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The Notch Delta-4 ligand helps to maintain the quiescence and the short-term reconstitutive potential of Haematopoietic Progenitor Cells through activation of a key gene network.

Running Title: Delta-4/Notch pathway retains the HPCs potential.

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Abbreviations: HPCs, haematopoietic progenitor cells; Dll4, Delta4 ligand; LSK, $Lin^{-}Sca^{+}c\text{-}Kit^{+}$

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Summary (141)

Understanding the role of Notch and its ligands within the different bone marrow niches could shed light on the mechanisms regulating haematopoietic progenitor cells (HPCs) maintenance and self renewal. Here, we report that murine bone marrow HPCs activation by the vascular Notch Delta4 ligand maintains a significant proportion of cells specifically in the G0 state. Furthermore, Delta4/Notch pathway limits significantly the loss of the *in vivo* short-term reconstitutive potential upon transplantation of Delta-4 activated HPCs into lethally irradiated recipient mice. Both effects are directly correlated with the decrease of cell cycle genes transcription such as *CYCLIN-D1*, *-D2*, and *-D3*, and the upregulation of stemness related genes transcription such as *BMI1*, *GATA2*, *HOXB4* and *C-MYC*. In addition, the transcriptional screening also highlights new downstream post-transcriptional factors, named *PUMILIO1* and *-2*, as part of the stem signature associated with the Delta4/Notch signalling pathway.

Keywords: Notch; Cell cycle; Haematopoietic Progenitor Cells, Dll4 protein, Pumilio

INTRODUCTION

Haematopoietic Progenitor cells (HPCs) reside predominantly in the bone marrow (BM), where blood cell development occurs. The BM microenvironment is a complex system comprising nonhaematopoietic components such as bone-forming cells including mesenchymal stem cells and osteoblasts, fibroblasts, adipocytes, innervation from the sympathetic nervous system, reticular cells, pericytes, and endothelial cells [1]. It has long been recognized that nonhaematopoietic BM-derived stroma cells are capable of supporting long-term haematopoiesis *in vivo* and *in vitro* [2] and understanding the precise mechanism by which niche cells interact to regulate HPCs is critical for the development and improvement of stem cell therapies based on HPC manipulation and administration.

The initial discovery that functional alterations in osteoblastic cells had consequences on HPC function attracted the interest of researchers in this field [3] [4]. A growing number of studies have subsequently implicated bone marrow vasculature, which consists of a vast network of thin-walled, fenestrated sinusoidal endothelial cells and perivascular stromal cells as well as small arterioles, in providing the proper milieu of prohaematopoietic factors needed to support HPCs pool [5] [6].

The role of Notch signalling in adult vertebrate HPCs maintenance remained controversial, largely because gain- and loss-of-function studies have not produced consistent results. It has been suggested to be dispensable for adult haematopoiesis through two complementary approaches blocking the canonical Notch signalling within HPCs (one with a dominant negative form of the mastermind-like protein, and the other inactivating the *RBP-J* gene) [7] [8]. However, recent studies support a role for Notch in the regeneration of HPCs after injury [5] [9] and more recently, a role of endothelial Jagged-1 was highlighted for homeostatic and regenerative

haematopoiesis [10]. The Notch ligand Dll4 (Dll4) (Delta like 4, mbDll4) is specifically expressed on endothelial cells of the arteries, arterioles and capillaries [11] [12] and also on the perivascular stromal cells, and pericytes [13]. The specific expression of Dll4 in the vascular niche, known to exert an essential role in HPCs maintenance, attracted our interest for the role of the Dll4/Notch pathway in the bone marrow HPCs homeostasis. We have previously reported that the membrane-bound Notch ligand Dll4 (mbDll4) counteracts the proliferation of human cord blood $CD34^+$ cells induced by cytokines and preserves a high LTC-IC potential in output $CD34^+$ cells, even in cells having performed a similar number of divisions, indicating that LTC-IC retention was mediated by mechanisms independent of the mitotic history [14] [15].

Here we report that Dll4 counteracts the proliferation of murine HPCs such as $Lin^-Sca^+c\text{-}Kit^+$ (LSK) cells, by keeping a higher fraction of cells in the G0 state. This event was correlated with a downmodulation of some key cell cycle genes transcription such as *D-CYCLINS*. Furthermore, Dll4 limits the loss of the short-term reconstitutive potential, as assessed by *in vivo* reconstitution assay, correlated with up-regulation of well-known self-renewal genes transcription such as *BMI1*, *HOXB4*, *GATA2* and *C-MYC* as well as *PUMILIO1* and *-2*, two RNA-binding proteins previously identified as regulators involved in stem cell proliferation in invertebrates. Altogether, these data reinforce the major role of the Notch ligand Dll4 in the vascular niche as a new player in HPCs maintenance.

MATERIALS AND METHODS

Mice

C57BL/6-Ly5.2, C57Bl/6-Ly5.1 (8-20 weeks old) were purchased from Janvier CERJ (Le Genest-St-Isle, France). All mice were maintained in the Gustave-Roussy Institute facilities under specific pathogen-free conditions.

Cytometric analysis.

Lineage negative Sca-1 and c-Kit positive (LSK) cell purification was performed as described by Spangrude and colleagues [16]. In some experiments, Lin^- and LSK cells were analysed using biotinylated mAbs raised against differentiation markers (Gr-1 clone RB6-8C5, Mac-1 clone M1/70, B220 clone RA3-6B2, CD3 clone 145-2C11 and Ter-119, mouse lineage panel, Becton Dickinson), revealed using streptavidin molecules, and stained with anti–Sca-1 mAb, (clone E13-161.7) anti–c-Kit mAb (clone 2B8), and anti-CD150 mAb (clone TC15-12F12.2). Biotinylated CD41 (clone ebioscienceMWReg30) and CD48 (HM48-1) mAbs were added in the lin cocktail to analyse the SLAM phenotype. CD44 (clone 515) and CD25 (clone M-A2-51) mAbs were used to phenotype lymphoid precursors. Notch receptor expression was performed using anti-Notch 1 (clone HMN-12), anti-Notch 2 (clone HMN2-35) and anti-Notch 4 (clone HMN4-14) antibodies (Biolegend). Isotype-matched antibodies were used as controls. Sorting of the LSK fraction was performed using a FACSvantage or an Aria III (Becton-Dickinson).

Culture experiments

Each of the S17 stroma cells (C/S17 and mbDll4/S17) were obtained and cultured as previously described [14]. LSK cells were cultured in 24-well plates coated with confluent C/S17 or

mbDll4/S17 in α-MEM containing 10% FCS (Stem Cell Technology, Vancouver, Canada). The following cytokines were added: murine Stem Cell Factor [muSCF], human Flt-3 ligand [HuFlt3-L], human interleukin-6 [IL-6], each at 100 ng/mL, and human interleukin-11 [IL-11] at 10 ng/mL. Some LSK cells were cultured on immobilized Dll4Fc (10 µg/ml) on plastic as previously described (Lauret et al., 2004), irrelevant human IgG1 served as a negative control. Some cultures were performed in the presence of 10mM DAPT (a gamma-secretase inhibitor: N-{3,5-difluorophenacetyl)-L-alanyl}-S-phenylglycine-t-bytyl ester; Calbiochem, San Diego) or DMSO as vehicle.

Cell-cycle analysis

Carboxyfluorescein Succinimidyl Ester (CFSE) Staining. Before coculture, sorted LSK cells were incubated with CFSE 1 µM for 10 minutes at 37°C allowing protein staining. Cell divisions were analysed on a FACSort cytometer after two days in culture (Becton Dickinson). *Ki-67 analysis*. Ki67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle (G1, S, G2 and M phases) but absent in resting cells (G0). Cells were labelled with APC-anti-CD45 mAb, incubated with a cytofix-cytoperm solution (Becton Dickinson), washed with a permwash solution (Becton Dickinson), and finally incubated with FITC-anti-Ki-67 mAb and DAPI before LSR cytometer analysis (Becton Dickinson). *Pyronin/Hoechst analysis*. FITC-anti-CD45 mAb labelled cells were incubated with 10 µg/ml Hoechst 33342 and 1 µg/ml Pyronin Y (RNA dye) (Sigma Chemical Co, St Louis, MO) at 37°C for 45 minutes in Hanks balanced salt solution (HBSS) medium supplemented with 2mM Hepes, 10% FBS and 1mg/l glucose [17].

Long-Term Culture-Initiating Cell (LTC-IC) and CFC assay

Long-term culture-initiating cell (LTC-IC) assay was performed as described by Sutherland and colleagues [18]. The cultures were recovered after 5 weeks and assayed for the presence of myeloid culture colony-forming unit (CFU-C) using a methylcellulose medium (M3234; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with cytokines. After 7 to 10 days of growth at 37°C, colonies were scored with an inverted microscope based on their morphology.

Long-term competitive repopulation assay

C57Bl/6 (Ly5.2) mice were used as recipients, whereas LSK cells were prepared from C57Bl/6 (Ly5.1) donors. Lethally irradiated recipients were cotransplanted with 1.5 x 10^5 Ly5.2 BM cells together with various amounts of cells exposed to S17 stroma or immobilized Dll4Fc protein for 7 days, injected into the retro-orbital sinus of mice. Haematopoietic reconstitution was assessed at 4 and 16 weeks after transplantation through analysis of blood or bone marrow respectively. Analysis of the presence of $CD45.1^+$ cells in blood and bone marrow of reconstituted mice at 4 and 16 weeks after transplant was performed using flow cytometry with a cut-off 0.1% $CD45.1^+$ (donor) cells for at least 10^5 analysed cells (FACS Sort). Animals displaying greater than 0.1% of $CD45.1^+$ positive cells were considered as positive for repopulation.

Microarray

Total RNAs from sorted cell populations were isolated using the RNAplus kit (Qbiogene) and further amplified using the MessageAmp aRNA kit (Ambion, Austin, TX). The integrity of the RNA samples was verified using an Agilent Bioanalyzer (Palo Alto, CA). The cDNAs were labelled and hybridized to the murine microarray manufactured in CEA microarray platform as described [19]. Slides were scanned with a Genepix 4000 microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA). Images were processed to acquire Cy5 and Cy3 fluorescence

intensities for each hybridized spot using Genepix Pro 4.0 software (Axon Instruments). Spots or areas of the array with obvious blemishes were flagged and excluded from subsequent analysis. Result files were imported into GeneSpring 6.1 software (Silicon Genetics, Agilent) for further analyses. To eliminate dye-related artefacts in 2-color experiments, intensity-dependent Lowess normalization was performed. The results represent the average of 8 independent measures and were deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with the GSE 5135 accession number. Differentially expressed genes were obtained by analysis of variance using ANOVA parametric test (P<0.01) followed by Benjamini and Hochberg multiple test correction. For stringent comparisons, only probes with a hybridization value in at least 6 of 8 replicates were considered for further analysis.

Quantitative reverse-transcriptase (RT)-PCR

Total RNA was extracted from cells using the Trizol reagent (InVitrogen). Synthesis of first-strand cDNAs was performed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using SYBRGreen PCR Master Mix (PE Applied Biosystems, Foster City, CA). PCR was monitored with a GeneAmp 5700 sequence detection system (PE Applied Biosystems). Quantification was performed using the C_t values method. The primers used are indexed in Supplementary data Table S1.

Statistical Analysis

All measurements were performed at least three times. Continuous variables were analysed using Student's t-test for paired values. The measured values were expressed as mean ± SEM. p-values of less than 0.05 were considered to be statistically significant.

RESULTS

Dll4 maintains a fraction of HPCs out of the cycle

We first examined Notch receptor expression on murine bone marrow HPCs. The majority of LSK cells expressed the Notch 2 receptor (79.7±1.8 %), while few of them expressed Notch 1 (29.1±9.3 %) and Notch 4 (13.9±3.0 %) (Figure S1). Our data confirmed the major role of Notch 2 in governing the rate of generation of mouse long- and short-term repopulating stem cells, as previously described [9, 20]. To assess mbDll4 activities on murine HPCs, LSK cells were cultivated with cytokines on control (C) and mbDll4 stromas (mbDll4). After a 7-day culture, the fold increase in total cell number was significantly reduced (1200±90 onto C versus 600±72 onto mbDll4, $p<0.05$, Figure 1A), indicating that mbDll4 reduced total cell expansion. Apoptosis analysis did not revealed any difference in the percentage of apoptotic cells between C and mbDll4 population neither at day 2 (12±5 % for C versus 13±4 % for mbDll4 culture) nor at 7 days of culture (data not shown), thus precluding a potential role of apoptosis in the reduction of cell expansion upon mbDll4 stimulation.

Labeling of cells with Carboxyfluorescein Diacetate Succinimidyl Ester to simultaneously monitor cell divisions at 2 days of culture when both populations were homogenous, still displaying 90% Lin^- cells (data not shown), showed that the proportion of cells not dividing or dividing once was significantly higher in the population exposed to mbDll4 as compared to C (3±0 % for C versus 6±0 % for mbDll4 culture at 0 division, $p=0.02$ and 15±0 % for C versus 19±0 % for mbDll4 culture at one division, $p<0.05$), while the proportion of cells having performed 4 divisions was lower in the population exposed to mbDll4, as compared to cells grown on C (18±2 % for C versus 12±2 % for mbDll4 culture, $p<0.05$, Figure 1B).

To precisely decrypt the modifications in the cell cycle and separate the G0 and G1 states, we used Ki-67/DAPI staining to estimate the frequencies of G0 ($Ki67^-$; 2N DNA content), G1 ($Ki-67^+$; 2N DNA content), and S/G2/M ($Ki-67^+$; >2N DNA content). According to these markers, we found a higher proportion of LSK cells exposed to mbDll4 in the G0 state (7±1 % for LSK exposed to C versus 13±2 % for LSK cells exposed to mbDll4 culture, $p<0.05$), with a significant modification in the proportion of cells in S/G2/M (47±2 % for C versus 37±3 % for mbDll4, $p<0.05$, Figure 1C). These modifications in the cell cycle were confirmed by Pyronin-Y (PY) and Hoechst 33342 (Ho) staining (Figure 1D). These data showed that mbDll4 counteracts the stimulatory proliferative effects of cytokines by maintaining out of the cycle a higher fraction of LSK cells.

Dll4 downmodulates expression of D-cyclins

To identify potential Dll4-dependent genes implicated in the cell cycle, we used a chimeric Dll4Fc protein for a stimulation set up independent of the stroma cell context. We first confirmed the antiproliferative activity of Dll4Fc on LSK cells. When cultured on immobilized Dll4Fc, HPCs displayed a reduced cell expansion as compared to control populations at day 7 of the culture (Figure S2A). Furthermore, analysis at day 2 of RNA/DNA contents by Pyronin-Y (PY) and Hoechst 33342 (Ho) staining revealed a higher proportion of cells in the G0 state (6±2 % for C versus 14±5 % for Dll4Fc, $p<0.05$, Figure S2B), with a significant modification in the proportion of cells in S/G2/M (49±3 % for LSK cells exposed to C versus 37±6 % for LSK cells exposed to Dll4Fc, $p<0.05$) (Figure S2B), as previously showed for mbDll4. Next, we assessed the expression of some key downstream cell cycle target genes 12h after exposure to Dll4Fc (before their first division). As shown in Figure 2, we observed a transcriptional upregulation of *p130* (1.33±0.11, p=0.02), *E2F4* (2.4±0.18, p=0.02) and *Rb* (2.7±0.41, p=0.04) genes and a

downregulation of *CYCLIN-D1* (0.26 ±0.01, p=0.02), *CYCLIN-D2* (0.27±0.02, p=0.03) and *CYCLIN-D3* genes (0.38±0.05, p=0.04) after exposure to Dll4Fc. In contrast, the level of transcriptional expression of $p18^{INK4c}$, $p19^{INK4d}$, $p21^{Cip1/Waf1}$, $p27^{Kip1}$, $p57^{Kip2}$, *cdk1, cdk4, cdk6, Dmtf1, p107* and *CYCLIN-E1, CYCLIN-E2* genes were not modified after exposure of HPCs to Dll4Fc (data not shown).

Dll4 limits the loss of HPCs primitive potential

We also examined the effects of mbDll4 on the LSK cells by following the proportion of LSK cells throughout a 7-day culture on C and mbDll4 stromas. A progressive decline in the % of LSK cells on the C stroma compared to the mbDll4 stroma became significant at day 2, and this difference increased progressively to reach 5-fold more LSK cells on mbDll4 stroma at day 7 (7±4% for C versus 38±0% on mbDll4, $p<0.05$, Figure 3A and B). We did not notice any significant difference in the proportion of the different lineages among Lin^+ cells, indicating that in our culture conditions, mbDll4 did not favour differentiation toward a particular myeloid lineage (Figure S3A). Nevertheless, we noticed at day 7 the appearance of a small number of $CD44^+/CD25^+$ (markers of early T cell differentiation) cells upon mbDll4 stimulation, as observed in previous studies (Figure S3B)[21].

To further characterize the primitive potential of cells generated after 7 days of culture on both stromas, we monitored their *in vitro* CFC and LTC-IC as well as their *in vivo* repopulating activity. Cells cultured on mbDll4 maintained a higher proportion of clonogenic colonies (48±8 CFC per 500 Lin^- on C versus 68±20 CFC per 500 output Lin^- cells on mbDll4, $p<0.05$, Figure 3C). The increased CFC number was the result of a general increase in all types of colonies (CFU-GM, BFU-E, and mixed colonies). Furthermore, colonies generated on mbDll4 displayed a

larger size, as compared to those derived from Lin^- cells on C (data not shown). The LTC-IC potential of output LSK cells generated on mbDll4 was preserved at day 7, compared to the one of input bone marrow LSK cells (18±4 LTC-IC-derived CFC/1000 for output LSK versus 24±0.5 for 1000 output LSK cells from mbDll4 culture), while this potential was strongly impaired in output LSK cells generated onto C (1.9±0.1 LTC-IC-derived CFC/1000 output LSK cells, p=0.03, Figure 3D). This leads to a strongly enhanced expansion of LTC-IC-derived CFC (251±7 LTC-IC-derived CFC for C versus 4825±110 LTC-IC-derived CFC for mbDll4 from 1,000 input LSK cells, $p<0.05$, Figure 3E).

The *in vivo* repopulating activity was assessed by examining the short-term reconstitution in the peripheral blood (4 weeks), and the long-term in the bone marrow (> 16 weeks) (Figure 4 and Figure S4). To cast off the expansion difference between control and mbDll4 cultures, we injected all the progeny of Ly5.1 LSK cells (ranging from 10 to 200 LSK seeded cells) grown for 7 days on C and mbDll4, independently on the number of cells grown in these two conditions, together with a fixed number of Ly5.2 bone marrow competitor cells into irradiated Ly5.2 recipients. While no chimerism was detected in mice infused with cells grown onto C stroma at 4 or 16 weeks, 14 to 40% of mice were chimeric at 4 and 16 weeks with cells grown onto mbDll4 stroma (Figure 4A), with a chimerism dependent on the amount of cells injected (from 0.06±0.03 for 10 LSK cells to 2.2±0.9 for 200 LSK cells, chimerism at 4 weeks with cells grown onto mbDll4 stroma, Figure 4B). When analysed at 16 weeks post-transplantation, low levels of bone marrow cell chimerism (ranging from 0.1 to 1%, data not shown) were observed in mice injected with the progeny of 10 to 200 LSK cells cultivated onto mbDll4, while no chimerism could be detected for mice injected with the progeny of control LSK cells. These data indicated that exposure to mbDll4 limits significantly the loss of the *in vivo* reconstitutive potential at least in short term analysis.

To confirm the implication of Notch signalling in mbDll4 activity, we analysed the requirement of the γ-secretase complex, which is an essential effector in the Notch signalling pathway. When LSK cells were cultured on mbDll4 for 7 days in the presence of DAPT (an inhibitor of the γ-secretase), cell expansion was restored (857±88 for cells onto mbDll4 in the presence of DAPT versus 298±48 for cells cultured on mbDll4 with DMSO, Figure S5A), and the maintenance of a high proportion of LSK cells on mbDll4 was abrogated (4±1 for cells onto mbDll4 in the presence of DAPT versus 26±5 % for cells cultured onto mbDll4 with DMSO, respectively, Figure S5B).

Identification of downstream target genes modulated by Dll4

To reveal new signalling pathways involved in the effects of Dll4 on HPCs, we performed a differential transcriptomic analysis of HPCs cultured for 6 or 12 h with or without Dll4Fc. First, we confirmed the similarity of the HPCs response between immobilized Dll4Fc and mbDll4. HPCs cultured for 7 days on immobilized Dll4Fc maintained a higher proportion of LSK cells (5±2 for C versus 22±7 % for Dll4Fc culture, $p<0.05$, Figure S6A), retained a primitive potential, evidenced by an LTC-IC potential identical to the input cells, leading to an enhanced expansion of LTC-IC-derived CFC (16±3 for C versus 323±19 for Dll4Fc culture, $p<0.05$, Figure S6B), and a higher *in vivo* reconstitution potential when inoculated into irradiated mice (78% of chimeric mice at 16 weeks post-transplant for Dll4Fc cultures versus 12% of chimeric mice for control cultures, Figure S6C).

Using the ANOVA parametric test ($p<0.01$) and Benjamini and Hochberg multiple testing correction, we found 559 modulated probes for Dll4Fc cultures and 64 modulated probes for control cultures after 12 hours of culture versus non-cultured LSK cells. To provide a biological

interpretation of these gene expression modifications, we looked for statistical significance of the enrichment of particular functional categories using Ingenuity pathways analysis (IPA) and DAVID resources (http://david.abcc.ncifcrf.gov/home.jsp), focusing particularly on Gene Ontology (GO) Biological Process terms. We found that most of the genes were included in three significant GO categories: "Cell Growth and Proliferation", "Cell Cycle Process" and "Translation Process" (Figure S7). Very few genes were modulated at 6hr (data not shown). Interestingly, one of the upregulated genes at this early time was *HOXB4* (1.8±0.4, p=0.01). Changes in gene expression observed at 12h were confirmed by RT-qPCR on a series of selected genes encompassing all the scale of modulations (Figure 5), validating thus the microarray data. Genes such as *GATA2* (2.4±0.4, p=0.04), *HES1* (4.7±1.8, p=0.01), *BMI1* (5.1±2.3, p=0.01), *EED* (1.7±0.2, p=0.002), and *C-MYC* (13.9±1.5, p=0.007) were already known to be involved in stem cell self-renewal [22-28]. The chaperone HSP90 protein (1.4±0.1, p=0.01), and the transcription factor MNT (6.9±1.2, p=0.03) were not previously identified as involved in this process. In addition, the post-transcriptional repressor gene *PUMILIO2* (*PUM2*) identified in invertebrates and previously described in other models to be involved in stem cell fate or expansion [29, 30], was also found highly upregulated (6.8±1.1, p=0.04). Therefore, we measured the transcription levels of *PUMILIO1* (*PUM1*) (absent in the microarray) by RT-qPCR. The results show that *PUM1* transcription was also upregulated in response to Dll4 (2.9±0.7, p=0.04), suggesting that both mammalian PUMILIO proteins might potentially play an important role in Notch-ligand Dl14-dependent cell development and differentiation. We also confirmed the down regulation of Osteopontin (OPN) (0.3±0.04, p=0.01) and the CCL12 chemokine (0.3±0.06, p=0.04).

DISCUSSION

In accordance with our previous work performed on human $CD34^+$ $CD38^{low}$ cord blood cells [14]), mbDll4 reduces murine bone marrow HPCs cell expansion correlated with the retention of their primitive potential *in vitro*. The present study further reveals that the reduced cell proliferation was mainly due to the maintenance of a higher proportion of HPCs in the G0 state. It also shows that cell culture on mbDll4-expressing stroma limits the loss of their short term repopulating capacity *in vivo*, and finally leads to the identification of the gene signature of HPCs in response to Notch/Dll4 signalling.

Maintenance of a higher proportion of LSK cells out of the cycle

The vascular niche has been demonstrated to maintain HPCs *in vivo* [5]. Endothelial and perivascular cells play a key role as main producers of SCF [31], and CCL12 [32] in the bone marrow. Furthermore, both of them support HPCs maintenance *ex vivo* [5]; [20]. The maintenance of quiescence is also a key feature for regulating HPCs homeostasis. Using the signalling lymphocyte attractant molecule (SLAM) family markers to identify quiescent HPCs, several studies report that most quiescent HPCs are localized adjacent to blood vessels in the bone marrow [31, 33, 34]. Only a few of the external factors governing HSCs quiescence in the vascular niche have been identified among which Tie2/Angiopoietin-1 play a major role [35]. Dll4 has been shown to be expressed in endothelial and perivascular cells [13]; [36]. We have previously shown that the exposure of human $CD34^+$ cells to mbDll4 maintains a high proportion of cells in the G0/G1 phase without distinguishing the G0 phase from the G1 phase. The present data further revealed a higher proportion of HPCs in the G0 phase, in spite of our cell culture conditions based on strong cytokine activation, suggesting that Dll4-expressing cells participate

to the maintenance of HPCs out of the cell cycle. To our knowledge, Dll4 is the only Notch ligand described as keeping HPCs out of the cycle.

To get insight into the mechanisms of Dll4 activity, we examined the transcriptional modulation of some cell cycle genes. The D-cyclin family, *CYCLIN-D1*, *CYCLIN-D2*, and *CYCLIN-D3* genes were all transcriptional expressed, albeit at different levels, in HPCs [37]. *CYCLIN D1*$^{-/-}$*D2*$^{-/-}$*D3*$^{-/-}$ embryos displayed a reduced numbers of fetal HPCs, with impairment in their ability to proliferate, revealing a unique requirement for the D-CYCLINS in the haematopoietic lineage [38]. The transcriptional downmodulation of the 3 *D-CYCLINS* genes observed in response to Dll4 is therefore in complete agreement with the key role of D-CYCLINS in HPCs proliferation. E2F4, which can bind to all 3 pRB family members to form the majority of cellular pRB family complexes, has been proposed to play a critical role in coordinating cell cycle exit [39]. The retinoblastoma (Rb) family of transcriptional repressors, including the pRb, p107 and p130 proteins, restricts cell cycle entry by regulating E2F gene transcription of positive cell cycle regulators. Conditional deletion of all three Rb family members in adult mice resulted in a robust cell-intrinsic myeloproliferation phenotype, with an increase in HPCs proliferation, and severe defects in self-renewal [40]. Therefore, the transcriptional upregulation of *E2F4* and *Rb* genes in response to Dll4 is consistent with their role in the cell cycle. Taken together, these findings indicate that Cyclins D and Rb family members may play a critical role in the activity of the Dll4/Notch pathway for the maintenance of the quiescent state.

mbDll4 limits the loss of HPC potential *in vitro* and *in vivo*

Exposure of LSK cells to mbDll4, not only preserves their *in vitro* CFC and LTC-IC potential, but also limits the loss of the *in vivo* short-term reconstitutive potential, as compared to LSK cells cultured in control conditions. A chimeric Delta1Fc (Dll1Fc), immobilized on the plastic, thus

mimicking the membrane-bound form, was previously used, showing an improvement of the long-term reconstitutive potential of BM LSK cells or human cord blood $CD34^+$ cells cultivated with Dll1 [41]; [42]. Exposure of LSK cells to mbDll4 showed a limited *in vivo* long-term reconstitutive potential, despite preserving the *in vitro* CFC and LTC-IC potential. We suggest that Dll4 contributes to the short-term bone marrow reconstitution, thus allowing rapid recovery of homeostasis in response to stress.

Exposure of LSK cells to Dll4 was correlated with an enhanced transcriptional expression of *HES1*, the well-known Notch target gene. A recent study suggests that γSE complex/Notch signalling controls early HPC commitment decisions in bone marrow, partly through the Hes family of transcriptional repressors [43]. Furthermore, overexpression of HES1 in $LSKCD34^-$ cells leads *in vivo* to an accumulation of the primitive SP cells and $LSKCD34^-$ cells in marrow of recipient mice, while maintaining the production of the differentiated blood cells [24]. However, its role on the cell cycle is still controversial: an overexpression of HES1 in human $CD34^+$ cells reduced their proliferation [44], while a comparable study led to an increase in the incorporation of BrdU in HES1-overexpressing cells, with a reduction in apoptosis and an increase in their capacity to expand in NOD-SCID mice [45]. Different levels of HES1 expression may be at the origin of these contrasting effects.

To identify the downstream mechanisms involved in Dll4 activity, we have compared the transcriptional response of HPCs exposed to Dll4 to control HPCs. Our comparative study of the transcriptome has provided us with a skeleton of an answer by revealed upregulation of some genes already identified to be implicated in the regulation of HPC self-renewal such as *BMI1* [25], *HOXB4* [22], *EED* [26], *GATA2* [23] and *C-MYC* [27]; [28]. Transcriptional screening has also highlighted new up-regulated candidate genes, *PUMILIO1* and *-2*, downstream of the

Dll4/Notch signalling pathway. Among the post-transcriptional regulators of stem cells, PUM proteins, which belong to the evolutionary highly conserved family of PUF proteins, have been shown to be central for the maintenance of germinal and somatic stem cells in invertebrate. They are able to bind specific motifs mainly present in the 3'-untranslated region (3'UTR) of target mRNAs, thus favoring sets of mRNA's to be degraded or kept untranslated. The fact that PUF genes are targets of Notch signalling in HPC was previously observed in C. elegans germline stem cells, the expression of the *FBF2* gene being under the control of GLP-1/Notch signalling [46]. Activation of *PUF* genes by Notch signalling in both C. elegans germ stem cells and murine HPCs enforces the link between these two major mechanisms regulating stem cell mitoses. However the implication of PUM proteins in the function of mammalian stem cells has been rarely addressed, only few reports have highlighted a possible role of PUM2 and two invertebrate PUM partners (NANOS and TRIM32) in the primitive potential of murine embryonic and adult spermatogonial and neural stem cells, respectively [29, 30]; [47]. Interestingly, a connection between PUF proteins and another main regulator of HSCs maintenance such as HOXB4/C4 was stressed through a previous study [48]. Comparative transcriptome analyses of human $CD34^+$ cells subjected or not to *HOXB4* or *HOXC4* genes have revealed that both homeoproteins upregulate *PUM1* and *PUM2* encoding genes. The link between Pumilio and Dll4/Notch pathway in the function of HPCs will be further investigated.

CONCLUSION

Our observations strongly indicate that the vascular Notch/Dll4 ligand exerts a dormant activity on HPCs, distinct from other Notch ligands, and displays clear-cut positive effects on the maintenance of the primitive functions of murine HPCs in culture. Furthermore, the link between

Pumilio and Dll4/Notch pathway in the function of HPCs is a new understanding step in the mechanism of the Notch signalling pathway. These newly identified factors, *PUM1* and *PUM2* could be implicated in the expansion and maintenance of HPCs.

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Authorship and Disclosures.

CC was the principal investigator and takes primary responsibility for the paper; CC, FM, AH, SPC, TK and EL performed the laboratory work for this study; EL, TLR and WV coordinated the research; CC, and EL wrote the paper. The authors report no potential conflicts of interest.

References

1. Silberstein LE, Lin CP. A new image of the hematopoietic stem cell vascular niche. Cell Stem Cell. 2013;13:514-516.

2. Frenette PS, Pinho S, Lucas D et al. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. Annu Rev Immunol. 2013;31:285-316.

3. Calvi LM, Adams GB, Weibrecht KW et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-846.

4. Mansour A, Wakkach A, Blin-Wakkach C. Role of osteoclasts in the hematopoietic stem cell niche formation. Cell Cycle. 2012;11:2045-2046.

5. Butler JM, Nolan DJ, Vertes EL et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell. 2010;6:251-264.

6. Ugarte F, Forsberg EC. Haematopoietic stem cell niches: new insights inspire new questions. Embo J. 2013;32:2535-2547.

7. Duncan AW, Rattis FM, DiMascio LN et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol. 2005;6:314-322.

8. Maillard I, Koch U, Dumortier A et al. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell Stem Cell. 2008;2:356-366.

9. Varnum-Finney B, Halasz LM, Sun M et al. Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. J Clin Invest. 2011;121:1207-1216.

10. Poulos MG, Guo P, Kofler NM et al. Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. Cell Rep. 2013;4:1022-1034.

11. Benedito R, Duarte A. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. Gene Expr Patterns. 2005;5:750-755.

12. Gale NW, Dominguez MG, Noguera I et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A. 2004;101:15949-15954.

13. Schadler KL, Zweidler-McKay PA, Guan H et al. Delta-like ligand 4 plays a critical role in pericyte/vascular smooth muscle cell formation during vasculogenesis and tumor vessel expansion in Ewing's sarcoma. Clin Cancer Res. 2010;16:848-856.

14. Lauret E, Catelain C, Titeux M et al. Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. Leukemia. 2004;18:788-797.

15. Lahmar M, Catelain C, Poirault S et al. Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human CD34+CD38low cell expansion and differentiation. Stem Cells. 2008;26:621-629.

16. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58-62.

17. Gothot A, Pyatt R, McMahel J et al. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. Blood. 1997;90:4384-4393.

18. Sutherland HJ, Eaves CJ, Eaves AC et al. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 1989;74:1563-1570.

19. Godefroy N, Bouleau S, Gruel G et al. Transcriptional repression by p53 promotes a Bcl-2-insensitive and mitochondria-independent pathway of apoptosis. Nucleic Acids Res. 2004;32:4480-4490.

20. Corselli M, Chin CJ, Parekh C et al. Perivascular support of human hematopoietic stem/progenitor cells. Blood. 2013;121:2891-2901.

21. Dallas MH, Varnum-Finney B, Martin PJ et al. Enhanced T-cell reconstitution by hematopoietic progenitors expanded ex vivo using the Notch ligand Delta1. Blood. 2007;109:3579-3587.

22. Amsellem S, Pflumio F, Bardinet D et al. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med. 2003;9:1423-1427.

23. Ezoe S, Matsumura I, Nakata S et al. GATA-2/estrogen receptor chimera regulates cytokine-dependent growth of hematopoietic cells through accumulation of p21(WAF1) and p27(Kip1) proteins. Blood. 2002;100:3512-3520.

24. Kunisato A, Chiba S, Nakagami-Yamaguchi E et al. HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. Blood. 2003;101:1777-1783.

25. Iwama A, Oguro H, Negishi M et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity. 2004;21:843-851.

26. Xie H, Xu J, Hsu JH et al. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. Cell Stem Cell. 2013;14:68-80.

27. Satoh Y, Matsumura I, Tanaka H et al. Roles for c-Myc in self-renewal of hematopoietic stem cells. J Biol Chem. 2004;279:24986-24993.

28. Wilson A, Murphy MJ, Oskarsson T et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev. 2004;18:2747-2763.

29. Lee MH, Hook B, Pan G et al. Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. PLoS Genet. 2007;3:e233.

30. Sada A, Suzuki A, Suzuki H et al. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science. 2009;325:1394-1398.

31. Ding L, Saunders TL, Enikolopov G et al. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature. 2012;481:457-462.

32. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature. 2013;495:231-235.

33. Kiel MJ, Yilmaz OH, Iwashita T et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109-1121.

34. Kiel MJ, He S, Ashkenazi R et al. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. Nature. 2007;449:238-242.

35. Arai F, Hirao A, Ohmura M et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004;118:149-161.

36. Shutter JR, Scully S, Fan W et al. Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev. 2000;14:1313-1318.

37. Passegue E, Wagers AJ, Giuriato S et al. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J Exp Med. 2005;202:1599-1611.

38. Kozar K, Ciemerych MA, Rebel VI et al. Mouse development and cell proliferation in the absence of D-cyclins. Cell. 2004;118:477-491.

39. Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol. 2002;3:11-20.

40. Viatour P, Somervaille TC, Venkatasubrahmanyam S et al. Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. Cell Stem Cell. 2008;3:416-428.

41. Varnum-Finney B, Brashem-Stein C, Bernstein ID. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. Blood. 2003;101:1784-1789.

42. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med. 2010;16:232-236.

43. Klinakis A, Lobry C, Abdel-Wahab O et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature. 2011;473:230-233.

44. Yu X, Alder JK, Chun JH et al. HES1 inhibits cycling of hematopoietic progenitor cells via DNA binding. Stem Cells. 2006;24:876-888.

45. Shojaei F, Trowbridge J, Gallacher L et al. Hierarchical and ontogenic positions serve to define the molecular basis of human hematopoietic stem cell behavior. Dev Cell. 2005;8:651-663.

46. Lamont LB, Crittenden SL, Bernstein D et al. FBF-1 and FBF-2 regulate the size of the mitotic region in the C. elegans germline. Dev Cell. 2004;7:697-707.

47. Schwamborn JC, Berezikov E, Knoblich JA. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell. 2009;136:913-925.

48. Auvray C, Delahaye A, Pflumio F et al. HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4. Haematologica. 2012;97:168-178.

LEGENDS OF FIGURES

Figure 1: mbDll4 reduces cell proliferation by keeping a fraction of cells in G0.

(A) Fold increase in total cell number. Sorted BM LSK cells were cultivated in 24-well plates (500-1000 cells per well) coated with C or mbDll4 stromas in the presence of mSCF, hFlt3-L, hIL-6 and mIL-11. Haematopoietic cells were harvested at day 7 and the fold increase in total cell number was calculated by dividing the number of output cells at day 7 by the number of input LSK cells, *= $p<0.05$. *(B) High resolution tracking of cell division.* Carboxyfluorescein Diacetate Succinimidyl Ester-labelled (CFSE) LSK cells were cultured in wells coated with C or mbDll4 stroma in the presence of the same cocktail of cytokines. Flow cytometry analysis of $CFSE^+$ cells was made after 2 days of culture. Percentage means represent the area of each peak (FACS analysis of one representative experiment). The percentages represent the mean of 3 experiments. Dashed lines illustrate single divisions, which are differentiated by losing CFSE fluorescence at each division. *(C) Distribution of cells in G0 versus G1 and S/G2M phase of the cell cycle using Ki-67 and DAPI staining.* Sorted BM LSK cells cultivated as previously described were harvested at day 2, and were stained with Ki-67 Ab (proliferating cells) and DAPI (DNA dye). Cells residing in G0 appear at the bottom of the G0/G1 peak, G1 cells are in the upper part and S/G2/M cells are at the right hand side of the G1 peak as indicated. *(D) Distribution of cells in G0 versus G1 and S/G2M phase of the cell cycle using Hoechst and Pyronin staining.* Sorted BM LSK cells were cultivated as previously described. Cells harvested at day 2 were stained with Pyronin (proliferating cells) and Hoechst (DNA dye). Cells residing in G0 appear at the bottom of the G0/G1 peak, G1 cells are in the upper part and S/G2/M cells are at the right hand side of the G1 peak as indicated. Results are presented as the mean ± SEM in triplicate assays**.** Data are expressed as mean ± SEM, n=3, $p< 0.05$.

Figure 2. Analysis of gene expression of regulators involved in cell cycle in response to Dll4.

Quantitative real time PCR was performed to validate the microarray data. PCR amplifications were carried out in triplicate on RNA prepared from LSK cells exposed or not to Dll4Fc for 12 hours (3 independent experiments). The mRNA expression of each gene was normalized to that of TF2D mRNA. The final ratios, expressed as a fold change, were generated by comparing expression levels of LSK cells exposed to Dll4Fc compared to control cells. Results are presented as the mean ± SEM in triplicate assays, $p<0.05$**.**

Figure 3: mbDll4 limits the loss of the *in vitro* primitive potential.

LSK cells were cultivated on C and mbDll4 stromas in the presence of mSCF, hFlt3-L, hIL-6 and mIL-11. *(A) LSK cell maintenance*. Cells harvested at the indicated days throughout the culture were labelled to identify the percentage of LSK cells in the total nucleated cell population. *(B) LSK cell analysis after 7 days.* FACS profiles of output LSK cells in one representative experiment (out of three) after seven days of culture. *(C) CFC potential of Lin$^-$ cells*. 500 sorted Lin$^-$ cells at day 7 were cultured in methylcellulose, and the number of erythroid (BFU-E), myeloid (CFU-GM) and mixed colonies were measured after 7 days. Results are expressed as mean ± SEM of ten different experiments, each performed in triplicate, *= $p<0.05$. *(D) LTC-IC potential of LSK cells.* 200 input and output LSK cells from each condition were cultured for 5 weeks on MS-5 cells, and cells from each well were then plated in methylcellulose and the number of CFC-derived LTC-IC was counted after 7 days (mean ± SEM of four independent experiments, *= $p<0.05$). *(E) Expansion of LTC-IC derived CFC.* Histogram represents the number of LTC-IC derived CFC per 1000 input LSK cells. The total number of LTC-IC-derived

CFC cells was calculated using the total cell count, and the percentages of LSK cells. Data are expressed as mean ± SEM, n=3, *= $p<0.05$.

Figure 4: mbDll4 limits the loss of the short-term reconstitution potential.

In vivo reconstitution potential. Progeny of 10 to 200 sorted Ly5.1 LSK cells cultivated in the conditions previously described, were harvested at day 7, mixed with fresh 1.5 x 10^5 Ly5.2 BM cells and transplanted into lethally irradiated C57B6 Ly5.2 mice (5 to 8 mice per group). Analysis of the presence of $CD45.1^+$ cells in blood and bone marrow of reconstituted mice at 4 and 16 weeks after transplant was performed using flow cytometry with a cut-off 0.1% CD45.1 $(donor)^+$ cells for at least 10^5 analysed cells (FACS Sort). Table A presents the frequency of reconstitution at 4 and 16 weeks after transplant, and panel B the average chimerism 4 weeks after transplant in recipient mice, *= $p<0.05$.

Figure 5. Analysis of gene expression of regulators involved in self-renewal in response to Dll4.

Quantitative real time PCR was performed to validate the microarray data. PCR amplifications were carried out in triplicate on RNA prepared from LSK cells exposed or not to Dll4Fc for 12 hours (3 independent experiments). The mRNA expression of each gene was normalized to that of TF2D mRNA. The final ratios, expressed as a fold change, were generated by comparing expression levels of cells exposed to Dll4Fc compared to controls. Results are presented as the mean ± SEM in triplicate assays, $p<0.05$**.**

Figure S1. Notch receptor expression on murine bone marrow LSK cells. Antibody staining with conjugated anti Notch-1, anti Notch-2, and anti Notch-4. Notch receptor expressions (red histograms) are overlaid on isotype controls (gray histograms) for the same population.

Figure S2. Dll4Fc reproduces mbDll4 activities by reducing cell expansion and keeping a fraction of cells in G0.

LSK cells were cultured for seven days in wells precoated with immobilized Dll4Fc as described in MM. Cultures with IgG1 served as a negative control. *(A) Fold increase in total cell number.* The fold increase in total cell number was calculated by dividing the number of output cells at day 7 by the number of input LSK cells. *(B) Distribution of cells in G0 versus G1 and S/G2M phase of the cell cycle.* LSK cells harvested at day 2 were stained with Pyronin (proliferating cells) and Hoechst (DNA dye). Cells residing in G0 appear at the bottom of the G0/G1 peak, G1 cells are in the upper part and S/G2/M cells are at the right hand side of the G1 peak as indicated. Results are presented as the mean ± SEM in triplicate assays, * $p<0.05$**.**

Figure S3: Effects of mbDll4/S17 on myeloid and lymphoid differentiation.

LSK cells were cultured in wells coated with C or mbDll4 in the presence of mSCF, hFlt3-L, hIL-6, and hIL-11 for 7 days. (A) Phenotype of nucleated cells after 7 days of culture on C and mbDll4 stromas was performed using monoclonal markers Gr-1, MAC-1, B220, CD4, CD8 and TER-119 specifically expressed by granulocytes, macrophages, B lymphocytes, T lymphocytes and erythrocytes, respectively, and immature markers, Sca-1 and c-Kit. Results are presented as the mean ± SEM in 3 experiments**.** (B) $CD44^+$ $CD25^+$ Lin^- cell phenotype of nucleated cells after 7 days of culture on C and mbDll4 stromas was performed using monoclonal markers GR-1,

MAC-1, B220, TER-119 (for Lin minus lineage), CD44 and CD25 (for T cell commitment). Results are presented as the mean ± SEM in 3 experiments**.**

Figure S4: Flow cytometric profiles of *in vivo* reconstitution potential.

Progeny of 10 to 200 sorted Ly5.1 LSK cells cultivated in the conditions previously described, were harvested at day 7, mixed with fresh 1.5 x 10^5 Ly5.2 BM cells and transplanted into lethally irradiated C57B6 Ly5.2 mice (5 to 8 mice per group). The chimerism of engrafted mice was analyzed using CD45.1 (clone A20), and CD45.2 (clone 104) mAbs. Flow cytometric profiles of $CD45.1^+$ and $CD45.2^+$ cells in peripheral blood at 4 weeks (A) and in bone marrow at 16 weeks (B) of representative engrafted mice, after transplant. Analyses were performed with a cut-off 0.1% $CD45.1^+$ (donor) cells for at least 10^5 analysed $CD45^+$ cells.

Figure S5. mbDll4 activates the Notch pathway.

Sorted LSK cells were cultivated with or without DMSO or DAPT (30 µM). Cells were harvested at day 7, and counted. (A) Effect of the presence of DAPT on the fold increase in total number of cells. (B) The percentage of LSK cells was estimated after 7 days of cultures on C and mbDll4 with or without DAPT. All data represent the mean ± SEM, n=3, *= $p<0.05$.

Figure S6. Dll4Fc reproduces mbDll4 activities by limiting the loss of the reconstitution potential.

LSK cells were cultured for seven days in wells precoated with immobilized Dll4Fc as described in MM. Cultures with IgG1 served as a negative control. *(A) LSK cell analysis after 7 days.* FACS profiles of output LSK cells in one representative experiment (out of three) after seven

days of culture. *(B) LTC-IC potential of LSK cells.* Expansion of LTC-IC-derived CFC after seven days of culture, *= $p<0.05$ (n=3). *(C) In vivo reconstitution potential*. Reconstitution potential of cells exposed to Dll4Fc. Progeny of 300 Ly5.1 LSK cells cultivated in the conditions previously described were harvested at day 7, mixed with $1.5x10^5$ Ly5.2 BM cells, and transplanted into lethally irradiated C57Bl6/J Ly5.2 mice (5 to 7 per group). Analysis of the presence of $CD45.1^+$ cells in bone marrow (4 and 16 weeks reconstitution) of reconstituted mice was performed using flow cytometry (FACS Sort).

Figure S7. Overrepresented functional categories modulated in response to Dll4.

Functional analysis of genes modulated in LSK cells exposed to Dll4Fc for 12 hr, using GO, terms from DAVID database (http://david.abcc.ncifcrf.gov/home.jsp).

Table S1. Sequences of RT-qPCR primers.

Figure 1

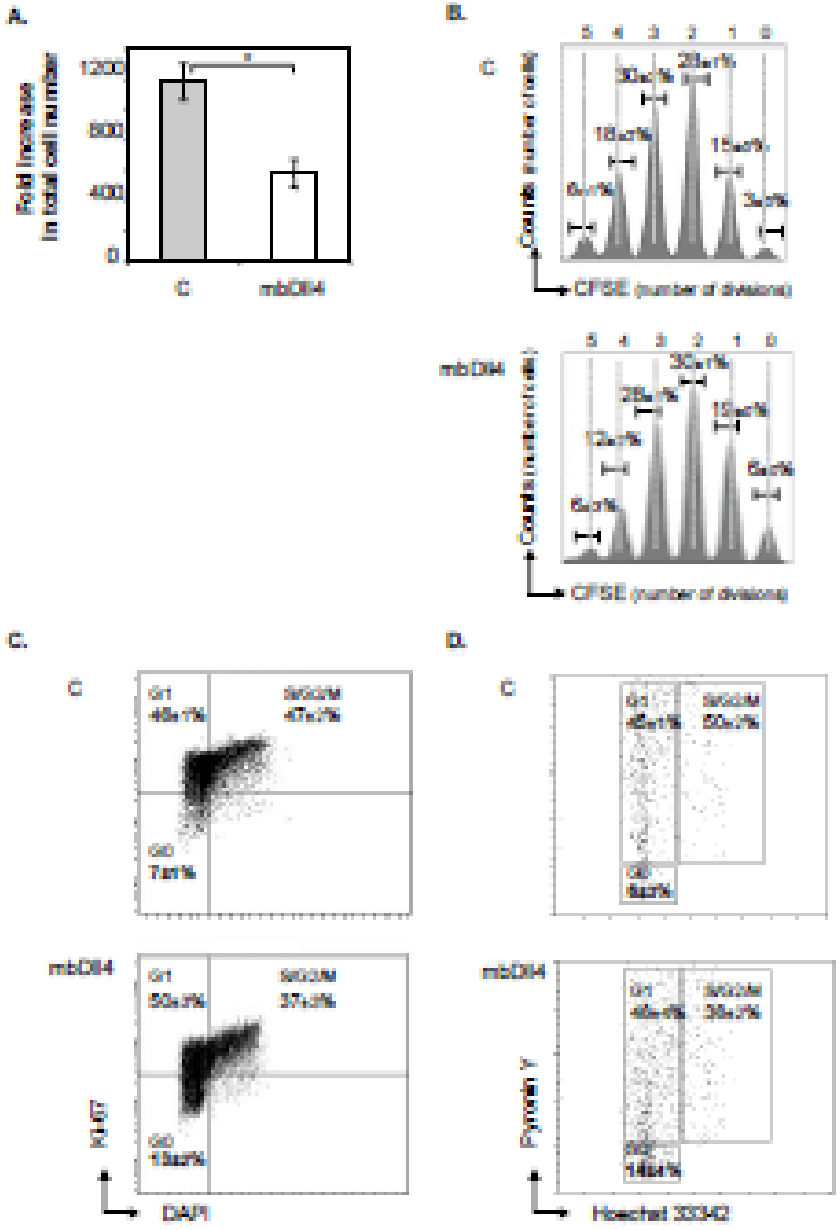


Figure 2

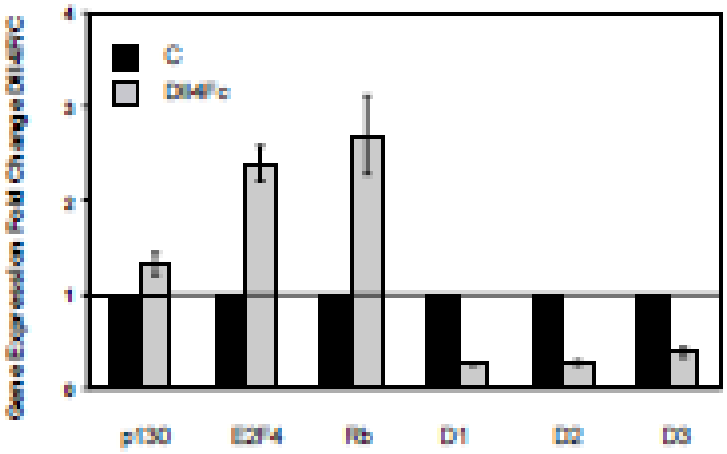


Figure 3

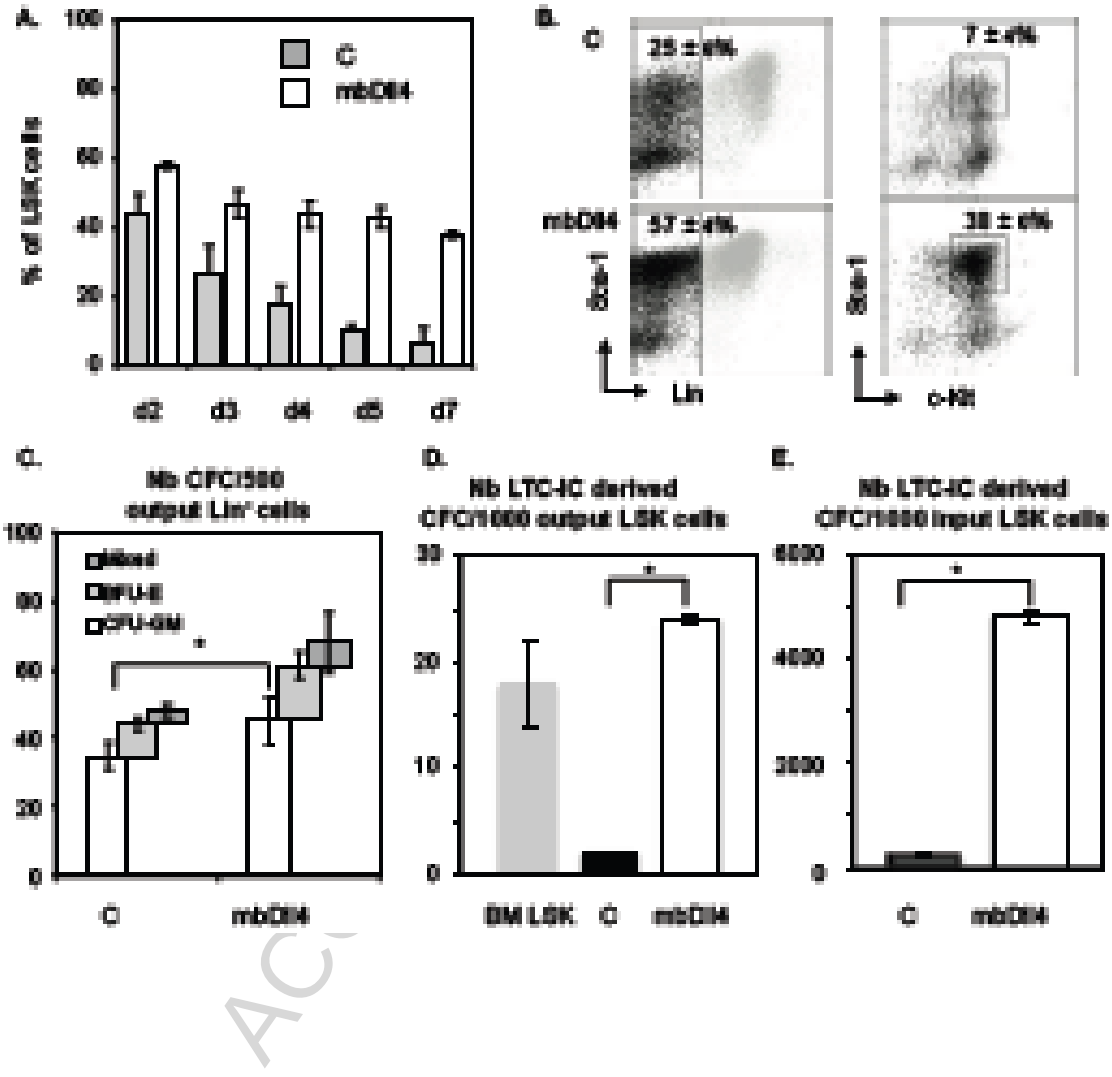


Figure 4

A.

No. of input HPCs per mouse	4 weeks after transplant			16 weeks after transplant	
	No. of mice engrafted with ≥0.1% CD45.1+ cells per total no. of mice				
	mbD84	Control	Fresh LSK	mbD84	Control
10 LSK	2/7	0/5	N.D.	1/7	0/5
30 LSK	2/7	0/5	N.D.	1/7	0/5
100 LSK	2/6	0/5	4/4	2/5	0/5
150 LSK	4/5	4/5	N.D.	3/5	0/5
200 LSK	7/7	4/7	N.D.	2/6	0/5

B.

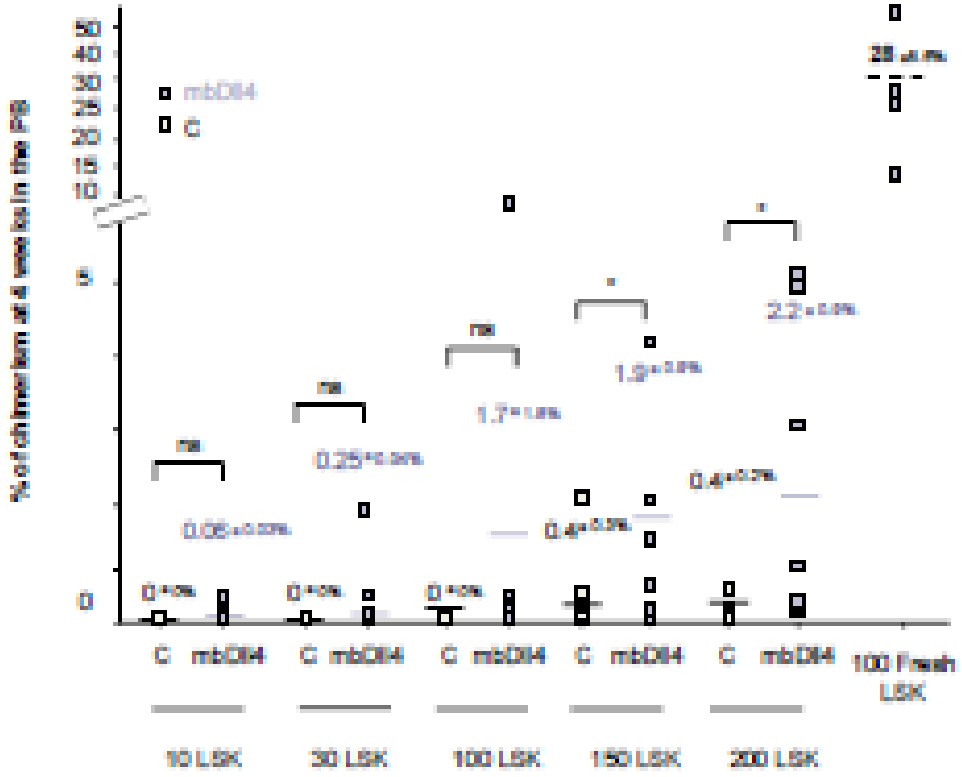
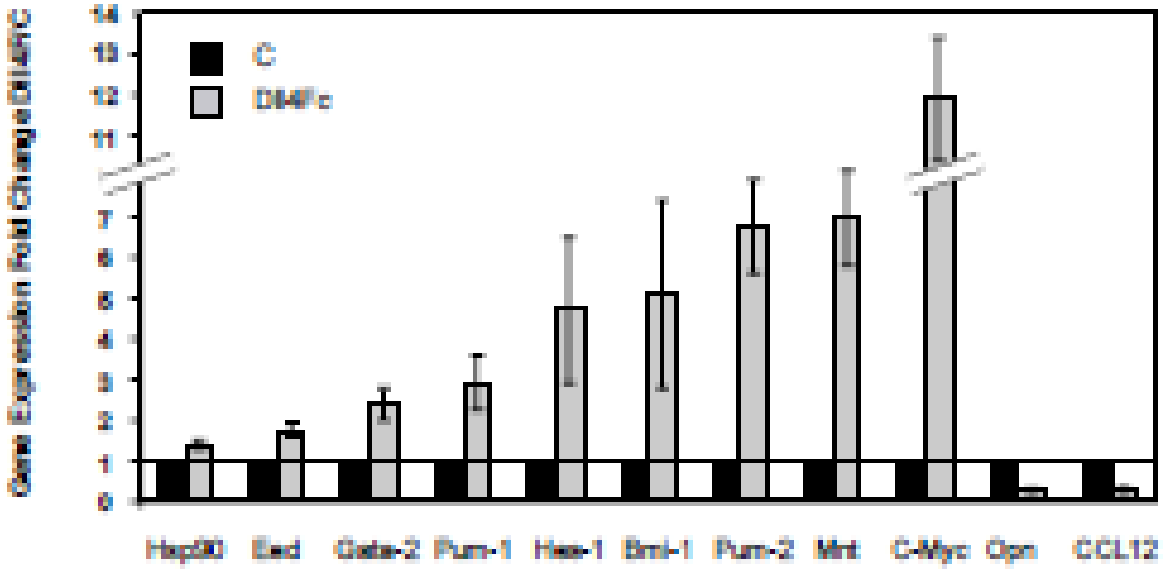


Figure 5



Highlights

- The Notch/Delta4 pathway maintains a proportion of HPCs out of the cell cycle.
- The Notch/Delta4 pathway limits *in vivo* the loss of HPCs primitive potential.
- PUMILIO-1 and -2 are new downstream factors of the Notch/Delta4 pathway.