Inflammation-mediated Notch signaling skews Fanconi Anemia hematopoietic stem cell differentiation

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Abstract

Hematopoietic stem cells (HSCs) can either self-renew or differentiate into various types of cells of the blood lineage. Signaling pathways that regulate this choice of self-renewal versus differentiation are currently under extensive investigation. Here we report that deregulation of Notch signaling skews HSC differentiation in mouse models of Fanconi anemia (FA), a genetic disorder associated with bone marrow failure and progression to leukemia and other cancers. In mice expressing a transgenic Notch reporter, deletion of the *Fanca* or *Fancc* gene enhances Notch signaling in multipotential progenitors (MPPs), which is correlated with decreased phenotypic long-term HSCs and increased formation of MPP1 progenitors. Furthermore, we found an inverse correlation between Notch signaling and self-renewal capacity in FA hematopoietic stem and progenitor cells. Significantly, FA deficiency in MPPs deregulates a complex network of genes in the Notch and canonical NF-ᴋB pathways. Genetic ablation or pharmacologic inhibition of NF-ᴋB reduces Notch signaling in FA MPPs to near wide-type level, and blocking either NF-ᴋB or Notch signaling partially restores FA HSC quiescence and self-renewal capacity. Taken together, these results suggest a functional crosstalk between Notch signaling and NF-ᴋB pathway in regulation of HSC differentiation.

Introduction

Hematopoietic stem cells (HSCs) are rare cell population which can either self-renew or differentiate into various types of mature blood cells. Delineating the signaling pathways that regulate this choice of self-renewal versus differentiation remains an enduring challenge of foremost importance. One of the key regulators is Notch, an extracellular signal plays a critical role in hematopoietic homeostasis (1). Gain or loss of Notch signaling components has been directly linked to multiple human disorders including hematologic malignancies (2, 3). NF-ᴋB is another known factor that controls cell fate, survival and differentiation (4, 5).

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NF-κB is indicated in various physiological processes, including immunity, inflammation, development and differentiation (4, 6). It has been shown that crosstalk between the canonical NF-κB and Notch signaling pathways may influence tissue homeostasis in certain cell types including hematopoietic progenitor cells (7, 8).

Fanconi anemia (FA) is an inherited disorder characterized by genome instability and an extremely high cancer predisposition (9–11). FA is genetically heterogeneous, with 15 complementation groups identified thus far. The genes encoding the groups A (FANCA), B (FANCB), C (FANCC), D1 (FANCD1/BRCA2), D2 (FANCD2), E (FANCE), F (FANCF), G (FANCG), I (FANCI/KIAA1794), J (FANCJ/BRIP1), L (FANCL), M (FANCM), N (FANCN/PALB2), O(FANCO/RAD51C) and P(FANCP/SLX4) have been cloned (9–13). The biological function of these FA proteins has been subjected to intensive investigation. The studies demonstrate that eight of the FA proteins (FANCA, B, C, E, F, G, L, and M) form a core complex, which functions as an ubiquitin ligase. In response to DNA damage or DNA replication stress, the FA core complex monoubiquitinates two downstream FA proteins, FANCD2 and FANCI, which then recruit other downstream FA proteins including FANCD1 (which is the breast cancer protein BRCA2), FANCJ, FANCN and other DNA repair factors, to nuclear loci containing damaged DNA and consequently influence important cellular processes such as DNA replication, cell-cycle control, and DNA damage response and repair (12, 13).

Two of the most important clinical hallmarks of FA are bone marrow (BM) failure and progression to leukemia caused by HSC depletion and malignant transformation (14). Indeed, there are fewer HSCs in FA patients and knockout mice compared to normal controls (11, 15). In fact, HSCs from FA patients or knockout mice display severe defects in *in vitro* survival and *in vivo* repopulating (14, 16–18). Although these hematologic phenotypes suggest that the FA pathway likely plays specific roles in hematopoiesis, the mechanisms by which FA proteins regulate HSC self-renewal and differentiation is unknown. In the present study, we show that FA deficiency enhances Notch signaling in multipotential progenitors (MPPs), which is correlated with decreased phenotypic long-term HSCs and increased formation of MPP1 progenitors. Mechanistically, we show that FA deficiency in MPPs deregulates genes controlled by the NF-κB pathway leading to enhanced Notch signaling. The study thus identifies a functional cross-talk between the NF-κB pathway and Notch signaling in HSC differentiation and establishes a role of FA proteins in the control of balance between renewal and lineage commitment. In addition, our data also lends support to the recent report that several members of FA core complex, including FANCA, FANCF, FANCG and FANCL functionally interact with HES1, a key player in the Notch signaling pathway (19).

Materials and Methods

Mice and treatment

Notch-eGFP;*Fanca*$^{-/-}$ or Notch-eGFP;*Fancc*$^{-/-}$ mice were generated by interbreeding the heterozygous *Fanca*$^{+/-}$ or *Fancc*$^{+/-}$ mice (20, 21) with Notch-eGFP transgenic mice (22). Notch-eGFP;*Fanca*$^{-/-}$;*p65*$^{f/f}$ mice were generated by interbreeding the *RelA/p65* mice (23) with Notch-eGFP-*Fanca*$^{+/-}$. *p65* gene deletion was accomplished by Cre-mediated deletion of floxed alleles by crossing the Notch-eGFP;*Fanca*$^{+/-}$;*p65*$^{f/f}$ mice with a Cre-ERT2 strain (24) and intraperitoneally (i.p.) injecting 100 μl of Tamoxifen (20 mg/ml, Sigma- Aldrich, St Louis, MO) daily for 3 days. Animals were maintained in the animal barrier facility at Cincinnati Children's Hospital Medical Center.

For *in vivo* treatment, mice were subcutaneously injected with γ-secretase inhibitor, DAPT in a range of doses (0, 10, 50, 100 and 200 mg/kg, Tocris, R&D, Minneapolis, MN) daily for

2 days followed by bone marrow cell isolation on day 3 (25–27). For TNF- treatment, a single injection (100 μg/kg) of mouse recombinant TNF- (Peprotech, Rocky Hill, NJ) was given by intraperitoneally (i.p.) injection 10 days before sacrifice (28). BAY 11–7082 (Calbiochem- Novabiochem Corporation, La Jolla, CA) was administered (i.p.) to mice at doses of 20 mg/kg/day for 20 days (29). Mice were then sacrificed for bone marrow cell isolation 12 hours after the final injection.

Flow Cytometry analysis and Cell Cycle analysis

The lineage marker (Lin) mixture (BD Biosciences, San Jose, CA) for BM cells from Notch-eGFP-WT and Notch-eGFP-$Fanca^{-/-}$ or Notch-eGFP-$Fancc^{-/-}$ mice included the following biotinylated antibodies: CD3 (145-2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), mouse erythroid cells Ly-76 (Ter119), Ly6G and Ly-6C (RB6–8C5). Other conjugated antibodies (BD Biosciences, San Jose, CA) used for surface staining included: CD45.1 (A20), CD45.2 (A104), Sca1 (D7), c-kit (2B8), CD34 (RAM34), Flt3 (A2F10.1), CD48 (HM48-1), CD150 (9D1), IL-7R (HIL-7R-M21). Biotinylated primary antibodies were detected by incubation of antibody coated cells with streptavidin-PerCP Cy5.5 (BD Biosciences, San Jose, CA) in a two-step staining procedure.

Intracellular staining for the active Notch1 (NICD) was conducted as previously described (30). Briefly, surface marker stained $CD150^+CD48^-$ LSK cells (LT-HSCs) from Notch-eGFP-$Fanca^{+/+}$ or Notch-eGFP-$Fanca^{-/-}$ mice pretreated with different doses of DAPT were first fixed and permeablized with a Phosflow kit (BD Pharmingen, San Jose, CA) and then stained with Notch1 antibody (mN1A) according to the manufacturer's instructions (BD sciences, San Jose, CA).

For cell cycle analysis, cells were surface stained, fixed and permeabilized in BD Cytofix/Cytoperm Buffer (BD Biosciences, San Jose, CA), then stained with 5 μg/ml Hoechst 33342 and 150 ng/ml Pyronin Y (Sigma-Aldrich, St Louis, MO). Cells were then subjected to Flow Cytometric analysis (BD Biosciences, San Jose, CA).

In vitro T cell differentiation assay

4000 LSK cells isolated from Notch-eGFP-$Fanca^{+/+}$ or Notch-eGFP-$Fanca^{-/-}$ were seeded to 24-well plate pre-coated with OP9-DL1 stromal cells (20,000 cells plated the day before the co-culture). The co-culture was conducted in -MEM medium (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum and the following cytokines: 5ng/ml IL-7, 5 ng/ml Flt3L (Peprotech, Rocky Hill, NJ).Suspension cells were subjected to Flow cytometric analysis using antibodies specific for CD3 (BD Pharmingen, San Jose, CA), CD4 (eBioscience, San Diego, CA), CD8, TCR (BD Pharmingen, San Jose, CA) and TCR (Beckman Coulter, Brea, CA) on day 14 and day 21.

CFU assay

The upper 30% of Notch-eGFP Lin$^-$Sca1$^+$c-kit$^+$ (LSK) cells or lower 30% of Notch-eGFP LSK cells were sorted from Notch-eGFP-WT, Notch-eGFP-$Fanca^{-/-}$ or Notch-eGFP-$Fancc^{-/-}$ mice. 100 LSK cells were seeded in duplicate and cultured in cytokine-supplemented methylcellulose medium (MethoCult 3434; Stem Cell Technologies, Vancouver, Canada) in 35 mm-gridded dishes. For CFU assay using Notch-eGFP$^-$ or Notch-eGFP$^+$ LT-HSCs or MMP1 cells, GFP positive and negative cells from LT-HSC or MPP1 gated cell compartment were sorted using FACS AriaII (BD Biosciences, San Jose, CA). Subsequently, 20 LT-HSCs or 200 MMP1 were plated. Colonies were counted on day 7, then isolated, replated and cultured for another 7 days.

Competitive transplantation

Competitive repopulation experiments were conducted by transplanting 500 Notch-eGFP[lo] or Notch-eGFP^{hi} LSK cells from Notch-eGFP-WT, Notch-eGFP-*Fanca*^{−/−} or Notch-eGFP-*Fancc*^{−/−} mice along with 0.3 million of congenic BM cells as competitors into lethally irradiated BoyJ recipients as previously described (31). For BMT using Notch-eGFP[−] or Notch-eGFP[+] LT-HSCs or MMP1 cells, 50 LT-HSCs or 1000 MPP1 (CD45.2^{+}) cells plus 0.3 million CD45.1^{+} cells were used. Donor-derived chimerism (CD45.2[+]) in recipient at 4, 8, 16 and 24 weeks post transplantation were determined by CD45.1-PE and CD45.2-FITC markers staining followed by Flow Cytometry FACSCanto I (BD Biosciences, San Jose, CA) analysis.

RNA isolation, Pathway-specific gene array analysis and Real time-PCR

Total RNA of purified MPP1 subsets from Notch-eGFP-WT, Notch-eGFP-*Fanca*^{−/−} and Notch-eGFP-*Fancc*^{−/−} mice was prepared with RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's procedure. To address the inhibitory effect of DAPT, total RNA was extracted from MPP cells isolated from Notch-eGFP-*Fanca*^{+/+} or Notch-eGFP-*Fanca*^{−/−} mice treated with or without DAPT (100 mg/kg body weight). Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen, Grand Island, NY) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. First-strand cDNA was used for pathway-specific arrays (SABiosciences, Valencia, CA). Genes involved in DNA repair, Cell cycle control, oxidative stress, inflammation, apoptosis and Notch signaling pathways were analyzed. Genes with folder changes>3 were picked up for confirmation by real time-PCR.

For real time-PCR, 1st cDNA described above were employed for PCR amplification using the primers listed in Table 1. Samples were normalized to the level of *GAPDH* mRNA, and the relative expression levels were determined by the standard curve method.

Preparation of cell extracts, Immunoblotting

To prepare whole cell extracts, Lin^{−} cells from Notch-eGFP-*Fanca*^{−/−} mice treated with indicated doses of DAPT were washed with ice-cold PBS, and resuspended in ice-cold lysis buffer containing 50mM Tris-HCL (pH 7.4), 0.1% NP40, and 1M NaCl supplemented with protease and phosphatase inhibitors (10μg/ml aprotinin, 25μg/ml leupeptin, 10μg/ml pepstatin A, 2mM PMSF, 0.1M NaP_2O_4, 25mM NaF and 2mM sodium orthovandate) for 30 min on ice. Cell lysates were resolved on SDS-PAGE, and immunoblots were analyzed with antibodies for the active form of Notch1 (NICD), Stat1 (Cell Signaling, Boston, MA), or -actin (Sigma-Aldrich, St Louis, MO). Each lane contains protein from 50,000 Lineage depleted cells (Miltenyi Biotec, Auburn, CA). Signals were visualized by incubation with anti-mouse or anti-rabbit secondary antibodies followed by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistical analyses

Paired or unpaired student's *t-test* was used for two-group comparison, and one-way ANOVA for more than two-group comparison. Values of p less than 0.05 were considered statistically significant. Results are presented as mean ± SD. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

Results

Enhanced Notch signaling in FA MPPs

To examine whether FA murine HSCs can balance self-renewal with differentiation, we crossed two FA mouse models (*Fanca*$^{+/-}$ and *Fancc*$^{+/-}$) with a reporter strain, in which Notch-driven eGFP expression acts as a sensor for HSC differentiation (22). We first used ImageStream to analyze Notch signaling in Notch-eGFP expression in Lin$^-$Sca1$^+$c-kit$^+$ (LSK) cells, a population containing HSCs and multipotential progenitors. We observed higher Notch-eGFP expression in LSK cells from FA mice compared to WT littermates (Fig 1A). Consistent with this, flow cytometric analysis showed that FA LSK cells expressed higher eGFP than their WT counterparts (Fig 1B, D). It appeared that Notch-eGFP was also expressed in Lin$^-$S$^-$K$^+$ cells (Fig 1C), a population consisting mostly of myeloid progenitors, from mice of either genotype, albeit at lower levels than more primitive LSK cells. However, no difference in Notch-eGFP expression was observed between WT and FA Lin$^-$S$^-$K$^+$ cells (Fig 1C, D).

Since Notch signaling favors lymphoid while interferes with myeloid differentiation (32–35), we next determined if increased Notch expression in FA LSK cells was associated with alteration in lineage differentiation. Surprisingly, we did not see reduced myeloid differentiation in FA mice. Specifically, when we looked further into the subsets in the stem/progenitor compartment, that is, granulocyte-macrophage progenitors (GMP), common myeloid progenitors (CMP) and megakaryocyte-erythrocyte progenitors (MEP), we saw no difference between WT and FA mice (Fig1E) although a slight increased portion of common lymphoid progenitors (CLP) was observed in FA mice (Fig 1F). Consistent with this, the frequencies of CD4$^+$ single positive (SP) T cells, CD8$^+$ SP T cells, CD4$^+$CD8$^+$ double positive (DP) T cells, as well as T cells bearing   TCR$^+$ or   RCT$^+$ cells derived from HSPCs at different stages of co-culture with OP9-DL1 feeder cells *in vitro* (36) were similar between the genotypes (Fig 1G). HSC differentiate through intermediate stages, generating at least two subsets of multipotential progenitors (MPP1 and MPP2; 37). Only LT-HSCs have the ability to self-renew. Therefore, we subdivided the LSK fraction using signaling lymphocytic-activation molecule markers (CD150 and CD48; SLAM; 38). We observed lower LT-HSC (LSKCD150$^+$CD48$^-$) number but higher MPP1 (LSKCD150$^+$CD48$^+$) cells in both *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice than in WT mice (Fig 1H). Therefore, reduction in LT-HSCs is associated with an increase in the number of MPP1 cells in the BM of FA mice.

Since we observed higher Notch-eGFP expression in FA LSK cells than in WT LSK cells, we next analyzed Notch signaling in more defined HSPC populations. Surprisingly, WT and FA mice showed strikingly different patterns of Notch-eGFP expression in LT-HSCs vs MPPs. Specifically, the percentage of WT LT-HSCs expressing Notch-eGFP was significantly higher than that of *Fanca*$^{-/-}$ LT-HSCs (Fig 2A). In contrast, FA MPPs expressed much higher Notch-eGFP than WT MPPs (Fig 2A). This was confirmed by analyzing the intracellular level of Notch-eGFP expression in single cells from these subsets (Fig 2B). Further, intracellular staining using antibody specific for the active Notch1 (NICD; 30, 39, 40) showed positive correlation between eGFP expression level and active Notch signal *in vivo* (Fig 2C). Thus, these results link Notch signaling to specific stages of HSPC frequencies.

Because loss of cellular quiescence contributes to HSC exhaustion (41), we next determined the cell cycle status of FA HSCs by PY/Hochest staining. We noticed that both total and eGFP-positive LSK cells from FA mice showed significantly decreased G$_0$ quiescent cells compared to those from WT mice (Fig 2D). To examine further the potential linkage between Notch signaling and HSC quiescence, we analyzed cell-cycle status of eGFP-positive LT-HSCs. Similar to the result obtained with the heterogeneous LSK cells, we

observed significantly reduced quiescent Notch-eGFP$^+$ LT-HSCs in FA mice compared to WT Notch-eGFP$^+$ LT-HSCs (Fig 2E). Therefore, the data indicate that Notch signaling fails to rescue loss of quiescence in FA HSCs.

Inverse correlation between Notch signaling and self-renew capacity in FA HSCs

The observations that Notch-eGFP expression was low in FA LT-HSCs but high in FA MPP cells suggested that alteration in Notch signaling in FA LT-HSCs and MPP cells could influence their capacity to self-renew and differentiate. To test this hypothesis, we used FACS to divide the Notch-eGFP$^+$ LSK population into Notch-eGFP^{hi} (MFI values: 49.41± 3.29 for $Fanca^{+/+}$ and 68.59 ± 3.07 for $Fanca^{-/-}$) and Notch-eGFP^{lo} (MFI values: 20.25 ± 2.68 for $Fanca^{+/+}$ and 26.4 ± 4.72 for $Fanca^{-/-}$) fractions (Fig 3A) and determined the self-renewal capacity of these two populations. Colony-forming unit (CFU) assay showed that although the first plating showed that both WT and the FA Notch-eGFP^{hi} fractions had similar capacity to generate colonies; replating of the FA Notch-eGFP^{hi} LSK cells yielded much fewer colonies (Fig 3B), suggesting a possible loss of *in vitro* self-renewing capacity. To complement these *in vitro* studies, we did competitive BM transplantation with 500 LSK cells purified from both fractions (expressing the marker CD45.2) along with 0.3 million CD45.1$^+$ competitor cells. Interestingly, we found that FA Notch-eGFP^{hi} LSK cells still displayed defects in hematopoietic repopulation compared to the same number of WT Notch-eGFP^{hi} LSK cells (Fig 3C).

This result prompted us to reevaluate the LSK population used to identify engrafting HSCs by dividing the LT-HSCs and MPP1 population into Notch-eGFP$^-$ and Notch-eGFP$^+$ cells (Fig 4A). As shown in Figure 4B, eGFP$^-$ LT-HSCs from $Fanca^{-/-}$ mice showed reduced colony forming units compared to WT eGFP$^-$ LT-HSC in the second plating, indicating an impaired *in vitro* self-renewal capacity. In contrast, $Fanca^{-/-}$ eGFP$^+$ LT-HSCs show comparable colony-forming ability to their WT counterparts in both platings (Fig 4B). On the other hand, both eGFP$^+$ and eGFP$^-$ MPP1 cells from $Fanca^{-/-}$ mice showed defective colony-forming capacity in the second plating (Fig 4B). These results suggest that Notch signaling may play distinct roles in self-renewal of LT-HSCs and MPP cells *in vitro*.

To examine the *in vivo* role of Notch signaling in self-renewal of LT-HSCs and MPP cells, we transplanted 50 LT-HSCs or 1000 MPP1 cells from each of the two fractions into lethally irradiated recipients to investigate their repopulating potential. $Fanca^{-/-}$ Notch-eGFP$^-$ LT-HSCs showed typical repopulating deficit at each time point post-transplantation (Fig 4C, D). Consistent with previous report (20), WT Notch-eGFP$^+$ LT-HSCs exhibited enhanced long-term repopulating capacity. However, $Fanca^{-/-}$ Notch-eGFP$^+$ LT-HSCs displayed quantitatively defective long-term repopulating activity with less than 20% donor-derived cells compared to more than 50% repopulated by WT Notch-eGFP$^+$ LT-HSCs at 16 and 24 weeks post-transplant (Fig 4C, D). All LT-HSCs, regardless of genotype status or Notch-eGFP expression, generate HSPCs as well as all mature lineages 24 weeks after transplantation (Fig S1). Neither WT nor FA MPP1 cells, regardless of Notch-eGFP expression, provided long-term reconstitution (Fig 4C, D). In fact, Notch-eGFP$^+$ FA MPP1 progenies were almost undetectable at 4 weeks after transplantation (Fig 4C, D). Taken together, these results indicate that Notch signaling fails to rescue repopulating defect of FA LT-HSCs and impairs the short-term repopulating ability of FA MPPs.

FA deficiency in MPPs deregulates the gene network of Notch and NF-κB pathways

To further understand the effect of enhanced Notch signaling mechanistically in FA MPPs, we purified MPP1 subsets and used pathway-specific gene array analysis to define the gene-expression signatures of each population (Fig S2A). Since FA cells have defects in DNA repair, Cell-cycle control, anti-oxidant defense, inflammatory response, and apoptotic

signaling, we focused on these pathways as well as the Notch signaling pathway (Fig S2A). A substantial increase in the expression of genes involved in the NF-κB and Notch signaling pathways was observed in the *Fanca⁻/⁻* or *Fancc⁻/⁻* MPP1 cells (Table 2, Fig S2B, S2C). These include NF-κB target genes *Gadd45b, Icam1, Irf1, Rela (p65) Sod2, Stat1, Tnfrsf1b (TNF receptor II)* and *Xiap*, as well as Notch signature genes *Hes1, Hey1, Hoxb4, Fzd1, Fzd2, Nfkb1, Notch1, Notch2* and *Numb* (Fig S2B, S2C).

Inflammatory stress-activated Notch signaling compromises HSC self-renewal

Because our gene expression analysis suggests a potential NF-κB-Notch crosstalk in FA HSPC cells and because FA HSPC cells are hypersensitive to inflammatory stress (42), we next determined whether TNF-α, a NF-κB activator and a major inflammation mediator in FA (43), would enhance Notch signaling in FA HSPCs. To this end, we treated Notch-eGFP-*Fanca⁻/⁻* mice and Notch-eGFP-WT littermates with TNF-α and examined Notch-eGFP expression in BM LSK cells. Interestingly, we found that TNF-α affected eGFP-positive HSPCs in both WT and FA mice. However, the effect of TNF-α was significantly exacerbated in FA mice compared to WT mice. Specifically, the frequency of GFP⁺ LSK cells was further increased in TNF-α-treated Notch-eGFP-*Fanca⁻/⁻* mice (Fig 5A). This was associated with a further decrease in HSC quiescence (Fig 5B). To substantiate these phenotypic analysis, we performed competitive BM transplantation by injecting 500 GFP⁻ LSK or GFP⁺ LSK cells isolated from the mice treated with TNF-α along with 0.3 million congenic WT BM cells into lethally irradiated recipients and determined donor-derived chimera 24 weeks post transplantation. We observed dramatically further impairment in hematopoietic repopulation of GFP⁺ LSK cells from TNF-α-treated Notch-eGFP-*Fanca⁻/⁻* mice (Fig 5C). In fact, donor-derived cells from recipients transplanted with TNF-α-treated FA eGFP⁺LSK cells were almost undetectable at 24 weeks after transplantation (Fig 5C). It is also noteworthy that Notch-eGFP⁺ HSPCs, regardless of genotypes, were more vulnerable to TNF-α treatment than Notch-eGFP⁻ HSPCs in the context of long-term hematopoietic repopulation (Fig 5C). To assess whether the enhanced expression of Notch-eGFP in TNF-α-treated *Fanca⁻/⁻* mice required canonical NF-κB signaling, we crossed Notch-eGFP-*Fanca⁺/⁻* mice with mice carrying a conditional gene encoding the p65 subunit of NF-κB (*p65^{f/f}*) (23). Inducible deletion of *p65* was accomplished by Cre-mediated deletion of floxed *p65* alleles using a Cre-ERT2 strain (24) and Tamoxifen (Fig S3). We found that inactivation of *p65* resulted in attenuation of *Hes1* and *Hey1* expression in LSK cells from TNF-α-treated Notch-eGFP-positive WT and Fanca⁻/⁻ mice (Fig 5D). Furthermore, *p65* deletion caused a significant reduction in Notch target gene expression in untreated Notch-eGFP-positive *Fanca⁻/⁻* LSK cells (Fig 5D). These results suggest that inflammatory stress activates Notch signaling and compromises hematopoietic repopulating capacity of HSPCs.

Inhibition NF-κB or Notch partially restores FA HSPC function

To further determine the functional relevance of Notch-NF-κB cross-talk_in FA HSPCs, we sought to test whether inhibition of NF-κB or Notch signaling could restore FA HSPC function. We took two approaches on NF-κB inhibition: genetic ablation of *p65*, a subunit of NF-κB transcription complex (23), and pharmacologic inhibition of NF-κB. We found that *p65* deletion in MPP1 cells of Notch-eGFP-*Fanca⁻/⁻* mice reduced Notch expression to nearly WT level (Fig 6A). To further substantiate this finding, we made use of BAY11–7082, an inhibitor of cytokine-induced IκB phosphorylation (29). Consistent with *p65* knockout, BAY11–7082 treatment also significantly reduced Notch-eGFP expression in FA MPP1 cells (Fig 6A). Phenotypic analysis of the HSPC subsets in the LSK compartment revealed that inhibition of NF-κB by either *p65* deletion or BAY11–7082 treatment increased LT-HSCs and decreased MPP1 cells in *Fanca⁻/⁻* mice to near WT levels (Fig 6B). Thus, these data provide molecular evidence that links NF-κB-Notch crosstalk to regulation of FA HSC differentiation.

To determine the functional consequence of inhibition of NF-κB and Notch signaling in FA HSPCs, we blocked either NF-κB or Notch signaling by conditional deletion of *p65* in *Fanca*^{−/−} mice or treated the mice with the γ-secretase inhibitor, DAPT that inhibits Notch signaling (25, 26), respectively, and assessed cell cycle status and repopulating capacity of HSPCs. Deletion of *p65* partially rescued the loss of quiescence of *Fanca*^{−/−} but not WT HSPCs (Fig 6C). For DAPT treatment, we found a dose-dependent (up to 100 mg/kg body weight) inhibition of Notch signaling evidenced by a decreased generation of the active form of Notch 1 (NICD) without affecting non-Notch signaling protein Stat1, which paralleled a decreased level of eGFP-positive MPP cells in *Fanca*^{−/−} mice (Fig S4A–B). Consistent with this, mice treated with DAPT at the dose of 100 mg/kg body showed effective inhibition of the expression of Notch target genes without affecting NF-κB transactivation in MPP cells (Fig S4C). Consequently, cell-cycle analysis demonstrated that DAPT treatment restored quiescence of LSK cells from *Fanca*^{−/−} mice (Fig 6C). Furthermore, LSK cells from *Fanca*^{−/−} mice subjected to *p65* deletion exhibited significant increase in long-term hematopoietic repopulation when transplanted to lethally irradiated recipients compared to untreated controls (Fig 6D). A notable increase, albeit not statistically significant, in hematopoietic repopulation was also observed in DAPT-treated *Fanca*^{−/−} LSK cells. These results indicate that blocking NF-κB or Notch signal can partially correct the functional defect of FA HSPCs.

Discussion

The current study identifies a potential interaction between the FA pathway and Notch signaling in HSC differentiation and establishes a role of FA proteins in the control of balance between renewal and lineage commitment. There are several findings that highlight the significance of our study: (1) Loss of murine FA proteins results in enhanced Notch signaling in MPPs, which is correlated with decreased phenotypic and functional LT-HSCs and increased formation of MPP1 progenitors; (2) Deletion of *Fanca* or *Fancc* gene deregulates genes in the Notch signaling and the NF-κB pathway; (3) TNF-α stimulation enhances Notch signaling in *Fanca*^{−/−} and *Fancc*^{−/−} LSK cells, leading to decreased HSC quiescence and compromised HSC self-renewal; (4) Inflammation-activated Notch target gene expression in *Fanca*^{−/−} and *Fancc*^{−/−} MPP cells requires NF-κB; (5) Genetic ablation or pharmacologic inhibition of NF-κB reduces Notch signaling in FA MPPs to near wide-type level and significantly increases LT-HSCs and decreases MPP1 cells in FA mice; (5) Blocking either NF-κB or Notch signaling partially restored FA HSC quiescence and self-renewal capacity.

Several previous studies also suggest a crosstalk between the FA proteins and Notch targets. Tremblay *et al.* identified the interaction between components of the FA core complex and HES1, a Notch target that participates in many of the Notch-assigned functions including proliferation, differentiation, apoptosis, self-renewal, and asymmetric cell division regulation (19, 44). Another study demonstrates that FA core complex interacts with HES1 and antagonizes HES1-mediated transcriptional repression (45). Here, we show that enhanced Notch signaling in FA MPPs skews HSC differentiation, evidenced by exhaustion of LT-HSC pool and increase in MPP1 frequency. Deregulated Notch signaling was further demonstrated by a marked increase in the transcription of Notch target genes (including *Hes1, Hey1, Notch1* and *Notch2*) in *Fanca*^{−/−} and *Fancc*^{−/−} MPP1 cells. Based on these results and the studies by others described above, we propose that one critical function of FA proteins in hematopoiesis is to regulate HSC differentiation, probably through modulating the integrity of the Notch signaling pathway.

Recent studies have identified the Notch pathway as a principal player on stem cell regulation and differentiation (46). While Notch plays a predominant role in the generation

of HSCs in the embryo, it appears that the maintenance of adult HSCs does not require Notch (47). However, our present study reveals a plethora of Notch functions in HSPC cell populations that is dependent on the cell context and environment. For example, we found that enhanced Notch signaling in murine FA MPP cells was associated with the reduction of LT-HSC pool and the increase in MPP production in the BM (Fig 1G and Fig 2A). Functionally, FA Notch-eGFP^{+} HSPC cells show hematopoietic repopulation defect upon transplantation into lethally irradiated recipients (Fig 3C and Fig 4C), which might result from increased HSC differentiation demanded by the expanded Notch-eGFP[+] MPPs. On the other hand, inflammatory stress generated by TNF-α stimulation enhanced Notch signaling of FA HSPC cells and led to decreased HSC quiescence and compromised HSC self-renewal (Fig 5). TNF-α is known to be a common activator for both Notch signaling and NF-κB pathways (47, 48). It has been shown that FA BM HSPC cells are hypersensitive to inflammatory cytokines including TNF-α (13). In addition, TNF-α is often found overproduced in FA patients (43, 49). Conceivably, increased TNF-α observed in FA patients could change the BM microenvironment and cause deregulation of the Notch and NF-κB signaling pathways. Our finding thus raises an important question as to whether deregulated Notch and NF-κB signaling caused by the elevated level of TNF-α in FA BM microenvironment is the mechanism that links to the defects in HSC self-renewal and differentiation.

Quiescence has been postulated to prevent HSC exhaustion (50). In the BM, HSCs are kept in a low proliferative, relatively quiescent state within the microenvironment (niche; 51). This dormancy is crucial to maintain the appropriate HSC pool. In this study, we demonstrate that HSPC (LSK) cells as well as LT-HSCs from $Fanca^{-/-}$ and $Fancc^{-/-}$ mice are less quiescent compared with WT controls, as evidenced by the reduced G_0 population (Fig 2C and 2D), indicating that FA proteins may play a role in HSC maintenance by regulating cell cycle entry. Although loss of quiescence in FA HSCs is independent of Notch signaling in the steady state, enhanced Notch signaling in FA HSPCs leads to impaired long-term repopulation abilities of FA HSCs (Fig 4). Notably, inflammatory stress (TNF-α stimulation) activated Notch signaling in both WT and FA HSPC (LSK) cells, which was correlated with decreased quiescence and impaired hematopoietic repopulation (Fig 5). Moreover, blockage of Notch signaling partially restored FA HSC quiescence and repopulating capacity (Fig 6). Therefore, our results suggest that while Notch signaling is dispensable for the maintenance of the adult HSCs, deregulated Notch signaling under inflammatory stress could tip HSCs toward less quiescent state leading to impaired stem cell function.

Recent evidence of interaction between Notch and NF-κB suggests that the crosstalk between these two important signaling pathways may influence tissue hemeostasis (7, 8, 52). On the one hand, it has been shown that transcriptional repressor CBF1, the essential transcription factor downstream in Notch signaling, interacts with a dual NF-κB/CBF1-binding site (κB2) in the IκBα promoter and de-repress IκBα gene transcription leading to NF-κB activation in hematopoietic progenitor cells (8, 53). On the other hand, it was found that activation of the canonical NF-κB pathway by TNF-α stimulation induced optimal expression of Notch targets such as $Hes1$ and $Hey1$ through a mechanism involving epigenetic activation of the Notch gene promoters by TNF-α stimulation (7). In this study, we provide several lines of evidence demonstrating the crosstalk between NF-κB and Notch pathways in the regulation of HSC maintenance. First, we found that TNF-α stimulation enhanced Notch signaling in $Fanca^{-/-}$ and $Fancc^{-/-}$ HSCP (LSK) cells, leading to decreased HSC quiescence and compromised hematopoietic repopulation (Fig 5). Second, although further study is required to reveal the underlying molecular mechanism, it is intriguing that FA deficiency in MPPs deregulates a complex network of genes in the Notch and canonical NF-κB pathways (Fig S2), suggesting a potential link between these two pathways. To

support this, we show that inflammation-activated transcription of Notch target genes in *Fanca*$^{-/-}$ MPP cells requires NF- B (Fig 5D and data not shown). Third, genetic ablation or pharmacologic inhibition of NF- B reduces Notch signaling in *Fanca*$^{-/-}$ MPPs to near wide-type level and significantly increases LT-HSCs and decreases MPP1 cells in *Fanca*$^{-/-}$ mice (Fig 6A–B). Finally, genetic ablation of NF- B or pharmacologic inhibition of Notch signaling partially restored FA HSC quiescence and hematopoietic repopulation (Fig 6C–D). Thus our results favor the mechanism by which NF- B regulates Notch pathway probably via influencing the expression of Notch target genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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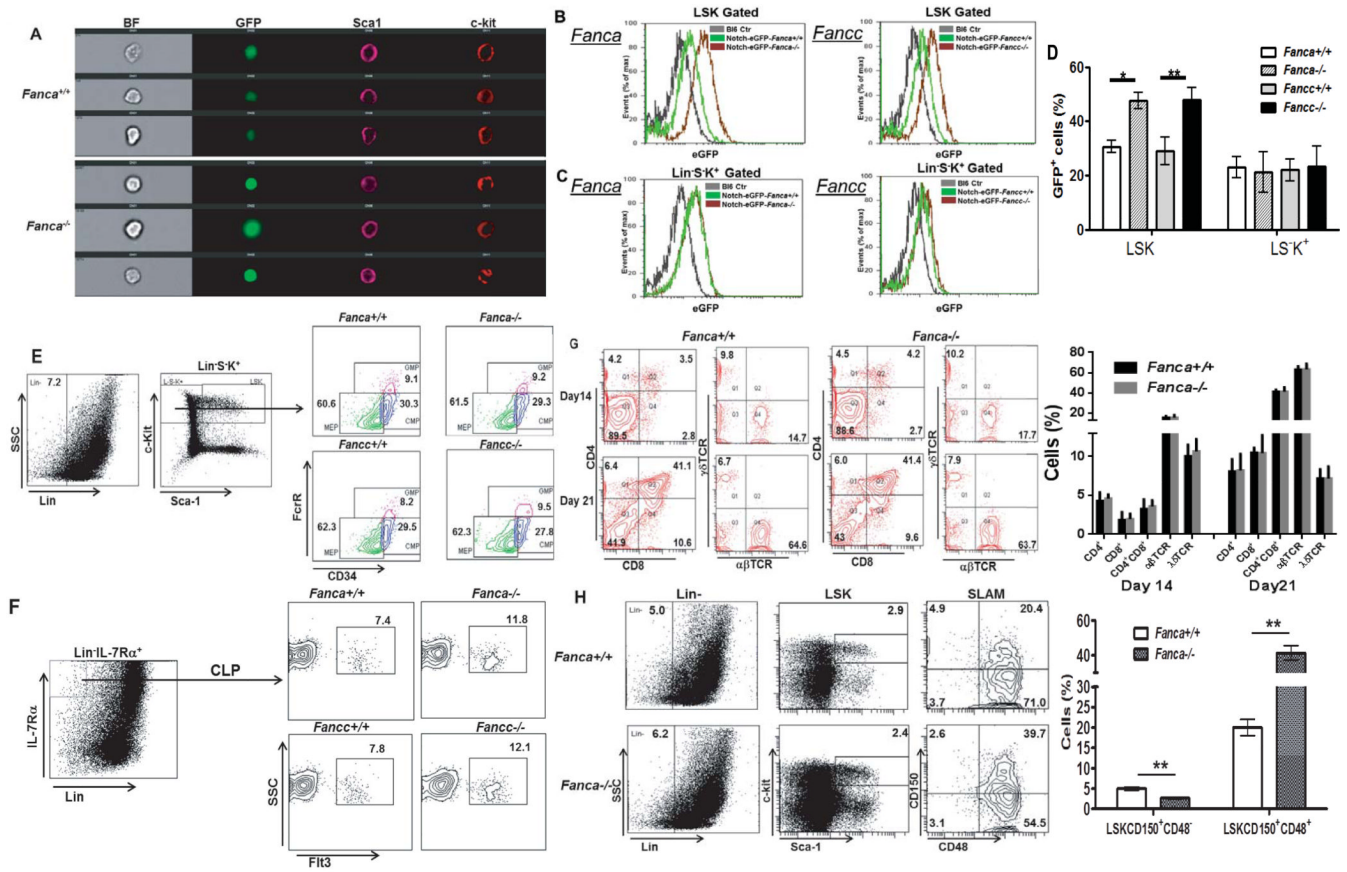
Figure 1. Increased Notch-eGFP expression in FA HSPCs
(A) eGFP+ LSK cells from *Fanca*−/− mice are brighter than those from WT. Whole bone marrow cells (WBMCs) from Notch-eGFP-*Fanca*+/+or Notch-eGFP-*Fanca*−/− mice were isolated followed by Lineage+ cell depletion. LinSca1+c-kit+ (LSK) cells were labeled and subjected to ImageStream analysis. Representative images were shown (n=5-7). (B) Enhanced Notch signaling in FA LSK cells. Whole bone marrow cells (WBMCs) from Notch-eGFP-*Fanca*−/−, Notch-eGFP-*Fancc*−/−, or WT littermates were isolated for cell surface marker staining. LSK cells were gated for eGFP expression analysis by Flow Cytometry. (C) No difference in Notch expression between FA and WT LinK+S cells. Cells described in (A) were gated for Linc-kit+Sca1 (LinK+S) population followed by Flow Cytometry analysis for eGFP expression. (D) Quantification of eGFP expression in LSK and LinK+S cells. Results are means plus or minus SD of 3 independent experiments (n=12 per group). (E–F) FA deficiency does not alter myeloid differentiation (E) or lymphoid differentiation (F). Cells described in (B) were subjected to Flow Cytometry analysis for myeloid or lymphoid progenitors. (G) FA deficiency does not affect T cell differentiation *in vitro*. 4000 LSK cells isolated from either *Fanca*+/+ or *Fanca*−/− mice were seeded to 24-well plate pre-coated with OP9-DL1 cells. Suspension cells were collected on day 14 and day 21 followed by Flow cytometric analysis for CD3, CD4, CD8, TCR and TCR. Representative image (Upper) and quantification (Lower) are shown. Results are means plus or minus SD of 3 independent experiments. (H) FA deficiency results in the reduction of LT-HSC and increased MPP frequency. Cells described in (A) were stained for SLAM markers followed by Flow Cytometry analysis. Representative image (Left) and quantification (Right) are shown. Results are means plus or minus SD of 3 independent experiments (n=5–7 per group).
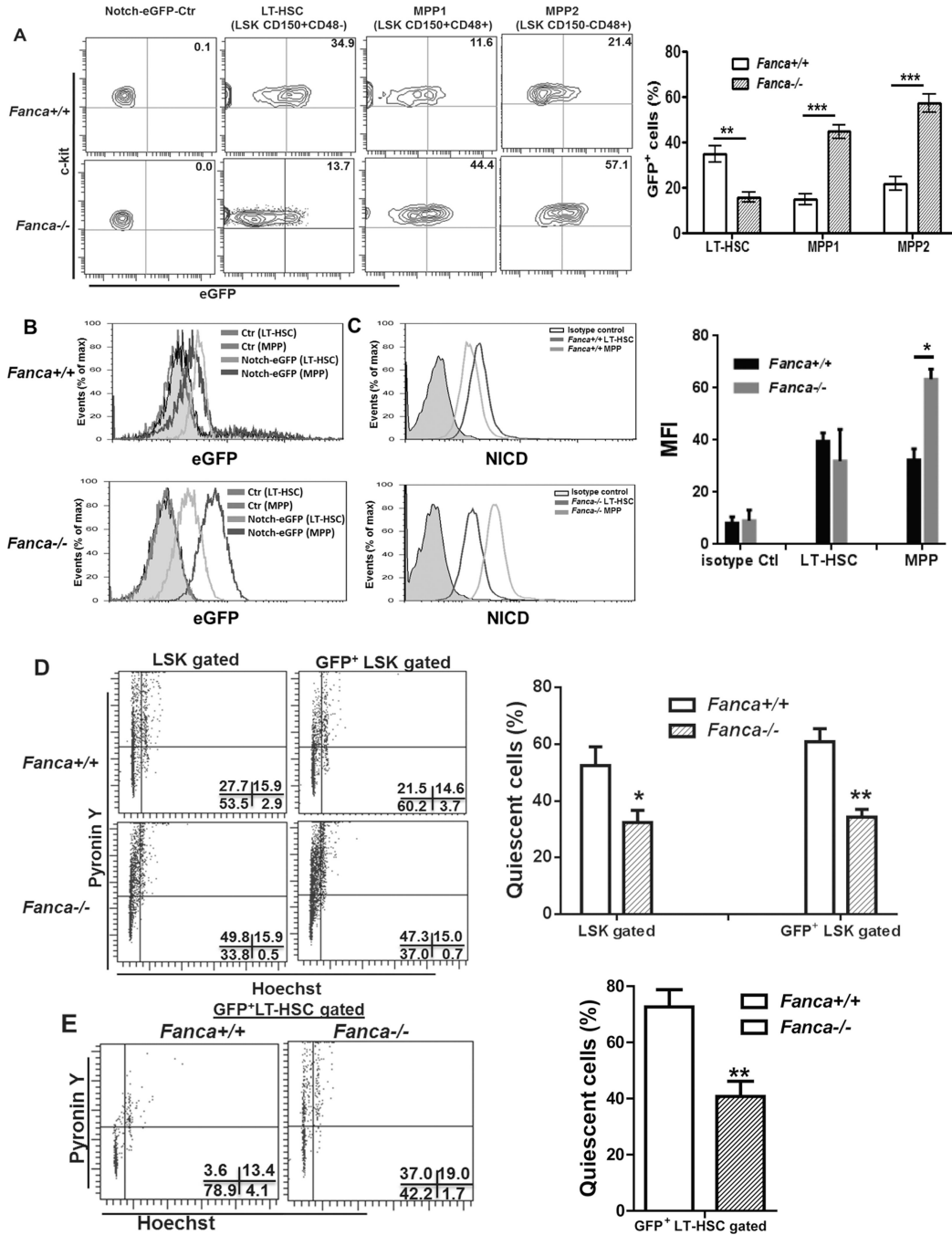
Fig 2. Enhanced Notch signaling in *Fanca*$^{-/-}$ MPPs

(A) Percentage of GFP$^+$ cells in LT-HSC and MPP populations. WBMCs from Notch-eGFP-*Fanca*$^{+/+}$ or Notch-eGFP-*Fanca*$^{-/-}$ mice were isolated and stained for SLAM markers followed by Flow Cytometry analysis. Representative GFP expression (Upper) from three cell populations: LT-HSC (LinSca1$^+$c-kit$^+$CD150$^+$CD48); MPP1 (LinSca1$^+$c-kit$^+$CD150$^+$CD48$^+$); or MPP2 ((Lin-Sca1$^+$c-kit$^+$CD150$^-$CD48$^+$) and quantification (Lower) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group). (B) Intracellular expression levels of GFP in LT-HSC and MPP subsets. The levels of GFP expression from cell populations described in (A) were plotted for histogram display. (C) eGFP expression is positively correlated with active Notch1 in HSPCs.

WBMCs from Notch-eGFP-*Fanca*^{+/+} or Notch-eGFP-*Fanca*^{−/−} mice were subjected to intracellular staining for active Notch1 (NICD). LT-HSC or MPP cell portions were gated for histogram display. (D) Decreased quiescence in *Fanca*^{−/−} HSPCs. Cell described in (A) were subjected to cell cycle analysis by Hochest 33342/Pyronin Y staining gated on total or eGFP+ LSK cells respectively. (E) Decreased quiescence of *Fanca*^{−/−} LT-HSCs. eGFP+ LT-HSCs from cells described in (A) were gated for cell cycle analysis. Representative flow graph (Left) and quantification (Right) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group).
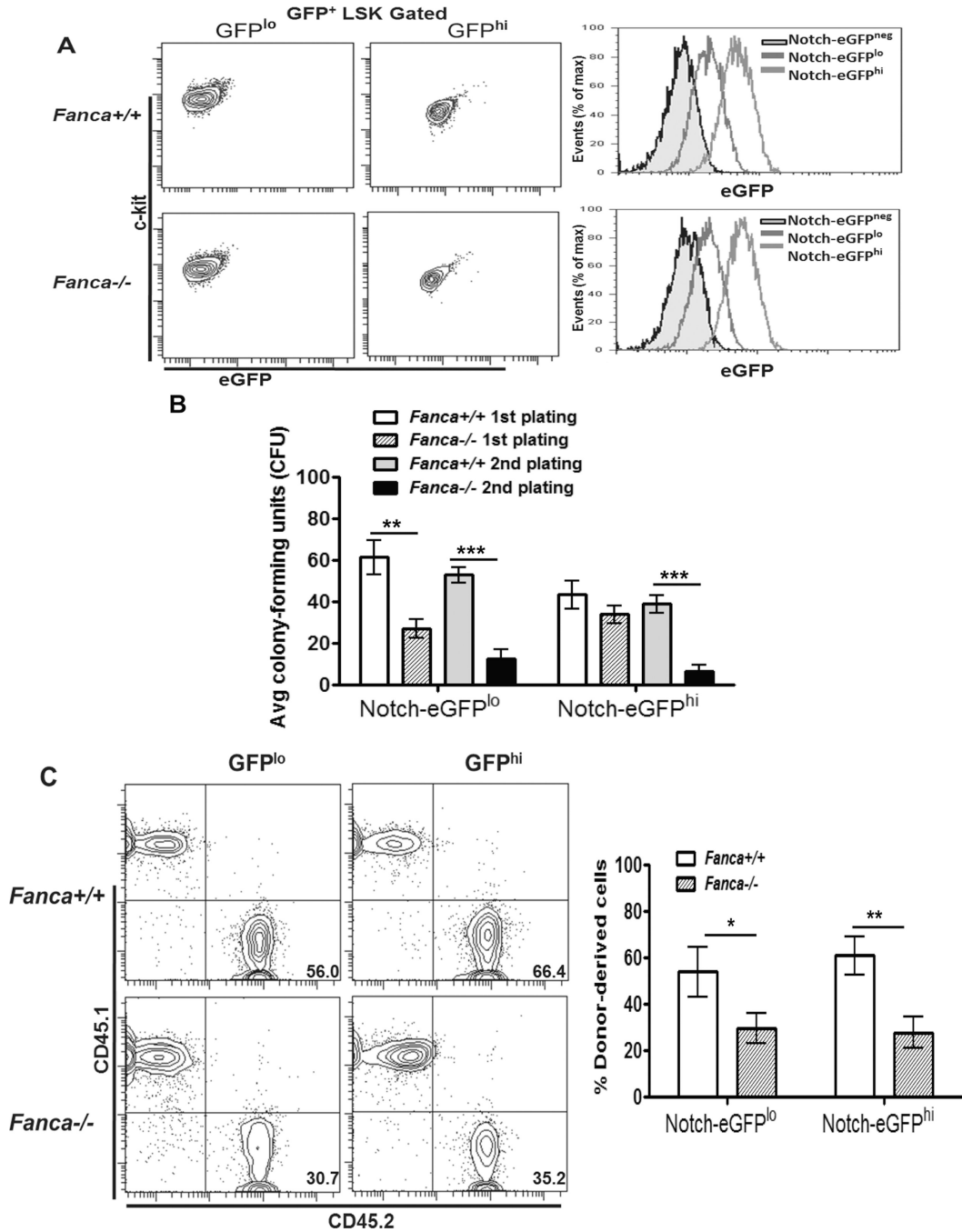
Fig 3. Self-renewal defect in *Fanca*$^{-/-}$ Notch-eGFP^{hi} HSPCs
(A) Flow-based purification of GFP^{lo} and GFP^{hi} subsets. The upper 30% of Notch-eGFP LSK cells (GFP^{hi}) or lower 30% of Notch-eGFP LSK cells (GFP^{lo}) were sorted from mice with the indicated genotypes. Representative flow images (Left) and histogram overlay (Right) are shown. (B) Replating defects of Notch-eGFP^{hi} LSK cells. 100 GFP^{lo} or GFP^{hi} LSK cells were seeded in duplicate and cultured in cytokine-supplemented methycellulose medium for 7 days. Colonies were then counted, isolated, replated and cultured for another 7 days. Error bars indicate standard deviation (std) of three experiments (n=9). (C) Hematopoietic reconstitution defects of *Fanca*$^{-/-}$ Notch-eGFP^{hi} LSKs. 500 GFP^{lo} or GFP^{hi} LSK (CD45.2$^+$) cells plus 0.3 million cells (CD45.1$^+$) were tail vein injected to lethally

irradiated recipient (BoyJ) mice. Donor-derived chimera was assessed by Flow Cytometry analysis 16 weeks post BMT. Representative dot graph (Left) and quantification (Right) are shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group).
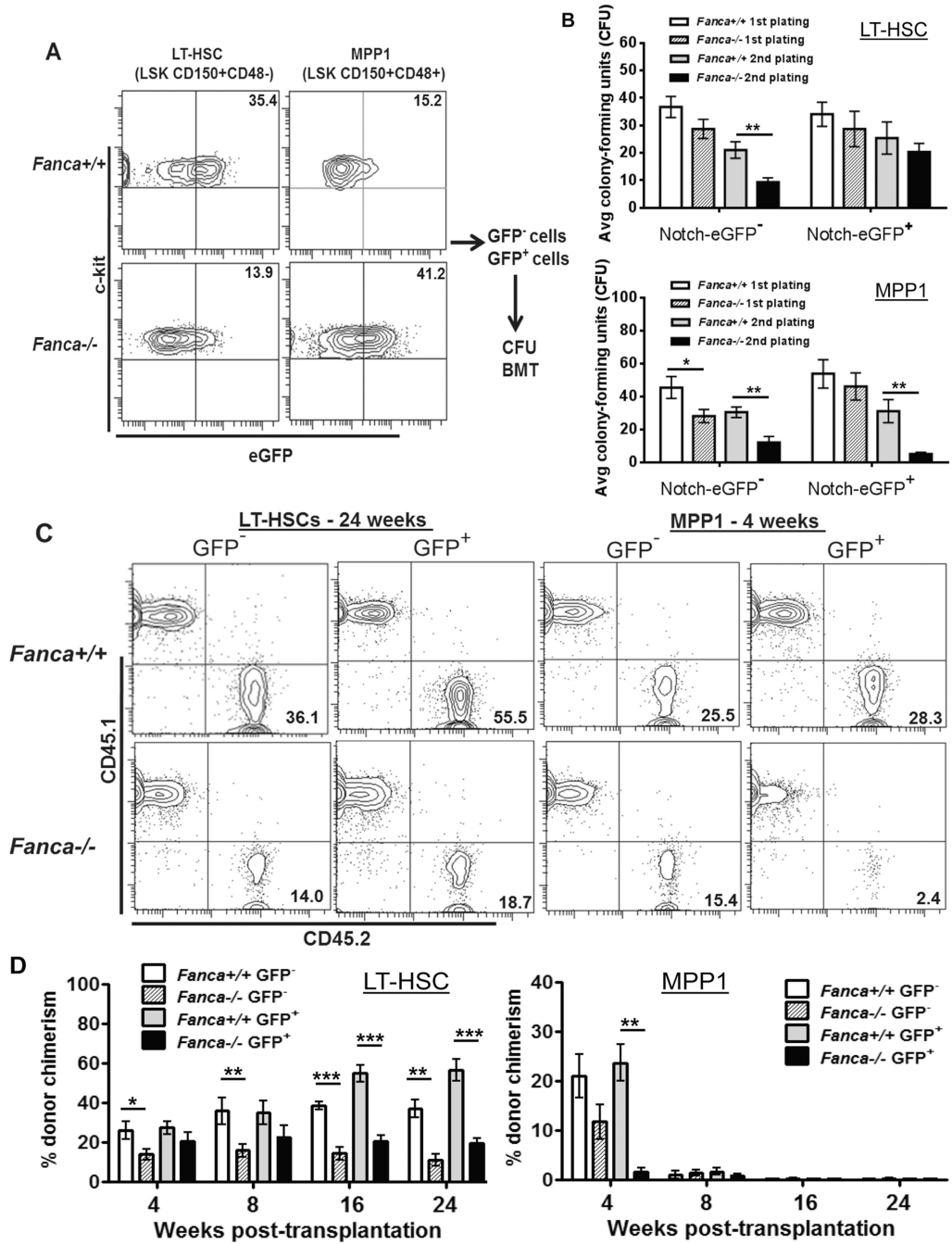
Fig 4. Inverse correlation between Notch signaling and self-renewal capacity of *Fanca*^{−/−} HSPCs
(A) Flow-based purification of GFP[−] and GFP[+] subsets. GFP[−] and GFP^{+} cells from LT-HSC or MPP1 compartments of Notch-eGFP-*Fanca*^{−/−} or Notch-eGFP-WT mice were sorted. Representative images (Left) and histogram overlay (Right) were shown. GFP[−] and GFP[+] subsets were then subjected to CFU assay (B) and BMT (C). (B) Increased Notch signaling results in replating defect in *Fanca*^{−/−} MPPs. 20 LT-HSCs or 200 MPP1 cells were seeded in duplicated and were cultured in cytokine-supplemented methylcellulose medium (MethoCult 3434). Subsequently, colonies were counted on day 7, then were isolated, replated and cultured for another 7 days. Error bars indicate standard deviation (STD) of three experiments (n=9). (C) Increased Notch signaling leads to self-renew defects in

Fanca$^{-/-}$ HSCs. 50 LT-HSCs or 1000 MPP1 (CD45.2$^+$) cells plus 0.3 million cells (CD45.1$^+$) were tail vein injected to lethally irradiated recipient (BoyJ) mice. Donor-derived chimera was assessed by Flow Cytometry analysis at 4, 8, 16 and 24 weeks after transplantation. (D) Quantification of self-renewal capacity of *Fanca*$^{-/-}$ HSCs. Experiments described in (C) were quantified. Results are means plus or minus SD of 3 independent experiments (n=9 per group).
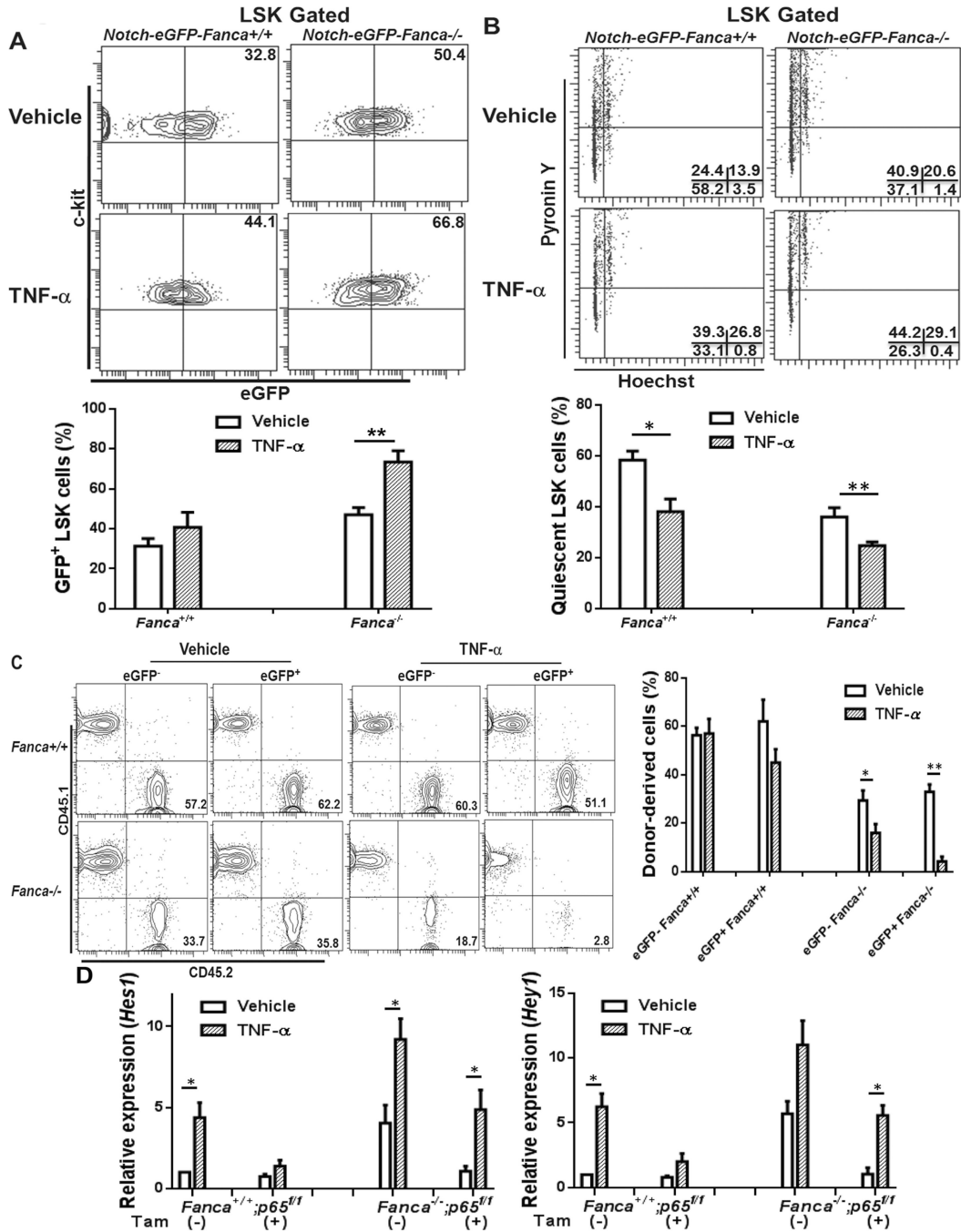
Fig 5. TNF- activates Notch signaling in HSCs

(A) Enhanced Notch signaling in *Fanca–/–* HSPCs by TNF- . Notch-eGFP-*Fanca$^{-/-}$* or Notch-eGFP-WT mice were treated with vehicle or TNF- (single injection; 100 μg/kg). Twenty-four hours later, WBMCs (whole bone marrow cells) were isolated and subjected to Flow Cytometry analysis for GFP expression in gated LSK cells. Representative flow graphs (Left) and quantification (Right) were shown. Results are means plus or minus SD of three experiments (n=9 per group). (B) TNF- further decreases HSC quiescence in *Fanca$^{-/-}$* mice. Cells described in (A) were subjected to cell cycle analysis using Hochest 33342/ Pyronin Y staining. Representative flow graphs (Left) and quantification (Right) were shown. Results are means plus or minus SD of three independent experiments (n=9 per

group). (C) Stress-activated Notch signaling compromises HSC self-renew. 500 GFP$^-$ or GFP$^+$ LSK cells (CD45.2$^+$) isolated from the mice treated with TNF- along with 0.3 million cogenic WT BM cells (CD45.1$^+$) were transplanted into lethally irradiated recipients and determined donor-derived chimera 24 weeks post BMT. Representative flow graphs (Upper) and quantification (Lower) were shown. Results are means plus or minus SD of three independent experiments (n=6 per group). (D) Inactivation of *p65* resulted in attenuation of *Hes1* and *Hey1* expression in TNF- -treated *Fanca*$^{-/-}$ mice. LSK cells from TNF- -treated *Fanca*$^{-/-}$*p65*$^{f/f}$ or *Fanca*$^{+/+}$*p65*$^{f/f}$*CreER* mice (plus or minus Tamoxifen injection for 3 days) were isolated for RNA extraction followed by 1st cDNA synthesis. cDNA were then subjected for Real time-PCR using primers for *Hes1* or *Hey1*. The results were plotted after normalization with *GAPDH* as internal control. Data are shown as mean plus or minus SD of 3 independent experiments.
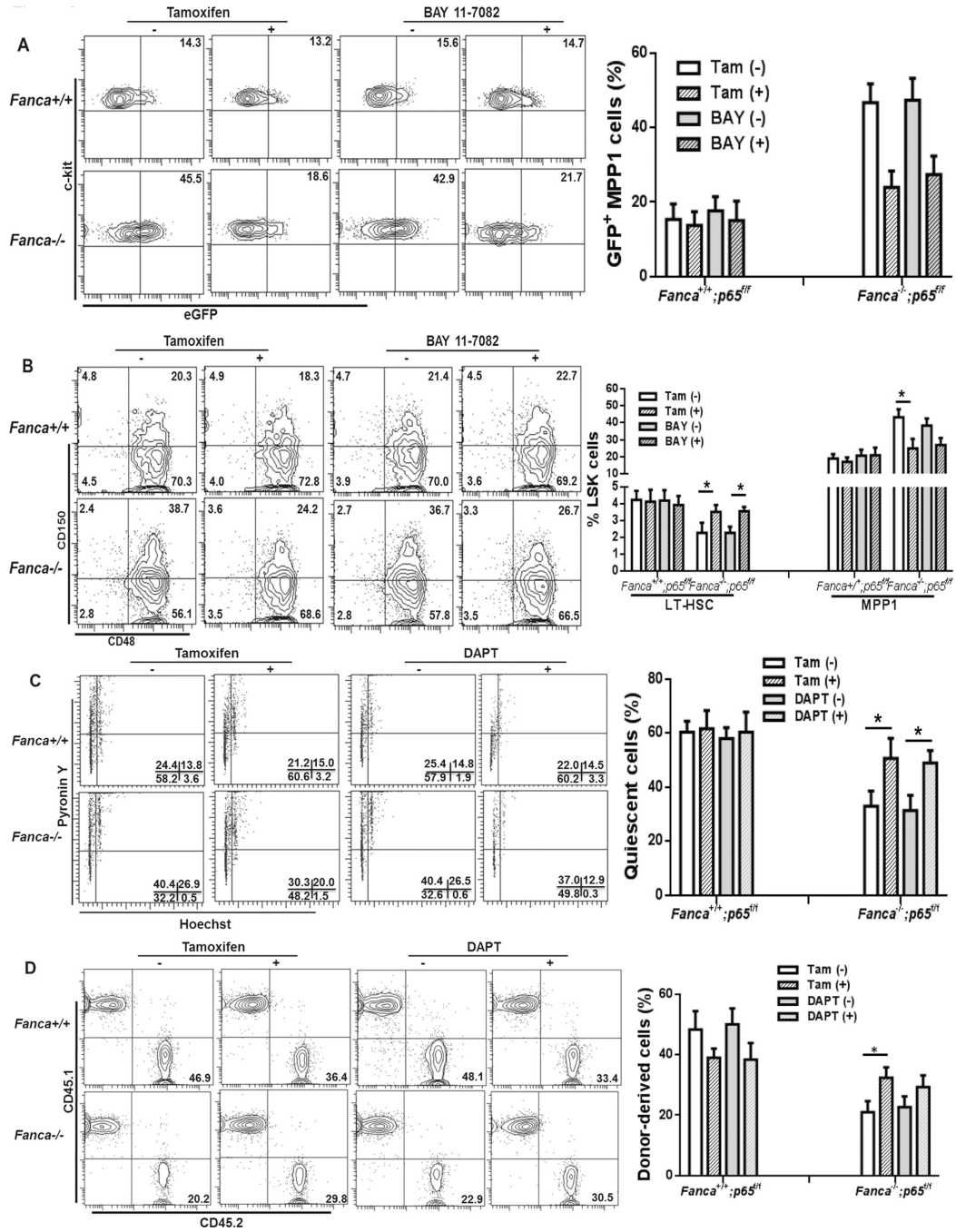
Fig 6. Inhibition of NF-KB partially rescues FA HSC phenotype

(A) Inhibition of NF-κB reduces Notch-eGFP expression in *Fanca*^{−/−} MPP1 to nearly WT level. WBMCs from genetic ablation (3 days vehicle or Tamoxifen-injected Notch-eGFP-*Fanca*^{+/+}*p65*[f/f]-*CreER* or Notch-eGFP-*Fanca*^{−/−}*p65*[f/f]*CreER* mice) or pharmacologic inhibition (BAY 11-7082-treated Notch-eGFP-*Fanca*^{+/+} or Notch-eGFP-*Fanca*^{−/−} mice) of NF-κB were isolated for Flow cytometry analysis. MPP1 cells were gated for GFP expression. Representative flow graphs (Upper) and quantification (Lower) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group). (B) Inhibition of NF-κB increases LT-HSCs and decreases MPP1 in *Fanca*^{−/−} mice. Cells described in (A) were subjected to SLAM analysis using Flow Cytometry. Representative

flow graphs (Lower) and quantification (Upper) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group). (C) Inhibition of NF-κB or Notch partially restores HSPC quiescence. BMCs from genetic ablation (3 days vehicle or Tamoxifen-injected Notch-eGFP-*Fanca*^{+/+} *p65*[f/f]*CreER* or Notch-eGFP-*Fanca*^{−/−} *p65*[f/f]*CreER* mice) or DAPT-treated Notch-eGFP-*Fanca*^{+/+} or Notch-eGFP-*Fanca*^{−/−} mice were isolated for cell cycle analysis. Representative flow graphs (Upper) and quantification (Lower) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group). (D) Inhibition of NF-κB or Notch partially restores HSPC self-renewal capacity. 500 LSK cells (CD45.2[+]) from mice described in (C) along with 0.3 million cogeneic WT BM cells (CD45.1[+]) were transplanted to lethally irradiated recipients. Donor-derived chimerism was determined 24 weeks after BMT. Representative flow graphs (Lower) and quantification (Upper) were shown. Results are means plus or minus SD of 3 independent experiments (n=9 per group).

Table 1

Primers used for Real-time PCR

Symbol	Forward primer	Reverse primer
Gadd45b	CTCCTGGTCACGAACTGTCA	GGGTAGGGTAGCCTTTGAGG
Rela	GCGTACACATTCTGGGGAGT	ACCGAAGCAGGAGCTATCAA
Sod2	GCCCCCTGAGTTGTTGAATA	AGACAGGCAAGGCTCTACCA
Tnfrsf1b	TGGCAGAGGAGCCTAGTTGT	CACACCCAGGAACAGTCCTT
Stat1	TGGTGAAATTGCAAGAGCTG	CAGACTTCCGTTGGTGGATT
Xiap	TTGGAACATGGACATCCTCA	TGCCCCTTCTCATCCAATAG
Hes1	CCCACCTCTCTCTTCTGACG	AGGCGCAATCCAATATGAAC
Hey1	AGCAGTGAGGTGAAGGGAGA	AACGGTGAAATCCGTGAGAC
Hoxb4	CTGGATCACAGCCTGGATTT	TGGAAGGGAGTGAGCAGTCT
Fzd1	CAAGGTTTACGGGCTCATGT	GTAACAGCCGGACAGGAAAA
Notch2	CCTGAACGGGCAGTACATTT	GCGTAGCCCTTCAGACACTC
Numb	CGGGAAAGAAAGCAGTGAAG	AGTGGTGCCATCACGACATA
GAPDH	AACI I IGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Table 2

Increased expression of genes involved in the NF- B pathway and Notch signaling pathway.

Pathway specific Array	Symbol	Accession #	Fold Up-Regulation
NF- B signaling Pathway	Ccr5	NM_009917	3.04
	Gadd45b	NM_008655	8.79
	Icam1	NM_010493	6.26
	Irf1	NM_008390	4.84
	Rela	NM_009045	6.17
	Sod2	NM_013671	9.10
	Stat1	NM_009283	9.96
	Tnfrsf1b	NM_011610	5.37
	Xiap	NM_009688	8.91
Notch Signaling Pathway	Fzd1	NM_021457	5.71
	Fzd2	NM_020510	4.27
	Hes1	NM_00823S	5.79
	Hey1	NM_010423	5.79
	Hoxb4	NM_010459	3.79
	Nfkb1	NM_008689	3.59
	Notch1	NM_008714	6.52
	Notch2	NM_010928	9.81
	Numb	NM_010949	3.28
	Pdpk1	NM_011062	3.52