itch^{-/-}$ groups had a similar abundance of donor-derived total bone marrow cells (Supplementary Fig. 5a). Although $Itch^{-/-}$ and wild-type donor cells had a similar multilineage-reconstitution capacity (analyzed by calculation of the frequency of CD45.2$^+$ lymphoid and myeloid lineage cells in the peripheral blood) at all time points analyzed (Supplementary Fig. 5b–d), the frequency of hematopoietic cells derived from Itch-deficient cells was significantly greater than the frequency of cells derived from wild-type cells (Fig. 2e). Similarly, we did competition experiments in which we lysed red blood cells in total bone marrow from $Itch^{-/-}$ and wild-type mice and mixed the resultant cells with competitor $Itch^{+/+}$ (CD45.1$^+$) cells at various ratios and injected the resultant mixture into lethally irradiated wild-type recipients. We found that hematopoiesis derived from $Itch^{-/-}$donor cells was greater in the peripheral blood of recipients at 4, 8, 12 and 24 weeks after transplantation (Fig. 2g).

We further strengthened our observations about the greater self-renewal properties of $Itch^{-/-}$ HSCs by radioprotection assays[27] in which we injected limiting dilutions of bone marrow cells from wild-type and $Itch^{-/-}$ mice into lethally irradiated wild-type recipients. Itch-deficient bone marrow cells were better in providing radioprotection to recipients, even at very low frequencies (Table 1).

Next we assessed the size of the HSCs pool in *Itch*$^{-/-}$ mice at later stages of life. The enhancement of the HSC pool was even greater at 20 weeks than it was at 4 weeks (Fig. 2h). In radioprotection experiments, bone marrow cells from 20-week-old *Itch*$^{-/-}$ mice were able to 'rescue' lethally irradiated mice at very low frequencies (Table 2). This proved that Itch-deficient HSCs retained their self-renewal properties even at later stages of life.

To investigate the long-term-repopulation abilities of *Itch*$^{-/-}$ HSCs, we did serial transplantation experiments under competitive settings[7]. We sacrificed primary recipients (wild-type mice that either received wild-type or *Itch*$^{-/-}$ BM cells) after 6 months of transplantation and injected their bone marrow cells into lethally irradiated wild-type secondary recipients. At 6 months after transplantation, we obtained blood from secondary recipient mice and analyzed hematopoiesis derived from donor (CD45.2^{+}) cells. Our analysis indicated that hematopoiesis derived from *Itch*$^{-/-}$ donor cells was significantly greater in the secondary recipients than was hematopoiesis derived from wild-type donor cells (Fig. 2i), most probably because of the greater number of LSK cells in the bone marrow (Fig. 2j). Together these data document a cell-intrinsic effect of Itch deficiency and suggest that *Itch*$^{-/-}$ HSCs are more competent than wild-type HSCs and have a greater ability to repopulate the hematopoietic system.

Hyperproliferation of HSCs in the absence of Itch

We next used the thymidine analog BrdU to assess whether the larger HSC pool in the Itch-deficient mice was due to more proliferation. After a single intraperitoneal injection of BrdU, we provided mice with BrdU in the drinking water for 3 d. Analysis of LSK cells on the fourth day showed augmented proliferation of LSK cells in the Itch-deficient mice (Fig. 3a) with more proliferation of LT-HSCs, ST-HSCs and MPPs (Fig. 3a). Next, we directly assessed the cell-cycle status of the LSK compartment under homeostatic conditions. Pyronin Y is a dye that stains total cellular RNA, and the intensity of the staining is directly related to cell-cycle status[28]. Accordingly, quiescent cells are usually negative to low for pyronin Y, while actively proliferating cells are positive for pyronin Y. In line with the BrdU experimental data, pyronin Y staining showed that Itch-deficient HSCs were less quiescent (Fig. 3b). In addition, we stained sorted LT-HSCs from *Itch*$^{-/-}$ and wild-type mice with the cytosolic dye CFSE and cultured the cells *in vitro* in the presence of a cytokine 'cocktail' that promotes HSC proliferation *in vitro* (interleukin 3, stem cell factor, interleukin 6, the hematopoietic growth factor Flt3L, and thrombopoietin). Flow cytometry of hematopoietic stem and progenitor cells (HSPCs) on days 3 and 6 suggested augmented proliferation associated with Itch deficiency (Fig. 3c). Itch-deficient HSPCs showed augmented *ex vivo* population expansion rates in response to cytokines (Fig. 3d). Of note, we observed the greater proliferation of *Itch*$^{-/-}$ HSCs only in the presence of the complete HSC cytokine 'cocktail', as *Itch*$^{-/-}$ HSCs cultured in the presence of cytokines neither survived nor proliferated for 10 d of culture (Supplementary Fig. 6). Together these data suggest that Itch deficiency is associated with augmented proliferation and population expansion of the HSC compartment.

Augmented recovery of the HSC pool in *Itch*$^{-/-}$ mice

To evaluate the *in vivo* consequences of Itch deficiency under physiological stimulation or stress, we analyzed hematopoietic recovery after hemoablation with 5-fluorouracil (5-FU). This reagent kills actively cycling cells and results in ablation of the progenitor pool but spares the quiescent stem cells responsible for long-term repopulation[29]. Analysis of the LSK compartment 2 d after injection of 5-FU showed that *Itch*$^{-/-}$ and wild-type mice had a similar proportion of LSK cells (Fig. 4a and Supplementary Fig. 7), which suggested that actively proliferating (5-FU-sensitive) LSK cells contributed to the larger HSC pool in the bone marrow of *Itch*$^{-/-}$ mice. At 8 days after 5-FU injection, there was augmented recovery of hematopoietic progenitors in the absence of Itch (Fig. 4a and Supplementary Fig. 7). We obtained blood from mice on days 0, 5 and 10 after 5-FU treatment and counted platelets, leukocytes and red blood cells and measured the hematocrit. Although we observed a similar drop in blood counts in both *Itch*$^{-/-}$ and wild-type mice on day 5, we noted augmented recovery of blood counts in Itch-deficient mice on day 10 (Fig. 4b).

To investigate whether the enhanced hematopoietic recovery in *Itch*$^{-/-}$ mice was a cell-intrinsic phenomenon, we transferred wild-type or *Itch*$^{-/-}$ bone marrow cells into lethally irradiated wild-type recipients. At 8 weeks after transplantation, we administered 5-FU intraperitoneally to the recipient mice on a weekly basis and monitored their survival. Recipients of *Itch*$^{-/-}$ bone marrow cells had better survival rates than did mice that received wild-type bone marrow cells (Fig. 4c). These data indicate that Itch-deficient HSCs show enhanced recovery of hematopoiesis under stress conditions.

Sustained progenitor properties of *Itch*$^{-/-}$ HSPCs

To investigate the ability of Itch-deficient HSPCs to maintain progenitor properties under *in vitro* culture conditions, we sorted LT-HSCs and cultured then in the presence of the HSC cytokine ‘cocktail’. We obtained aliquots of cells on days 5, 10, 15 and 20 of culture and analyzed their expression of lineage markers (CD11b, Gr-1, B220, Ter119 and CD3ε). Starting from day 10, Itch-deficient HSPCs showed lower expression of these lineage-associated markers (Fig. 5a). The proportion of cells that maintained the progenitor immunophenotype (LSK) was much higher in *Itch*$^{-/-}$ cell cultures than in wild-type cell cultures (Fig. 5b). To assess whether these cells also maintained the functions of progenitor cells after *in vitro* culture, we sorted LSK cells after 20 d of *in vitro* culture and assessed colony-forming units (CFU). Although the wild-type cells generated modest number of colonies in the semisolid medium, *Itch*$^{-/-}$ cells generated an augmented number of colonies (Fig. 5c). We also assessed the ability of LT-HSCs cultured *in vitro* to repopulate the hematopoietic system. After 20 d of culture, we sorted wild-type or *Itch*$^{-/-}$ cells with an LSK phenotype and injected the cells into sublethally irradiated congenic (CD45.1^{+}) recipient mice. As a control, we injected freshly isolated (naive) wild-type or *Itch*$^{-/-}$ LSKs into sublethally irradiated recipients. At 4 weeks after transplantation, we killed the recipient mice and calculated the frequency of CD45.2^{+} cells in the peripheral blood. Whereas only ~5% cells were CD45.2^{+} in recipients that received wild-type cells, ~36% cells were CD45.2^{+} in recipients that received Itch-deficient LSKs (Fig. 5d). Analysis of the multilineage differentiation of donor cells showed that wild-type cells cultured *in vitro* had an greater myeloid differentiation capacity and a lower lymphoid differentiation

capacity than did naive wild-type LSKs. *Itch*$^{-/-}$ cells cultured *in vitro* showed multilineage-differentiation capacities similar to those of naive Itch-deficient LSKs (Fig. 5e). Overall, these experiments suggest that Itch-deficient HSPCs maintain their progenitor properties even after prolonged *in vitro* culture.

Augmented Notch1 signaling in *Itch*$^{-/-}$ HSPCs

Overexpression of the intracellular domain of the oncogenic transcription factor Notch1 (ICN1) in HSCs results in augmented proliferation, more competence and enhanced *ex vivo* expansion rates without the loss of self-renewal[30,31]. Our analysis here has identified many phenotypic similarities between Itch-deficient HSCs and Notch1-overexpressing HSCs. Moreover, Itch is directly involved in Notch1 ubiquitination[17,32,33]. On the basis of those observations, we hypothesized that Itch deficiency might result in defective ubiquitination of Notch1 and, thus, in augmented Notch1 signaling in HSCs. To test our hypothesis, we did biochemical analysis of Lin$^-$ hematopoietic progenitor cells (HPCs) from wild-type and Itch-deficient mice. First, with a Notch1-specific antibody, we detected more full-length Notch1 and a cleaved form consisting of the transmembrane and intracellular domain in HPCs (Fig. 6a). We also measured Notch1 protein in LT-HSCs sorted from *Itch*$^{-/-}$ and wild-type mice by flow cytometry. Itch-deficient LT-HSCs isolated from the bone marrow (Fig. 6b,c) and the fetal liver (Supplementary Fig. 8) had more Notch1. To further substantiate and independently document the augmented Notch1 signaling in Itch-deficient HSCs, we crossed *Itch*$^{-/-}$ mice with transgenic Notch reporter mice[34]. In these reporter mice, the expression of green fluorescent protein is directly proportional to the activity of Notch signaling *in vivo*[34]. Suggestive of the fact that Notch signaling is augmented in HSCs in the absence of Itch, HSCs from *Itch*$^{-/-}$ transgenic Notch reporter mice had higher expression of green fluorescent protein (Fig. 6d,e). Itch may also be involved in the regulation of various cellular proteins, such as c-Jun, JunB, PLC-γ1, Smad2 and p73 (ref. 15). Because of their critical roles in hematopoiesis and signal transduction, we analyzed the expression of these proteins in HPCs; however, we found similar expression of all in the absence of Itch (Supplementary Fig. 9).

Notch1 signaling is initiated by engagement of the Notch ligand. Ligand binding leads to two successive proteolytic cleavage events that result in the separation of the intracellular domain from the transmembrane domain. The cleaved ICN1 translocates from the cell membrane to the cytoplasm and subsequently to the nucleus, where it interacts with the transcription factor CBF (RBP-j*k*) and activates the expression of target genes such as *Hes1* and *Myc*[35]. Using antibodies specific for cleaved Notch1 (that recognize cleavage between Gly1743 and Val1744 of the intracellular domain), we detected more cleaved Notch1 in *Itch*$^{-/-}$ HPCs (Fig. 6f), which suggesting that more total Notch1 led to more release of ICN1. In addition, both the nuclear and cytoplasmic fractions of Itch-deficient HPCs had more ICN1 (Fig. 6g), which correlated with the augmented *Hes1, Myc* and *Dtx1* mRNA in the LT-HSCs of *Itch*$^{-/-}$ mice detected by real-time PCR (Fig. 6h and Supplementary Fig. 10). Of note, *Itch*$^{-/-}$ and wild-type LT-HSC cells had similar amounts of *Notch1* mRNA (Fig. 6i), which suggested that the greater abundance of Notch1 protein in Itch-deficient cells might have been a consequence of defective degradation.

To evaluate whether Itch interacts with Notch1 in HPCs, we transduced prestimulated HPCs with retrovirus overexpressing ICN1 and coimmunoprecipitated proteins from lysates with antibody to Notch1 (anti-Notch1). Our analysis showed interaction between Notch1 and Itch (Fig. 6j) and that Notch1 protein was ubiquitinated only in the presence of Itch (Fig. 6k). Finally, knockdown of Notch1 by small hairpin RNA in LT-HSCs in Itch-deficient mice resulted in a lower frequency of HSCs (Fig. 7a and Supplementary Fig. 11) and diminished radioprotection function (Table 3), which demonstrated that Notch1 activation was responsible for the phenotype of *Itch*$^{-/-}$ HSCs. Furthermore, knockdown of Notch1 in *Itch*$^{-/-}$ LSK cells led to modest proliferation *in vivo* (Fig. 7b) and *in vitro* (Fig. 7c) and lower expression of *Hes1* mRNA and *Myc* mRNA (Fig. 7d,e). These data suggest that the phenotype of *Itch*$^{-/-}$ HSCs was at least in part due to augmented Notch1 signaling.

DISCUSSION

Here we have shown that Itch, a member of the HECT family of E3 ligases, is a negative regulator of HSC development and function. The self-renewal of stem cells is tightly controlled by various cell-intrinsic and cell-extrinsic factors[1–6,36]. Throughout their lifespan, HSCs respond to signals mediated by a spectrum of cytokines and growth factors that can promote their quiescence, proliferation, migration and differentiation. Under steady-state conditions, HSCs constantly replenish the entire hematopoietic system. During an immune insult, HSCs must proliferate and differentiate into specific cell types that accomplish the required effector functions[37]. Whenever HSCs switch from one physiological state to another, a tight balance between protein synthesis and protein degradation is probably vital for their proper function. Here we have shown that defective cellular pathways that resulted in deficient ubiquitination resulted in altered HSC self-renewal properties, and we have demonstrated an important role for Itch in this process. Other E3 ubiquitin ligases, such as the RING finger ligase c-Cbl, have been shown to control the development and function of HSCs[7,8,38]. Together, these studies highlight the pivotal roles of E3 ubiquitin ligases and the importance of post-translational modifications in the physiology of HSCs and the molecular control of HSC self-renewal.

The hallmark feature of adult stem cells is their relative proliferative quiescence[2]. Published studies suggest that two distinct HSC subsets—dormant HSCs and active HSCs—can exist in the HSC pool under steady-state conditions[23,39]. In the mouse, dormant HSCs are generally quiescent, with a division rate of approximately once every 150 d and are believed to divide only five times during the lifespan of the mouse, whereas active HSCs are thought to be more proliferative and to produce the progenitors and mature cells required for the maintenance of normal hematopoiesis[23,39]. Quiescence is critical for the maintenance, survival and self-renewal of HSCs. Published studies of mice deficient in p21, Gfi1, Pten, FoxO1, 3 and 4, Pbx1, Mi2β, TSC1, PML and Fbw7 have proven that unscheduled HSC proliferation results in loss of self-renewal or stem cell exhaustion[2,6,39]. We found that Itch-deficient mice had a larger HSC pool due to their hyper-proliferative properties but did not show the phenomenon of ‘stem cell exhaustion’. Instead, Itch-deficient HSCs were more competent and faster in repopulating the hematopoietic system under both steady-state and stress conditions, which indicatedthat in certain situations, self-renewal of HSCs is not compromised even after excessive rounds of proliferation. Similar observations have been

obtained with c-Cbl-deficient mice[7] and Egr1-deficient mice[40], in which HSC functions are not compromised despite continuous and accelerated proliferation. Studies of these mice will be very useful in understanding the physiology and molecular mechanisms behind inexhaustible stem cell properties even after excessive rounds of proliferation.

In mammalian hematopoiesis, Notch signaling is essential for definitive hematopoiesis in the developing embryo, as well as for the development of T cells, marginal zone B cells and megakaryocytes[41–43]. Although the role of Notch in the differentiation of multiple hematopoietic lineages has become increasingly clear, its role in the development and maintenance of adult HSCs has remained controversial. The idea of Notch1 as an important modulator of adult HSCs has been provided by several gain-of-function studies through overexpression of the active form of Notch1 (ICN1) or its downstream target Hes1 in HSCs. ICN1 overexpression in HSCs results in *ex vivo* HSC population expansion without compromise of their self-renewal properties[30,43,44]. However, genetic studies of mice that are deficient in Notch1, Jagged1 or Rbp-jκ or express a dominant negative form of the mastermind-like protein DN-MAML, which functions as a total Notch inhibitor, have shown that canonical Notch signaling is dispensable for HSC homeostasis in the bone marrow[42–44]. A possible interpretation of these paradoxical findings might be that although Notch signaling might not be required for the homeostasis of adult HSCs, augmented Notch signaling may result in enhanced HSC self-renewal. In line with that idea, it has been proposed that Notch signaling is maintained at a minimal basal amount in HSCs[42,43]. As exaggerated Notch signaling results in altered self-renewal, pathways that negatively regulate Notch signaling should remain active in HSCs. Itch is known to directly participate in the degradation of Notch1 through ubiquitination[15,17,32,33]. Our analysis has provided direct evidence that the lack of Itch resulted in augmented Notch1 signaling in HSCs, leading to altered self-renewal.

LRF has also been identified as a negative regulator of Notch signaling in early hematopoiesis[45]. LRF-deficient mice have more HSCs, perturbed B cell development and ectopic T cell development in the bone marrow due to inappropriate activation of Notch signaling in the hematopoietic progenitor cells[45]. These observations are in line with the earlier idea that Notch signaling needs to be suppressed during the early stages of B cell development in the bone marrow[46]. We propose that Itch deficiency results in augmented Notch signaling, thus leading to enhanced self-renewal of HSCs. Although the phenotype of LRF-deficient and Itch-deficient HSCs was similar, we did not detect extrathymic T cell development or perturbed B cell development in the bone marrow of young (4-week-old) Itch-deficient mice. A possible explanation for the distinct phenotypes is that LRF deficiency might induce inappropriate Notch expression and activation in cells that usually lack Notch signaling, such as cells of the B lineage[45]. In contrast, Itch deficiency resulted in sustained activation of Notch signaling only in hematopoietic cells that usually express Notch.

Nonetheless, the phenotype of Itch-deficient HSCs should be interpreted with care, as it differs in several ways from the ICN1-overexpression phenotype[47]. Unlike HSCs with retrovirus-mediated, constitutive expression of ICN1, *Itch*$^{-/-}$ HSCs expressed physiological amounts of *Notch1* mRNA and Notch1 protein, and Notch1 activation strictly required

Notch engagement by its ligands expressed on neighboring cells in the niche. Those and other results may explain any differences that can be observed between *Itch*$^{-/-}$ HSCs and ICN1-overexpressing HSCs. Nevertheless, this is the first report to our knowledge describing a phenotype associated with augmented Notch signaling in HSCs under physiological settings.

Several human patients have been identified with mutations in *ITCH* that result in deficiency in Itch expression[48]. These patients have multiple defects, including multisystem autoimmunity, morphological and developmental abnormalities[48]. Although there is no information available yet on the HSC defects in these patients, our study warrants consideration of such a possibility. In essence, our study has identified a previously unknown function for Itch in the restriction of the HSC pool size and emphasizes the importance of post-translational modifications in the maintenance of HSC homeostasis. A better understanding of the roles of Itch in regulation of hematopoiesis might aid the development of new therapies for stem cell–based disorders.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

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

ONLINE METHODS

Mice.

The *Itch*$^{-/-}$ mice have been described[16]. Transgenic Notch reporter mice (Jackson Laboratories) were crossed with *Itch*$^{-/-}$ mice to obtain *Itch*$^{+/-}$ (heterozygous) transgenic Notch reporter pups; those mice were interbred to generate *Itch*$^{-/-}$ and Itch$^{+/+}$ transgenic Notch reporter mice. All mice were kept under specific pathogen–free conditions in the animal care facility at Yale University. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Yale University.

Competitive-repopulation and bone marrow–transplantation studies.

For competitive-repopulation experiments, various numbers of donor (CD45.2^{+}) LSK cells (1×10^3, 2×10^3, 1×10^4, 1.8×10^4 or 2×10^4) were sorted from 8-week-old wild-type and *Itch*$^{-/-}$ mice and mixed with defined numbers of competitor LSK (CD45.1^{+}) cells (2×10^4, 1.9×10^4, 1.8×10^4, 1.7×10^4 and 2×10^3) and the mixtures were transplanted intravenously into lethally irradiated (11 Gy) congenic (CD45.1^{+}) recipients. For serial transplantation, 2×10^6 bone marrow cells from the primary transplants (6 months after transplantation) were injected into lethally irradiated congenic (CD45.1^{+}) recipients. In some experiments, *Itch*$^{+/+}$ and *Itch*$^{-/-}$ bone marrow was depleted of red blood cells and 1×10^6 total cells were transplanted into lethally irradiated (CD45.1^{+}) recipient mice.

For radioprotection assays, red blood cells were lysed in *Itch*$^{+/+}$ and *Itch*$^{-/-}$ donor bone marrow and limiting dilutions of the resulting bone marrow cells (1×10^2, 1×10^3, 5×10^3,

1×10^4, 5×10^4 and 1×10^5) were injected into lethally irradiated wild-type ($CD45.1^+$) recipient mice. All mice survived at least 10 d, which suggested that fatalities were due to hematopoietic failure.

Cell culture.

Purified $CD150^+CD48^-$ LSK cells or LSK cells were cultured *in vitro* in the presence all or some of the following recombinant cytokines: mouse interleukin 3 (10 ng/ml), mouse interleukin 6 (10 ng/ml), mouse stem cell factor (50 ng/ml), mouse thrombopoietin (10 ng/ml) and human Flt3L (50 ng/ml; all from Peprotech). Cells were cultured in Iscove's modified Dulbecco medium supplemented with 10% FCS, 2 mM L-Glutamine, 1% Penicillin-Streptomycin and 1 mM non-essential amino acids.

Cell-proliferation studies.

In vivo proliferation experiments with BrdU and *in vitro* proliferation experiments with CFSE (carboxyfluorescein diacetate succinimidyl ester) were done as described[7].

For assay of colony-forming units, cells cultured *in vitro* were sorted on day 20 on the basis of their LSK immunophenotype and were cultured in semisolid Methocult M3534 medium supplemented with cytokines to promote the proliferation of progenitor cells; colonies were detected on day 12 according to the manufacturer's instructions (StemCell Technologies).

Analysis with 5-FU.

Mice were given a single intraperitoneal dose of 5-FU (150 mg per kg body weight) on day 0. Blood was obtained from mice or mice were killed on days 0, 2, 5 and 10 after injection, and their bone marrow LSK compartments were analyzed by flow cytometry. The Hemavet 950 LV system (DREW scientific) was used for differential blood count analysis.

In some experiments, wild-type $CD45.1^+$ recipients were lethally irradiated and given transplantation of $Itch^{+/+}$ or $Itch^{-/-}$ bone marrow cells. At 8 weeks after transplantation, 5-FU was administered intraperitoneally to recipients (150 mg per kg body weight on a weekly basis) and survival was monitored.

Pyronin and Hoechst staining.

The cell cycle status of the freshly isolated HSCs was determined with Hoechst 33342 (Molecular Probes) and pyronin Y staining (Sigma). Sorted LT-HSCs were resuspended in PBS containing 2% (vol/vol) FCS and 10 μM Hoechst 33342. Cells were then incubated for 30 min at 37 °C and then were washed and resuspended in PBS supplemented with 20 mM HEPES, pH 7.4, glucose (1 mg/ml), 10% (vol/vol) FCS, 10 μM Hoechst 33342 and pyronin Y (1 μg/ml). Cells were incubated for an additional 30 min at 37 °C, then were washed and analyzed by flow cytometry. Pyronin Y fluorescence was detected at 575 nm in the linear range.

Flow cytometry.

Single-cell suspensions were analyzed by flow cytometry with a FACSScan, FACSCalibur or LSR II (BD) and CellQuest software (BD Biosciences), FACSDiva software (BD

Biosciences) or FlowJo software (Tree Star), respectively. Cells were sorted into defined subpopulations with a FACSAria (BD Biosciences). Cells incubated with biotinylated monoclonal antibodies were incubated with fluorochrome-conjugated streptavidin-phycoerythrin, streptavidin–peridinin chlorophyll protein, streptavidin-allophycocyanin and streptavidin-allophycocyanin-indotricarbocyanine (BD Pharmingen). The following monoclonal antibodies were used: CD3e (145-2C11; BD Biosciences), CD-4 (GK1.5; BD Biosciences), CD8 (53-6.7; BD Biosciences), CD11b (M1/70; BD Biosciences), CD11c (HL3; BD Biosciences), CD19 (1D3; BD Biosciences), CD34 (RAM34; BD Biosciences), CD45.1 (A20; BD Biosciences), CD45.2 (104; BD Biosciences), CD48 (HM48-1; BD Biosciences), CD117 (2B8; BD Biosciences), CD150 (TC15-12F12.2; Biolegend), B220 (RA3-6B2; BD Biosciences), Flt3 (A2F10.1; BD Biosciences), Gr-1 (RB6-8C5; BD Biosciences), Sca-1 (D7; BD Biosciences) and TER119 (TER119; BD Biosciences). Cells reacted with biotinylated monoclonal antibodies were incubated with fluorochrome-conjugated streptavidin-PE (#554061; BD Biosciences), streptavidin-PerCP-Cy5.5 (#551419; BD Biosciences), streptavidin-APC (#554067; BD Biosciences) and streptavidin-APC-Cy7 (#554063; BD Biosciences). All fluorescence intensity plots are presented in log scales.

Intracytoplasmic staining.

For detection of Notch1 by flow cytometry, $CD150^{+}CD48^{-}$LSK cells were first fixed and made permeable with a Phosflow kit (BD Pharmingen) and then were stained with phycoerythrin–anti-Notch1 (mN1A; BD Biosciences) according to the manufacturer's instructions (BD Biosciences).

Immunoblot analysis and Immunoprecipitation.

Immunoblot analyses were done as described[7]. Primary antibodies were specific for c-Jun (60A8; Cell signaling), JunB (C37F9; Cell signaling), Fyn (# 4023; Cell signaling), SMAD2 (86F9; Cell signaling), p73 (#4662; Cell signaling), HDAC1 (#2062; Cell signaling), c-Myc (#9402; Cell signaling), ubiquitin (#3933; Cell signaling), Notch1 (full-length + transmembrane domain(C44H11; Cell signaling) and cleaved Notch1 (Val1744; Cell signaling), Hes1 (#Q14469; Millipore) and GAPDH (L18; Santa Cruz Biotechnologies) and actin (I-19; Santa Cruz Biotechnologies). Proteins were immunoprecipitated with antibody to cleaved Notch1(Val1744) with a commercially available kit according to the manufacturer's instructions (Upstate).

RNA extraction and real-time PCR.

Total RNA was isolated with the RNeasy Mini kit ('Qiagen), then cDNA was synthesized with oligo(dT) primer and Expand reverse transcriptase (Roche). PCR was done in duplicate with a 7500 RealTime PCR system and Power SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems).

Retrovirology.

For short hairpin RNA (shRNA) studies, Notch1-specific shRNA was designed through VectorNTI (Invitrogen) software. Two shRNA constructs recognizing different regions of

Notch1 mRNA (shRNA1 and shRNA2) were cloned into the pSicoR retroviral backbone (Addgene). Retroviruses were generated with PhoenixGP cell lines. Confirmation of knockdown of *Notch1* mRNA with shRNA showed efficient downregulation with both shRNA1 and shRNA2; however, the efficiency of knockdown was better (~70%) with shRNA1 and thus only shRNA1 was used for the knockdown experiments. Cells transduced with control shRNA served as controls.

For immunoprecipitation studies, retrovirus overexpressing ICN1 was prepared as described[30].

Ubiquitination studies.

For ubiquitination assays, human embryonic kidney 293T cells were cotransfected with plasmid that expressing c-Itch, Notch1 and ubiquitin, then, 48 h after transfection, proteins were immunoprecipitated with anti-Notch1 (C44H11; Cell signaling) as described above. Precipitates were analyzed by immunoblot, probed with anti-ubiquitin (#3933; Cell signaling).

Statistical analysis.

Statistical significance was assessed with a two-sided Student's *t*-test. *P* values above 0.05 were considered not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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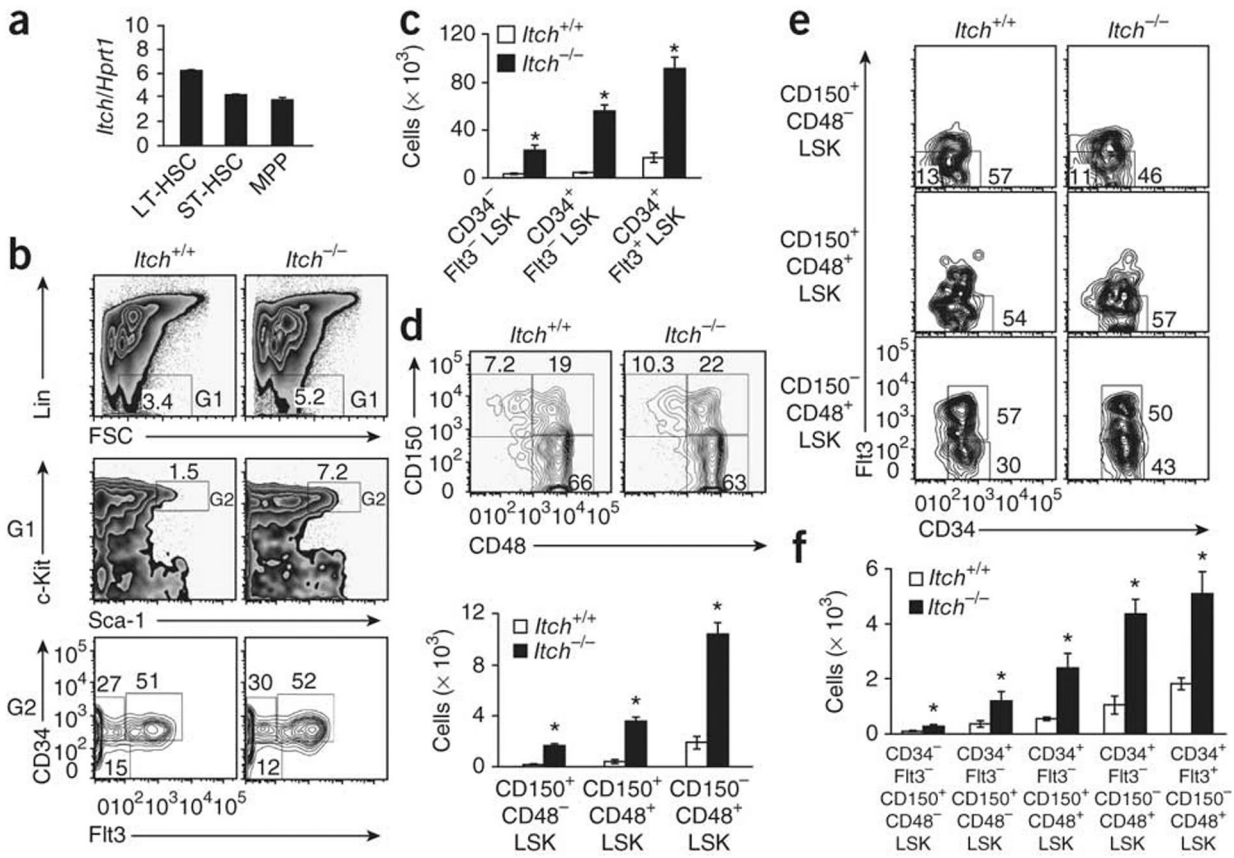

Figure 1.

Itch deficiency results in a greater frequency of HSCs in the bone marrow. (**a**) Real-time PCR analysis of *Itch* mRNA expression in sorted LT-HSCs (LSK $CD150^{+}CD48^{-}$), ST-HSCs (LSK $CD150^{+}CD48^{+}$) and MPPs (LSK $CD150^{-}CD48^{+}$), presented relative to the expression of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase). (**b**) Distribution of Lin^{-} cells (top), LSK cells (middle) and LSK subsets defined on the basis of the expression of CD34 and Flt3 (bottom), among total wild-type and $Itch^{-/-}$ bone marrow cells. Numbers adjacent to outlined areas indicate percent cells in each throughout. (**c**) Absolute number of cells in HSC subsets in 4-week-old wild-type and $Itch^{-/-}$ mice ($n = 5$ per group) based on the gates presented in **b**. (**d**) Distribution (top) and absolute number (bottom) of LSK subsets in 4-week-old wild-type and $Itch^{-/-}$ mice ($n = 5$ per group), assessed on the basis of expression of CD150 and CD48. (**e**) Expression of Flt3 and CD34 on cells of wild-type and $Itch^{-/-}$ bone marrow LSK subsets identified as in **d**. (**f**) Absolute number of cells of LSK subsets from 4-week-old wild-type and $Itch^{-/-}$ mice ($n = 5$ per group), stained as in **e**. $*P < 0.05$ (Student's *t*-test). Data are representative of two independent experiments (**a**; mean and s.e.m. of duplicates) or ten (**b**–**e**) or three (**f**) independent experiments (mean and s.e.m. in **c**,**d**,**f**).

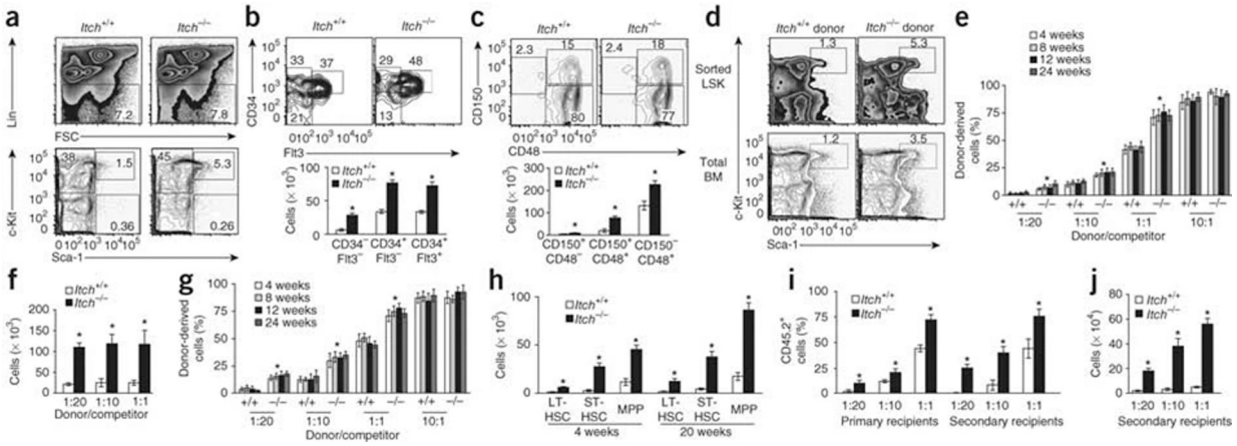

Figure 2.

Cell-intrinsic defects and greater competence of Itch-mutant HSCs. (**a**) Distribution Lin^- cells (top) and LSK cells (bottom) in wild-type and $Itch^{-/-}$ fetal livers ($n = 5$ mice per genotype). FSC, forward scatter. (**b**,**c**) Distribution (top) and absolute number (bottom) of cells of the LSK subsets in wild-type and $Itch^{-/-}$ fetal livers ($n = 5$ mice per genotype), assessed on the basis of the expression of CD34 and Flt3 (**b**) or CD150 and CD48 (**c**). (**d**) Distribution of donor-derived sorted LSK cells (top) or total bone marrow cells (bottom) from wild-type and $Itch^{-/-}$ ($CD45.2^+$) mice in the total bone marrow of wild-type ($CD45.1^+$) congenic recipient mice 18 weeks after transplantation. (**e**,**f**) Hematopoiesis of donor-derived ($CD45.2^+$) cells in the peripheral blood (**e**) and absolute number of LSK cells in the bone marrow (**f**) of wild-type ($CD45.1^+$) recipients ($n = 10$ per group) of donor wild-type or $Itch^{-/-}$ ($CD45.2^+$) LSK cells mixed at various ratios (horizontal axes) with wild-type competitor ($CD45.1^+$) cells. (**g**) Hematopoiesis of donor-derived ($CD45.2^+$) cells in the peripheral blood of wild-type ($CD45.1^+$) recipients ($n = 10$ per group) of total wild-type or $Itch^{-/-}$ ($CD45.2^+$) bone marrow cells mixed at various ratios (horizontal axis) with wild-type competitor ($CD45.1^+$) cells. (**h**) Absolute number of cells of various LSK subsets in 4- and 20-week-old wild-type and $Itch^{-/-}$ mice ($n = 5$ per genotype). (**i**,**j**) Hematopoiesis of either wild-type or $Itch^{-/-}$ donor-derived ($CD45.2^+$) cells in primary and secondary wild-type recipients (**i**; $n = 10$ per group) or number of either wild-type or $Itch^{-/-}$ donor-derived LSK cells in secondary wild-type recipients (**j**). $^*P < 0.05$ (Student's *t*-test). Data are representative of three (**a**–**c**,**h**) or two (**d**–**g**,**I**,**j**) independent experiments (mean and s.e.m.).

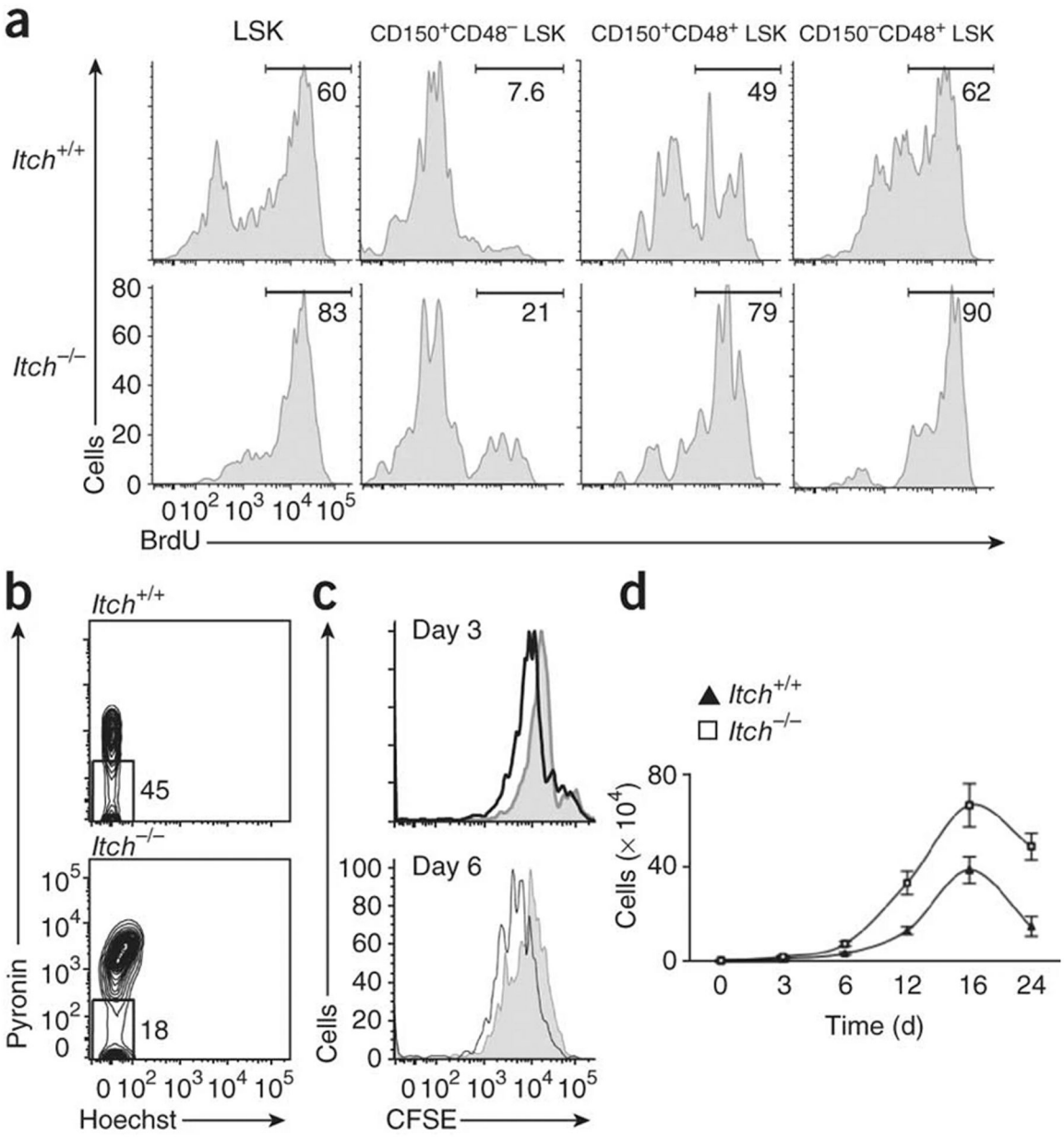

Figure 3.

Accelerated proliferation of Itch-mutant HSCs. (**a**) *In vivo* proliferative potential of wild-type and *Itch*$^{-/-}$ LSK subsets, as assessed by BrdU incorporation. Numbers above bracketed lines indicate percentages of proliferating (BrdU+) cells. (**b**) *Ex vivo* cell cycle status of wild-type and *Itch*$^{-/-}$ LSK cells, as assessed by pyronin Y and Hoechst staining. (**c**) *In vitro* proliferative potential of wild-type (shaded histograms) and *Itch*$^{-/-}$ (black lines) CD150^{+}CD48^{-} LSK cells, as assessed by CFSE dilution. (**d**) *Ex vivo* population expansion of wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells in response to a cytokine ‘cocktail’. Data are representative of two (**a**,**b**) or five (**c**,**d**) independent experiments (mean ± s.e.m. of duplicates in d).

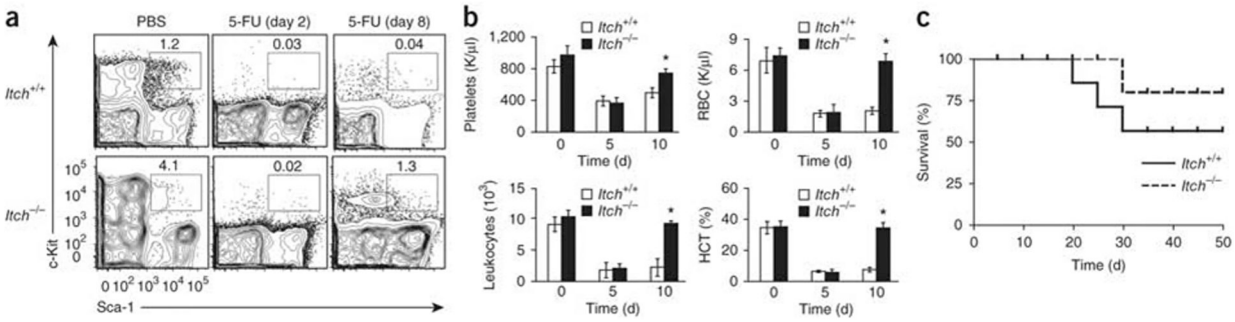

Figure 4.

Augmented repopulation activity of *Itch*$^{-/-}$ HSCs show after myeloablation. (**a**) Distribution of LSK cells in wild-type and *Itch*$^{-/-}$ bone marrow ($n = 5$ mice per group) after injection of 5-FU. (**b**) Differential blood counts and hematocrit (HCT) of wild-type and *Itch*$^{-/-}$ mice ($n = 5$ per group) after injection of 5-FU. RBC, red blood cell. *$P < 0.05$ (Student's *t*-test). (**c**) Survival of wild-type recipients ($n = 10$ per group) of wild-type or *Itch*$^{-/-}$ bone marrow cells, assessed with a log-rank nonparametric test after sequential 5-FU treatment and presented as a Kaplan-Meier survival curve. $P = 0.0082$ (Student's *t*-test). Data are representative of two independent experiments (mean ± s.e.m. in **b**).

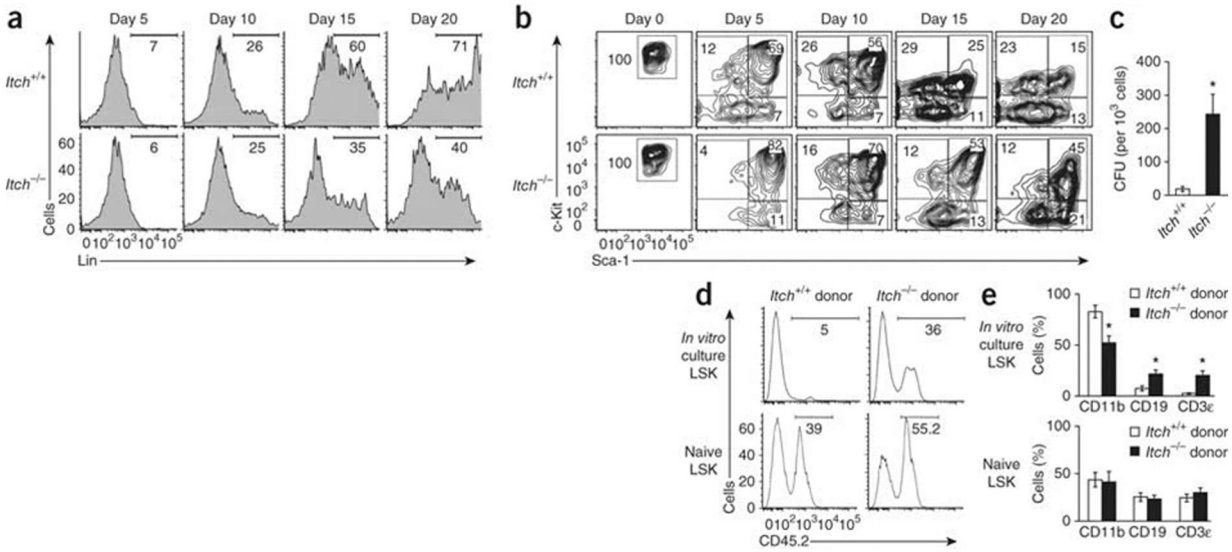

Figure 5.

Less spontaneous differentiation by Itch-deficient HSCs *in vitro*. (**a**) Expression of lineage markers in wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells after *in vitro* culture in the presence of a cytokine 'cocktail'. Numbers below bracketed lines indicate percent Lin^{+} cells. (**b**) Expression of Sca-1 and c-Kit in wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells after *in vitro* culture in the presence of a cytokine' cocktail'; Lin^{-} cells were pre-gated. (**c**) Colony-forming unit (CFU) potential of wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells. (**d**) Hematopoiesis of donor-derived (CD45.2^{+}) cells in the peripheral blood of wild-type (CD45.1^{+}) recipients (n = 8 mice per group) that received LSK cells cultured *in vitro* (top) or freshly isolated LSK cells (Naive; bottom). Numbers below bracketed lines indicate percent CD45.2^{+} cells. (**e**) Multilineage reconstitution by donor-derived (CD45.2^{+}) cells (myeloid, CD45.2^{+}CD11b^{+}; B lineage, CD45.2^{+} CD19^{+}; T lineage, CD45.2^{+}CD3ε^{+}) in recipients (n = 8 mice per group) of cells as in **d**. *P < 0.05 (Student's t-test). Data are representative of five (**a**,**b**) or two (**c**–**e**) independent experiments (mean and s.s.m. in **c**,**e**).

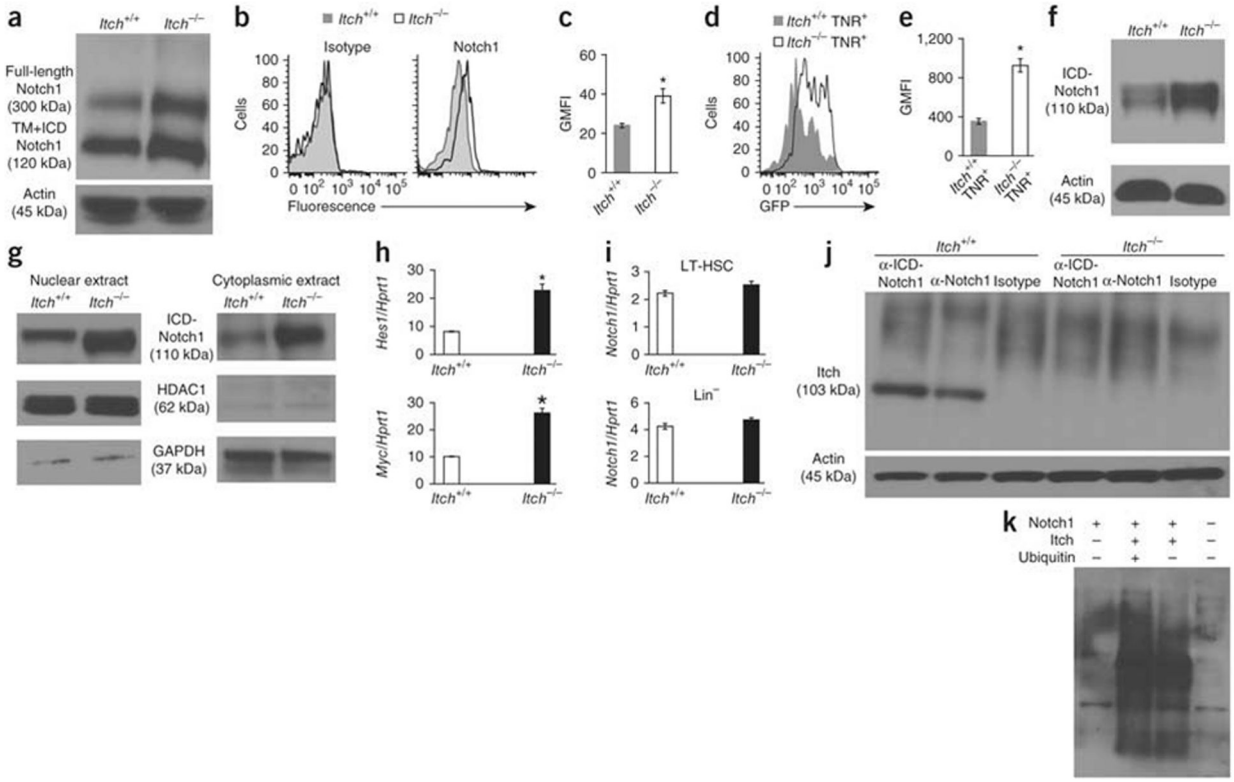

Figure 6.

Itch deficiency results in more Notch1 protein and signaling in HPCs. (**a**) Full-length Notch1 and cleaved Notch1 (transmembrane and intracellular domains (TM+ICD)) in wild-type and *Itch*$^{-/-}$ Lin^{-} bone marrow cells. Actin serves as a loading control throughout. (**b**) Intracellular Notch1 expression in wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells; left, cells stained with isotype-matched control antibody (controls). (**c**) Geometric mean fluorescence intensity (GMFI) of the results in **b**. (**d**) Expression of green fluorescent protein (GFP) in CD150^{+}CD48^{-}LSK cells from Itch$^{+/+}$ and *Itch*$^{-/-}$ transgenic Notch reporter (TNR^{+}) mice. (**e**) Geometric mean fluorescence intensity of the results in **d**. (**f**) Cleaved Notch1 in wild-type and *Itch*$^{-/-}$ Lin^{-}bone marrow cells. (**g**) cleaved Notch1 in the nucleus (left) and cytoplasm (right) of wild-type and *Itch*$^{-/-}$ Lin^{-} bone marrow cells. HDAC1 (histone deacetylase) and GAPDH (glyceraldehyde phosphate dehydrogenase) serve as controls. (**h**,**i**) Real-time PCR analysis of *Hes1* and *Myc* in wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK cells (**h**) and of *Notch1* in wild-type and *Itch*$^{-/-}$ CD150^{+}CD48^{-} LSK or Lin^{-}cells (**i**); results are presented relative to *Hrpt1* expression. (**j**) Immunoprecipitation analysis of the interaction between Itch and Notch1 in Lin^{-} cells. (**k**) Ubiquitination assay of the involvement of Itch in the ubiquitination of Notch1 protein. kDa, kilodaltons. *$P < 0.05$ (Student's *t*-test). Data are representative of five (**a**,**f**), three (**b**–**e**) or two (**g**–**i**) independent experiments (average and s.e.m. in **c**,**e**; mean and s.e.m. in **h**,**i**).

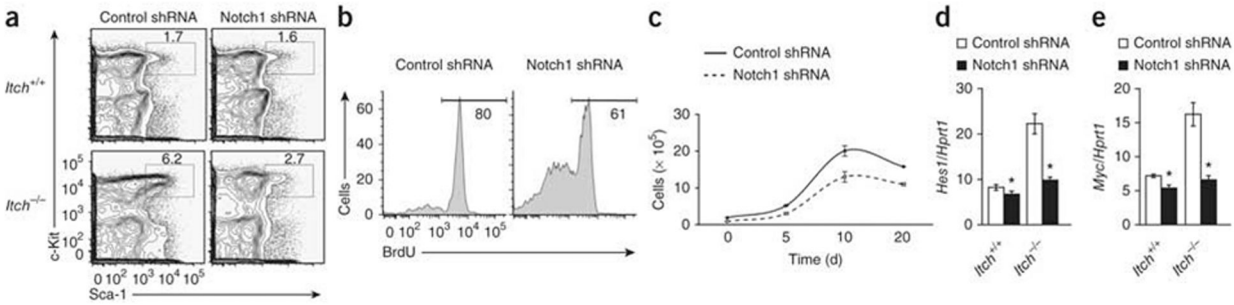

Figure 7.

Knockdown of Notch1 in Itch-deficient HSCs results in reversion of the phenotype. (**a**) Distribution of donor-derived (CD45.2$^+$) LSK cells in total bone marrow from wild-type (CD45.1$^+$) congenic recipients 8 weeks after transplantation of wild-type and *Itch*$^{-/-}$ (CD45.2$^+$) CD150$^+$CD48$^-$ LSK cells transduced with control or Notch1-specific short hairpin RNA (shRNA). (**b**) Incorporation of BrdU *in vivo* by donor-derived (CD45.2$^+$) LSK cells from wild-type (CD45.1$^+$) congenic recipients 8 weeks after transplantation of cells as described in **a**. (**c**) *Ex vivo* population expansion of LSK cells obtained from wild-type (CD45.1$^+$) congenic recipients 8 weeks after transplantation of cells as described in **a**, then cultured *in vitro* in the presence of an HSC cytokine 'cocktail'. (**d**,**e**) Real-time PCR analysis of *Hes1* (**d**) and *Myc* (**e**) in wild-type and *Itch*$^{-/-}$ CD150$^+$CD48$^-$ LSK cells obtained from wild-type (CD45.1$^+$) congenic recipients 8 weeks after transplantation of cells as described in **a**; results are presented relative to *Hrpt1* expression. **P* < 0.05 (Student's *t*-test). Data are representative of two independent experiments (mean ± s.e.m. of duplicate samples in **d**,**e**).

Table 1

Radioprotective functions of young bone marrow cells

Donor cells	Itch$^{+/+}$ donor	Itch$^{-/-}$ donor
0	0/10	0/10
1×10^2	0/10	0/10
1×10^3	0/10	0/10
5×10^3	0/10	4/10
1×10^4	2/10	8/10
5×10^4	3/10	9/10
1×10^5	9/10	10/10

Survival of lethally irradiated wild-type (CD45.1$^+$) recipients (n = 10 per group) of limiting numbers of bone marrow cells from 4-week-old Itch$^{+/+}$ or Itch$^{-/-}$ mice; results are presented as number of surviving mice/total mice. Data are representative of two independent experiments.

Table 2

Radioprotective functions of older Itch$^{-/-}$ bone marrow cells

Donor cells	Itch$^{+/+}$ donor	Itch$^{-/-}$ donor
0	0/10	0/10
1×10^2	0/10	0/10
1×10^3	0/10	2/10
5×10^3	0/10	8/10
1×10^4	0/10	10/10
5×10^4	5/10	10/10
1×10^5	8/10	10/10

Survival of lethally irradiated wild-type (CD45.1$^+$) recipients of limiting numbers of bone marrow cells from 20-week-old Itch$^{+/+}$ and Itch$^{-/-}$ mice; results are presented as number of surviving mice/total mice. Data are representative of two independent experiments.

Table 3

Radioprotection by Itch$^{-/-}$ cells expressing Notch1-specific shRNA

Donor cells	Control	Notch1
0	0/10	0/10
1×10^3	0/10	0/10
1×10^4	0/10	0/10
5×10^4	0/10	0/10
1×10^5	5/10	0/10
5×10^5	8/10	4/10
1×10^6	10/10	10/10

Survival of lethally irradiated wild-type (CD45.1$^+$) recipients (n = 10 per group) of limiting numbers of bone marrow cells from wild-type recipients transplanted with CD150$^+$CD48$^-$ LSK Itch$^{-/-}$ cells expressing control (middle) or Notch1-specific (left) shRNA; results are presented as the number of survivors/total mice. Data are repre- sentative of two independent experiments.