The intracellular Domains of Notch1 and 2 Are Functionally Equivalent During Development and Carcinogenesis

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Abstract

Although Notch1 and Notch2 are closely related paralogs and function through the same canonical signaling pathway, they do contribute to different outcomes in some cell and disease contexts. To understand the basis for these differences we examined in detail mice in which N1ICD and N2ICD were swapped. Our data point to the conclusion that strength (defined here as the ultimate number of intracellular domain molecules reaching the nucleus, integrating ligand-mediated release and nuclear translocation) and duration (half life of NICD/RBPjk/MAML/DNA complexes, integrating cooperativity and stability dependent on shared sequence elements) are the factors that underlies much of the differences between Notch1 and Notch2 in all the contexts we examined including T cell development, skin differentiation and carcinogenesis, the inner ear, the lung, and the retina. We were able to show that phenotypes in the heart, endothelium, and marginal zone B cells are attributed to haploinsufficiency but not intracellular domain composition. Tissue-specific differences in NICD stability were most likely caused by alternative scissile bond choices by tissue specific γ-secretase complexes following the ICD swap. Reinterpretation of clinical finding based on our analyses suggests that differences in outcome segregating with Notch1 or Notch2 are likely to reflect outcomes dependent on the overall strength of Notch signals.
Introduction

The Notch signaling pathway is active in all metazoan, with a single receptor present in *Drosophila*, two in *C. elegans*, and four in mammals (Kopan and Ilagan, 2009). Multiple lines of evidence suggest that the two most related mammalian Notch paralogs (*Notch1* and *Notch2*) have different and at times opposite biological functions, most notably, in predicting survival of cancer patients (Boulay et al., 2007; Chu et al., 2011; Fan et al., 2004; Graziani et al., 2008; Parr et al., 2004). This latter observation presents an interesting challenge therapeutically, the resolution of which requires a mechanistic framework that can explain this apparent divergence in function between Notch1 and Notch2.

Notch proteins are large transmembrane receptor that become activated by transmembrane ligands of the DSL family (reviewed within (Kopan, 2010)). Ligand binding unfolds a protective juxtamembrane domain (Kovall and Blacklow, 2010) to expose the protein to ADAM10 cleavage (Groot et al., 2013; van Tetering et al., 2010). The remaining peptide undergoes cleavage by the γ-secretase complex (Jorissen and De Strooper, 2010), releasing the Notch intracellular domain (NICD). NICD stability is determined by the identity of the amino terminal amino acid (Blat et al., 2002) and by a degron(s) that is phosphorylated after assembly of a transcription complex (Chiang et al., 2006; Fryer et al., 2004). In considering how Notch1 and Notch2 proteins could contribute differentially to disease outcome, the simplest models evoke differential NICD composition. This model assumes that the evolution of N1ICD and N2ICD resulted in preferred association with other cellular proteins or transcription factors, which would modify the outcome differentially.

Motivated by these observations and by the apparent asymmetric functions of *Notch1* and *Notch2* in renal development (Cheng et al., 2007), we created two new strains of mice harboring rearranged *Notch1* and *Notch2* loci (Liu et al., 2013): *Notch12* (N12) and *Notch21* (N21). In response to ligands, the *Notch1* extracellular and transmembrane domain of the N12 allele is sequentially cleaved to release the *Notch2* ICD (N2ICD; Fig. 1A, B); reciprocally, cleavage of the *Notch2* extracellular and transmembrane domain of the N21 allele releases the *Notch1* ICD (N1ICD; Fig. 1A, B; (Liu et al., 2013)). Using these strains we analyzed kidney development and found no support for the hypothesis that NICD composition was driving the differences between the two receptors. Instead, we identified a different mechanism that explained the divergent function of
Notch1 and Notch2 in kidney development (Liu et al., 2013). We discovered that in the kidney, Notch2 constituted the larger proportion of Notch receptors at the cell surface and was cleaved in response to ligands more efficiently, and that these differences were coded by the amino acid composition of the extracellular domain (ECD). N1ICD and N2ICD were perfectly equivalent: 100% of nephrons were rescued by the N1ICD when it was expressed from the Notch2 locus, whereas no nephron formed when N2ICD was solely produced from the Notch locus (in Pax3-Cre, N2^f/f, N1^{12/12} animals which lack endogenous Notch2 in the developing kidney; See Fig. 3 of Liu et al, 2013). This mirrors the absolute requirement for Notch2 in kidney development (Cheng et al., 2007). The importance of the ECD in transporting Notch and ensuring cleavage efficiency in vivo was confirmed recently by comparing N1 and NIP1::Cre proteolysis (Liu et al., 2015).

As conclusive and unequivocal as these results are, they did not rule out the possibility that NICD composition does play a role in other tissues. To address this possibility, we analyzed a multitude of cell types and organs in the N12 and N21 strains with special attention given to those in which either Notch1 or Notch2 plays dominant roles, reasoning that this could reflect nuclei where NICD composition plays an important role. We also examined the role of NICD composition in skin carcinogenesis. Overall, we still could not demonstrate one case where composition played a significant role. Instead, we uncovered multiple lines of evidence indicating that the Notch signaling pathway is exquisitely sensitive to NICD dosage. The significance of these data to disease outcome will be discussed.

Results.

Outbred N12 And N21 Homozygous Mice Display No Overt Phenotype.

We have analyzed the N12 and N21 strains of mice for overall morphological and growth parameters. When the lines were maintained on a mixed genetic background, both N12/12 and N21/21 homozygous pups were born at the expected Mendelian frequencies (Fig. 1C, D), despite the presence of Notch receptors releasing exclusively N1ICD (N1^{+/+}; N2^{21/21}) or N2ICD (N1^{12/12}; N2^{+/+}). Since Delta-like1 (Dll1) haploinsufficiency affected metabolic pathways and growth (Rubio-Aliaga et al., 2009; Rubio-Aliaga et al., 2007), we wondered if the monotone composition of NICD in N21 and N12 homozygote animals resulted in similar deficiencies. We monitored weight gain as a
surrogate for metabolic state over an eight-week period after birth and concluded that these mice were indistinguishable from gender-matched wild type littermates in their growth rates (Fig. 1E, F).

Some Organs Where Either Notch1 Or Notch2 Are Dominant Develop Normally.

We have recently shown that the cell surface distribution and the efficiency of activation upon ligand binding, but not NICD composition, resulted in a dominant contribution of Notch2 to kidney development (Liu et al., 2013). To extend this analysis to other organs where one receptor is acting alone or is dominant over its paralog, we examined the development of T-cells (Fiorini et al., 2009), the inner ear (Kiernan et al., 2005; Zhang et al., 2000), the endocardium (MacGrogan et al., 2010) and skin (Demehri et al., 2008; Rangarajan et al., 2001), where Notch1 is dominant, and the liver (Geisler et al., 2008), eye (Geisler et al., 2008; McCright et al., 2002), cardiac neural crest cells (McCright et al., 2001; Varadkar et al., 2008) and lung (Morimoto et al., 2012), where Notch2 is dominant. Analysis of hair cell distribution within the cochlea of N12 homozygote mice demonstrated the same infrequent perturbations in patterning as seen in their wild type littermate (Fig. 2A-C). In Notch1 deficient animals, a block in T-cell development manifests by accumulation of double negative CD4/CD8 cells (DN cells) with DN1 characteristics (Radtke et al., 1999), demonstrating an essential role for Notch1 in T cell development and lineage commitment in the thymus. We detected similar proportions of DN, double and single positive CD4/CD8 T-cells in the thymus of N12 homozygous and WT mice (Fig. 2D). A more detailed analysis of DN subtypes confirmed a normal transition through the Notch1-dependent DN1-DN3 gate (Figure 2E). Together, these observations establish that the amino acid composition of N2ICD conserved all the key residues necessary for full function in organs that develop only in the presence of N1ICD. A similar conclusion emerged in five Notch2-dependent organs: the kidney (Liu et al., 2013), the lung (Fig. 2F), the liver, the eye and the heart (Fig. 2G).

Whereas the N2ICD could provide all the necessary functions for proper T-cell development in the thymus of N1^{12/12} mice, it is possible that N1ICD could induce ectopic T-cell development from common lymphoid progenitors (CLP) in the bone marrow (BM) of N2^{21/21} mice, where normally only B-cells develop and Notch2 is expressed in a Jagged1-dominated environment (Stanley and Guidos, 2009). We therefore analyzed BM cells for the presence of ectopic T-cells by examining the surface marker CD4, CD8, CD44 and CD25 signatures and uncovered no evidence for ectopic
T-cell development in the BM, indicating no effect of swapped ICD on promoting early T cell differentiation (data not shown). To extend the analysis and examine the impact of specific ligands, we used the in-vitro system to titrate Notch signal strength as described previously (Dallas et al., 2005; Varnum-Finney et al., 2011). No significant differences were seen in the ability of WT and N21/21 HSC to generate SK+CD11b- progenitors or CD25+ (DN2 cells) at any ligand concentration (Fig. S1). Combined, these analyses established that ICD1 was equivalent to ICD2 for inducing SK+ cells self-renewal and inhibiting myeloid differentiation in vivo and in vitro, which we have previously shown is dependent on Notch2 (Varnum-Finney et al., 2011). Thus, amino acids unique to N1ICD were not selected to favor cooperative interactions with T-cell specific inducers.

Disruption in skin barrier function results in the production of thymic stromal lymphopoietin (TSLP), which in turn leads to a dramatic expansion in B-cell proliferation during the first three weeks of life (Demehri et al., 2008). To better define the role of NICD in skin homeostasis, we took advantage of the availability of antibodies specific to Notch1 and Notch2 (characterized in (Liu et al., 2013)). Careful examination of Notch1 and Notch3 expression during development revealed expression in the ectoderm prior to stratification. Expression in the basal layer was expanded into the supra basal layers after epidermal stratification (Fig. 3 and Fig. S2). By contrast, Notch2 was only detected in supra-basal cells (Fig. 3, Fig. S2). This distribution suggested that perhaps a dominant role for Notch1 in skin development (Demehri et al., 2008; Pan et al., 2004) and homeostasis (Demehri et al., 2009) reflected its function in the basal cell population (Tadeu and Horsley, 2013). To test if N1ICD composition contributes any unique function in the skin, we examined the levels of the cytokine TSLP and performed complete blood count (CBC) in the serum of animals with various combinations of Notch1 and Notch2 alleles (Fig. 4A-B). The presence of one N12 allele (Fig. 4Ad, 4Bd) was sufficient to maintain normal skin differentiation as judged by TSLP levels and white blood cell (WBC) counts, even in the absence of Notch2 (Fig. 1Af). We noted however that in this sensitized background the N2ICD protein was not as potent as N1ICD and a very weak hair phenotype was noticeable along the dorsal midline (Fig. 4Be, Msx2cre; N1+/f; N2f/f vs. Fig. 4Bf, Msx2cre; N112/f; N2f/f). Conversely, restoring the expression of N1ICD from the Notch2 locus in animals lacking Notch1 (Fig. 4Ah and Bh, Msx2cre; N1f/f; N2r21/f; Fig. 4Ai and Bi, Msx2cre; N1f/f; N221/21; Fig. 4Ak and Bk, Msx2cre; N1f/f; N221/f) provided no relief as judged by TSLP levels,
WBC counts as well as skin pathology (Fig. S3, compare the K14 staining pattern among Msx2cre; N1f/f; N2\textsuperscript{21/21} and Msx2cre; N1f/f and Msx2cre; N2f/f).

A slight difference in activity might be amplified in a chemical carcinogenesis assay (Demehri et al., 2009). To test this possibility, we compared tumor latency and tumor burden in mice lacking Notch1 but with wild type (Msx2cre; N1f/f; N2\textsuperscript{+/+}) or N2\textsuperscript{1/2} proteins expressed at the Notch2 locus (Msx2cre; N1f/f; N2\textsuperscript{+/21}) (Fig. 4C-E, Fig. S3). We observed no differences in the time to tumor formation and all animals developed at least one tumor. However, we noted a significant difference in the number of tumors that developed by week 25 post-DMBA treatments. Instead of protecting from chemical carcinogens, the N21 allele seems to be slightly weaker than the wild type allele. Because N12 and N21 were created in B6 ES cell lines, this difference was not attributable to strain contributions (Quigley et al., 2009). Combined, this report and the results in Fig. 4Bf and 4E suggested that ICD composition was not likely to be important.

We next tested the possibility that the N12 and N21 alleles differ slightly in their stability due to the selection of cleavage sites by γ-secretase, which can be affected by the composition of charged amino acids near the transmembrane domain (Tagami et al., 2008). In turn, shifting cleavage sites will alter the identity of the amino-terminal residue, affecting stability of some NICD species (Blat et al., 2002). To examine this possibility, we compared the amount of N2ICD released from N12 alleles in N1\textsuperscript{12/12} homozygotes to that of N1ICD released from N1 alleles in wild type (N1\textsuperscript{+/+}) in various organs, including the kidney, brain, heart and epidermis, in newborn pups (Fig. 5). This is possible because N2ICD released from N12 protein and N1ICD that are released from N1 protein share the same six N-terminal amino acids and therefore can be recognized by the anti-cleaved Notch1 V1744 antibody with equal affinity (see Fig. S4D). We noted differences in the reactive amount of the V1744 antigen in these tissues between wild type and N1\textsuperscript{12/12} mice, with the epidermis containing the least relative amount of V1744 antigen in N1\textsuperscript{12/12} mice. This result could imply that the selection of S3 cleavage sites varied from tissue to tissue in accordance with tissue-specific composition of γ-secretase (Jorissen and De Strooper, 2010). To access the overall amounts of NICD, we analyzed C-terminal tagged N1 and N12 in HEK293 cells (Fig. S4). The identity of the band containing the NICD was confirmed by its sensitivity to DAPT, a γ-secretase inhibitor (Fig. S4A). Next, the total amounts of NICD produced from N1, N12, and N1\textsuperscript{V1744G} (Huppert...
et al., 2000) were evaluated in the absence or presence of the proteasome inhibitor Lactacystin (Lac). The amount of NICD accumulated in N12 expressing cells was half of that present in HEK293 expressing N1 in the absence of Lac, independent of plasmid concentration (Fig. S4B, C). The addition of the inhibitor Lac significantly increased the overall amount of myc-tagged N1ICD in HEK293 cells expression wild type N1, N12 or N1^{V1744G}, a mutation producing a degradable NICD species that, when homozygous, lead to a strong phenotype in endothelial cells (Huppert et al., 2000) but not in the somite (Huppert et al., 2005). Nevertheless, the level of N2ICD in N12-expressing HEK293 cells in the presence of Lac only reaches about 75% of N1ICD levels (Fig. S4C), suggesting that both the cleavage of N12 and the stability of N2ICD released from such an allele is reduced when compared to the wild type N1. Unfortunately, due to the lack of an epitope-specific antibody recognizing cleaved Notch2, we could not perform similar experiments for N1ICD in N21 cells.

**Differential Accumulation Of NICD Impacts Notch Signaling In Two Dosage-Sensitive Tissues: Cardiac/Endothelial Cells (N12) And Marginal Zone B Cells (N21).**

Based on these results, we reasoned that dosage-sensitive tissues might not tolerate a single N21 or N12 allele if the respective production of N1ICD and N2ICD from these loci is reduced. To test this, we crossed N1^{12/12} sire with either N1^{+/−} or N1^{+/V1744G} dams (Table 1). Out of 21 pups born to N1^{+/−} mothers, not one had the genotype N1^{12/−}. Further analysis revealed that these embryos were recovered at expected frequency before E10, but displayed phenotypes in endothelial cells (pale yolk sac and lack of vascular development) from E10 onwards (Fig. 6A, Table 1; (Huppert et al., 2000; Krebs et al., 2000; Liu et al., 2011)). Furthermore, if the N1^{12} allele provides less NICD than N1, but more NICD than N1^{V1744G}, one would expect survival of N1^{12/V1744G} embryos to be better than N1^{V1744G/V1744G}, which die at E10.5 (Huppert et al., 2000). Indeed, these embryos fared better, with a third (23/79) of N1^{12/V1744G} pups surviving to birth (but only 2/23 surviving at weaning). *In utero*, a high fraction of N1^{12/V1744G} embryos displayed vascular phenotypes, several surviving past E15.5 but failing to thrive shortly thereafter (Table 1 and data not shown).

These data could indicate that N12 is a weak allele of *Notch1*, which, though stronger than N1^{V1744G}, is insufficient in dosage-sensitive endothelial cells (Theodoris et al., 2015). Alternatively,
the composition of the N2ICD may result in failure to activate key target genes (or resulted in ectopic activation of deleterious genes). To differentiate between these two different hypotheses, we isolated ToPro 3-, CD31+, CD45− endothelial cells by FACS from E9.5 embryos of the following genotypes: N1+/+, N1+/12, N1+/V1744G, N112/V1744G, N1+/−, N112/−, purified total RNA and performed RNA sequencing (RNA-Seq) to determine the gene expression profile from each of the genotypes (see methods). We reasoned that if the latter hypothesis is true, the transcriptome of endothelial cells containing N2ICD will cluster together and that N1ICD and N2ICD “signatures” will be present in the dataset. Alternatively, all the alleles will be clustered according to “strength” of signal and will not contain a NICD paralog-specific “signature”. We used GeneSpring Bioinformatics software to analyze the RNA-SEQ data and an unsupervised hierarchical clustering algorithm resulted in the formation of an allelic series ordered based on decreasing strength: The samples containing N1+/+, N1+/12, and N1+/V1744G clustered near each other, while those with the genotypes N1+/−, N112/V1744G and N112/− showed altered gene expression and increased hypoxic signature (996 genes, Fig. 7A).

Given the role of Notch signaling to control endothelial stalk-tip cell fate decisions, we analyzed the RNA-SEQ to identify if there were any changes in stalk-tip gene expression present in our samples. Using the tip cell marker VegfR3 as an anchor, we identified 155 genes trending with a tip cell signature (including VegfR2, VegfR3, Unc5b, PdgfRb) in the samples N1+/+, N1+/12, and N1+/V1744G, which also clustered according to “strength” (Fig. 7B). This hierarchy, and the failure to identify a N2ICD transcriptional signature in this dataset, supported the hypothesis that the endothelial phenotype may be due to reduced N2ICD levels and not composition. Interestingly, when we clustered genes that also display dosage sensitivity in human iPS-derived endothelial cells (Theodoris et al., 2015), no clear pattern emerged (Fig. 7C).

To further establish that the N12 allele is hypomorphic, we asked if haploinsufficient cardiac phenotypes associated with Notch1 (de la Pompa, 2009; de la Pompa and Epstein, 2012; Garg et al., 2005; Nus et al., 2011) are evident in the surviving N1+/12, N112/V1744G and N112/12 mice (Table 2, Fig. 6B). Surviving N112/V1744G and N112/12 mice displayed various cardiac phenotypes that included, with variable penetrance, pulmonary valve stenosis, ventricular septal defects (VSD) and right ventricular hypertrophy (Table 2). To define the penetrance and severity of the cardiac phenotypes on an inbred background, we crossed B6 males with N1+/12 F1 females for 10 generation at which time we bred N1+/12 pairs. Strikingly, only one N112/12 pup survived in 114 live births (74 were N1+/12
and 39 were $N1^{+/+}$). By contrast, $N1^{12/12}$ embryos constituted ~25% of the litters examined at E9.5, E14.5 and E18.5. All five E18.5 $N1^{12/12}$ embryos from this background we examined histologically had VSD (Fig. 6D). These results are consistent with N12 being a hypomorphic allele, most likely due to dosage sensitivity that can be modified by the strain background.

Marginal zone B (MZB) cells development and homeostasis in the marginal zone (MZ) of the spleen are Notch2 dependent processes (Fig. 8A) (Arnon et al., 2013; Hao and Rajewsky, 2001; Simonetti et al., 2013; Tanigaki et al., 2002) displaying dosage sensitivity: only ~10% of the expected numbers of MZB cells were present in Notch2 heterozygote spleens (Saito et al., 2003; Witt et al., 2003). To ask where Notch2 is activated and determine what choices are made by Notch2 heterozygote cells, we analyzed the distribution of YFP + cells isolated from NIP2::CreLO; ROSA YFP mice (Liu et al., 2013), which are heterozygous to Notch2 but the inactive allele releases Cre recombinase when its ECD is activated by ligand. Cre recombines and activates YFP in cells that experience Notch2 activation and YFP expression is inherited by their decedents. We used flow cytometry to examine YFP expression in T1 (CD23-, CD21-, IgM+), T2 (CD23+, CD21-, IgM+), Follicular B cell (Fo) (CD23+, CD21-, IgM), MZP (CD23+, CD21+, IgM+) and MZB (CD23-, CD21+, IgM+) populations (Fig. 8B and B'). As expected, we found YFP expression in MZP and MZB cells but surprisingly, FoB cells were labeled instead of T2 cells (Fig. 8E, F).

To ask if NICD composition made a contribution, we analyzed MZB cell numbers in the spleens of wild type, $N2^{21/21}$, $N2^{-/-21}$, $N2^{-/-}$, and $N2^{21/-}$ mice (Fig. 8B and B'). Although variable, $N2^{21/21}$ spleens contained as few MZB cells as N2 heterozygotes (~20% of wild type, Fig. 8C and D) and $N2^{21/-}$ contained even fewer. A similar reduction in MZP cells is also detected (Fig. 8C). To ask if the defects we detected reflected the composition of N1ICD, we quantified number of MZB cells generated from $CD19$-Cre; Rosa-NICD animals in which N1ICD was overexpressed (Murtaugh et al., 2003). If N1ICD were not competent to interact with MAML1 and drive the T2 to MZP transition, we would not expect to see an increase in the number of MZB/MZP cells. Instead, we observed a ~3 fold increase in MZP cells and a ~2.5 fold increase in MZB cells (Fig. S5), similar to the effect of N2ICD (Hampel et al., 2011). These finding further strengthens the notion that the NICD are interchangeable and the N1ICD is efficient in driving the MZP cell fate.
Discussion

An understanding of how qualitative and quantitative aspects of Notch signaling govern gene expression output remains elusive (see (Boareto et al., 2015; Shaya and Sprinzak, 2011; Sprinzak et al., 2010)). Notch1 and Notch2 have been shown to have different contributions in certain cellular, developmental and disease contexts, while having equal contributions in others. In this and a previous study (Liu et al., 2013) we failed to find support of the notion that NICD composition differentiates Notch1 from Notch2. Our data instead point to the conclusion that strength (defined here as the ultimate number of intracellular domain of NICD molecules reaching the nucleus, integrating ligand-mediated release and nuclear translocation) and duration (half life of NICD/RBPjk/MAML/DNA complexes, integrating cooperativity and stability dependent on shared sequence elements) are most likely the factors that underlies much of the differences between Notch1 and Notch2 in many contexts. This conclusion does not rule out the possibility that the composition of N3ICD (and N4ICD) can deliver functions (e.g., (Zheng et al., 2013)). The differences in Notch1/2 signaling cannot be explained by virtue of differences in their amino acid composition.

We show that on a mixed genetic background, mice were insensitive to the composition of the NICD. Even organs or cell types that evolved using predominantly one receptor, like T cells, hematopoietic stem cells, lung, or the inner ear can effectively utilize the orthologous ICD, arguing against the possibility that sequence diversity between Notch1 and Notch2 arose to accommodate tissue specific partners. In addition to this general conclusion, several interesting details have emerged. First, these alleles are deficient relative to the parental Notch allele because the choice of scissile bond by $\gamma$-secretase appears to be impacted by the composition of intracellular juxtamembrane amino acids, affecting the overall steady state level of N1ICD. The degree of this impact varies based on the cell type, perhaps due to the tissue-specific composition of $\gamma$-secretase (Jorissen and De Strooper, 2010). This led us to examine the swap alleles across a null allele and in tissues known for dosage sensitivity. The $N1^{12}$ allele was unable to support endothelial development across a null, but in trans-heterozygote combination with another weak Notch1 allele ($N1^{V1774G}$) viability was somewhat improved. The existence of Notch1 alleles differing in strength allowed us to ask whether introduction of the N2ICD resulted in a specific gene signature. Our transcriptome analyses failed to detect such a signature, instead characterizing the $N1^{12}$ allele as a weak Notch1
allele. It is worth noting that our analysis in mouse endothelial cells did not support the conclusion that Notch heterozygote iPS-derived endothelial cells activate an ossification program (Theodoris et al., 2015), similar to the response of smooth muscle cells that have lost Notch signals (Briot et al., 2014). Instead we observe an elevated tip cell signature in Notch heterozygotes, consistent with the majority of published studies exploring the role of Notch in endothelium (Benedito et al., 2009; Hellstrom et al., 2007; Jakobsson et al., 2010).

Notch2 activation within the follicular compartment by Dll1-expressing fibroblastic reticular cells (Fasnacht et al., 2014) triggers a Notch2 signal strength-dependent conversion of precursor cells to MZB cells and the subsequent migration to the marginal zone. The \(N^{2f}\) allele behaved as a weak Notch2 allele during MZB cell differentiation. This observation can fit any one of three models. First, apoptosis eliminates marginal zone progenitor cells (MZP) with low Notch2 activity, resulting in fewer MZB cells. Second, a developmental fate switch relying on Notch2 shunts MZP cells with low Notch2 activity to follicular B cells (FoB) instead of MZB cells. Third, Notch2 is required for the retention of MZB cells in the marginal zone and for the maintenance of MZB cell identity. In the third model, after the effects of Notch2 activation declines the cells return to the follicle and become indistinguishable from FoB cells, or are re-stimulated and move back to the MZ (Simonetti et al., 2013). Evidence for this option comes from the rapid turnover kinetics within the MZ (Arnon et al., 2013; Simonetti et al., 2013), the persistence of MZB cells in conditional Rag2\(^{-/-}\) mice, suggesting replenishment of MZB cells from a FoB cell pool (Hao and Rajewsky, 2001; Srivastava et al., 2005) in a Notch2 dependent process. The rapid expulsion of MZB cells into the circulation when Notch2 blocking antibodies are added (Simonetti et al., 2013) is consistent with this possibility as well. Our lineage tracing of NIP2::Cre\(^{LO}\) B cells show labeling in the FoB cell compartment, consistent with either models 2 (shunting of cells with lower Notch2 activity to FoB) or 3 (Notch2-dependent cycling between FoB and MZB cells) but not with apoptosis removing cells with lower Notch2 activity. Lack of YFP expression in T2 cells indicates that they do not experience substantial NIP2::Cre\(^{LO}\) activation (Liu et al., 2015).

Importantly, the strain background modified dosage effects in endothelial and cardiac tissue. \(N^{12/12}\) mice are viable and display no overt phenotype on a mixed background (Figure 1). However, moving the \(N^{12}\) allele into the congenic B6 background increased the penetrance and severity of VSD in \(N^{12/12}\) animals to such a degree that only one of 114 live birth survived to
adulthood. This genetic enhancement of weak Notch1 alleles may play an important role in disease etiology in human as well. Identifying Notch1 cardiac modifiers could prove relevant both as a predictor of cardiac health and as a path to the identification of therapeutically exploitable pathways protecting these mice from VSD in the mixed background. In addition, our data warrant reinterpretation of clinical findings reporting differential outcomes segregating with Notch1 or Notch2 (Boulay et al., 2007; Chu et al., 2011; Fan et al., 2004; Graziani et al., 2008; Parr et al., 2004). Our data suggest that such differences likely reflect the overall strength of Notch signals, and indicate that a careful evaluation of which allele is “stronger” may indicate the preferred direction of an intervention- to increase or decrease pathway strength –instead of a focus on allele composition.
Material and Methods

Mice

The generation and genotyping of the N12 and N21 mice is described in (Liu et al., 2013). N1V1744G (Huppert et al., 2000), Notch1 null (N1-; (Conlon et al., 1995)), Notch2 null (N2-; BayGenomics gene trap lines LST103), N1f/f (Notch1tm2Rko, (Yang et al., 2004)), N2f/f (Kiernan et al., 2005), and Msx2-Cre mice (Pan et al., 2004) and Rosa\textsuperscript{Notch} (Murtaugh et al., 2003) were genotyped as described. All mice were harvested either in the animal facility of Washington University or in the animal facility at Cincinnati Children’s Medical Center (CCHMC). Washington University and CCHMC Animal Studies Committees approved all experimental procedures.

Immunohistochemistry

All immunohistochemistry procedures, including H&E staining, were performed as described in (Liu et al., 2011). Briefly, tissues were dissected from euthanized animals, fixed with 4% paraformaldehyde in 1XPBS at 4°C overnight (newborn hearts were fixed for around one week) with constant agitation and washed thoroughly with 1XPBS. For paraffin sections, the tissues were further dehydrated with 30%, 50% and 70% ethanol and embedded in paraffin. Before antibody staining, the paraffin sections were deparaffinized with xylene substitute and rehydrated with 95%, 70%, 50% and 30% ethanol. Heat-mediated antigen retrieval was achieved by autoclaving sections at 121°C for 20 minutes in 10mM sodium citrate (pH 6.0). For frozen sections, tissues were soaked in 30% sucrose in 1XPBS overnight and embedded in Tissue-Tek OCT compound. Antigen retrieval was achieved by permeabilizing sections at room temperature in 1XPBS with 0.1% Triton X-100 for 20 minutes. The following primary antibodies were used: anti-Keratin 19 (Abcam) on liver paraffin sections; rabbit anti-Notch1 (Cell Signaling), rabbit anti-Notch2 (Cell Signaling), rabbit anti-Notch3 ICD (Gift from Dr. Urban Lendahl), chick anti-K14, K1, filaggrin (generous gift from Dr. Julie Segre) and rat anti-ß4 integrin (BD Bioscience) on frozen skin sections. The primary antibodies were further detected with the following secondary antibodies: HRP-conjugated anti-rabbit followed by DAB color reaction (for Keratin 19); Cy3-conjugated donkey anti-rabbit; Alexa Fluor 488-conjugated donkey anti-chick; Cy5-conjugated donkey anti-rat (all from Jackson Immuno Research). Inner ears were dissected under a stereomicroscope, fixed and stained with FITC-conjugated phalloidin (R&D Systems). All immunofluorescence images were captured with ApoTome microscope (Zeiss) and...
bright-field images taken with a Z3 microscope (Zeiss). Images were further processed with Adobe Photoshop CS2 and Canvas X.

**Diagnosis of congenital heart defects**

Neonatal pups were collected within hours of birth to prevent cannibalization of animals that have serious congenital heart defects. The pups were euthanized. The thorax was fixed in 10\% neutral buffered formalin. The hearts were then dissected, embedded in paraffin, and entirely sectioned in the frontal plane at 6 \( \mu \)m thickness. A pediatric cardiologist inspected all the sections to phenotype the hearts, as previously described (Winston et al., 2010).

**Cell Culture, Transfection and Western blot**

Cell culture, Transfection and Western blot from tissue lysate or cultured cells were performed as described in (Liu et al., 2013). The following primary antibodies were used: Rabbit monoclonal anti-V1744 antibody (Cell Signaling); mouse anti-6Xmyc tag antibody (9E10 ascites); mouse anti-ß-actin antibody (AC-15, Sigma).

**Flow Cytometry Analysis**

Flow cytometry was performed as previously described (Liu et al., 2013). Briefly, the spleen and the thymus were mechanistically disrupted and filtered using a nylon mesh. Bone marrow cells were flushed from femur and tibia bones. The red blood cells in the samples were lysed with 1X Lysis buffer (0.826\% NH4Cl, 0.1\% KHCO3, 0.002\% EDTA) by pipetting up and down for 45 seconds. The samples were pelleted at 2000rpm for 5 minutes, resuspended in 1X FACS staining buffer (1XPBS+3\% BSA), incubated with proper antibodies on ice for 30 minutes, washed and filtered through 40\( \mu \)m membrane for flow cytometry analysis. The following antibodies from either Biolegend or BD Bioscience were used: anti-CD4, CD8, CD44, CD25, CD21, CD23, B220, IgM, Thy1.2, Sca-1 and c-Kit. Data were collected on BD FACScan with FlowJo Collectors’ Edition and analyzed with FlowJo software (TreeStar).

**Fluorescent Activated Cell Sorting (FACS) of Embryonic Endothelial Cells**

9.5-day-old embryos were dissected, washed in 1XPBS and digested in 1ml pre-warmed Williams E medium containing 1mg Collagenase (Sigma) at 37\( ^\circ \)C with vigorous shaking for 30 minutes; at the
same time, genotyping PCR was performed with yolk sac. Digested embryo samples were further lysed with 1X Red Blood Cell lysis buffer (0.826% NH₄Cl, 0.1% KHCO₃; note that EDTA is omitted to avoid activating Notch), pelleted, re-suspended in 1XFACS staining buffer (1XPBS+3% BSA), then stained with FITC-conjugated CD31 (PECAM) and PerCP-Cy5.5-conjugated CD45 on ice for 30 minutes, washed and re-suspend in 1XFACS staining buffer containing 2µM To-Pro 3 (Life Science) and left on ice until genotyping results were obtained. Samples from embryos with the same genotype were then pooled for FACS to increase the cell number and reduce FACS time. To-Pro 3⁺ (live), CD45⁻ (non-lymphocytes) and CD31⁺ endothelial cells were sorted directly into lysis buffer from Qiagen RNeasy Micro Kit that was supplemented with 2-mercaptoethanol using a highly modified Beckman Coulter MoFlo and were then kept at -80°C until ready for RNA purification.

**HSC Culture**

LSKSLAM cells were sorted from adult murine bone marrow depleted of cells expressing the following lineage markers: CD1, CD3, CD8a, CD5, CD11b, B220, GR1, and TER-119. LSKSLAM cells were then obtained using fluorescence-activated cells sorting (FACS) on an ARIA Cell Sorter (BD), based on positive Sca-1, c-Kit and CD150 expression but negative CD48 expression. 250-500 LSKSLAM cells were cultured in non-tissue culture wells (Falcon, BD) previously incubated with PBS plus fibronectin fragment CH-296 (Takara Shuzo Co.) at 5µg/ml solutions, and containing either Notch ligand Delta1<sup>ext-IgG</sup> at 5 µg/ml, Jagged1<sup>ext-IgG</sup> at 20 µg/ml, or HumanIgG<sub>1</sub> (Sigma). Construct, protein generation, and quantitative evaluation in cell culture of these ligands have all been described previously (Dallas et al., 2005). LSKSLAM cells were cultured for 14 days as previously described (Varnum-Finney et al., 2011), stained with monoclonal antibodies, and analyzed on a Canto-2 cytometer (BD).

**Total RNA purification and RNA-seq Analysis**

Total RNA from sorted cells was purified using the Qiagen RNeasy Micro kit following manufacturer’s protocols. RNA quality and concentration was determined using an Agilent 2100 Bioanalyzer. The RNA-seq libraries were generated by the CCHMC Gene Expression Core using a Nugen Ovation RNA-Seq System V2 and Nextera DNA Sample prep kit according to manufacturer’s protocols. Sequencing was carried out using the Illumina HiSeq 2000 system according to Illumina protocols. To analyze the RNA-Seq data we used an approach that is similar to the model
developed by Mortazavi et al. in their ERANGE (http://woldlab.caltech.edu/rnaseq/) RNAseq analysis pipeline. Per-spot sequence reads were aligned allowing up to 2 mismatches and 10-multiple mappings to both genome and transcriptome targets. We used Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) and Tophat (http://tophat.cbcb.umd.edu/manual.html) for genome and transcriptome alignments. Analysis of the data was conducted using Genespring 12.6 and the RNA-SEQ data was RPKM normalized and filtered on expression, removing those that failed to have a minimum of 5 RPKM in at least two samples. An ANOVA statistical test was applied to find differentially expressed genes, \( P \) value \( \leq 0.05 \), The data have been deposited in the GEO database (GSE69276).

**Quantitative RT-PCR Analysis**

Quantitative RT-PCR analysis to compare the expression levels of various marker genes in E18.5 embryonic lung were performed as described in (Morimoto et al., 2012).

**Chemical Skin Carcinogenesis Study**

This was performed as described in (Demehri et al., 2009) and (Nicolas et al., 2003). Briefly, 15 animals for each genotype were treated once at the dorsal skin after shaving with 200\( \mu l \) acetone containing 25\( \mu g \) DMBA (Sigma) at the age of 6-10 weeks. The appearance and number of tumors was monitored and recorded weekly thereafter.

**TSLP Measurement and WBC count**

Serum TSLP level was determined with Quantikine mouse TSLP ELISA Kit (R&D Systems). WBC counts were determined with Hemavet 950 Analyzer (Drew Scientific) after fresh blood was diluted in equal volume of 10mM EDTA in 1XPBS.

**Echocardiography**

Echocardiography was performed on conscious, adult mice using a Vevo 2100 Imaging System (VisualSonics, Toronto, Canada) equipped with the MS-400 30 MHz linear-array transducer. Parasternal views in the long and short-axis of the heart were obtained with a handheld technique to collect 2D images. Additional color and pulsed-wave Doppler images of the right ventricular outflow
tract, pulmonary valve and artery were obtained to diagnose and quantify the degree of pulmonic valve stenosis.

**Acknowledgment**

We thank Washington University Siteman Cancer Center Flow Cytometry Core for allowing us to use their flow cytometer and for their assistance with FACS, Genome Technology Access Center (GTAC) for assistance with Agilent Bioanalyzer analysis, Mouse Cardiovascular Phenotyping Core for performing echocardiography. We also thank the CCHMC Gene expression core for assistance in generating the RNA-SEQ libraries. We also want to thank Dr. Sung-Ho Huh for technical help in analyzing inner ear samples. ZL, CZ, AZ, MM and RK were supported by National Institutes of Health grant RO1G55479, RK and EB were supported in part by the William K. Schubert Chair for Pediatric Research, B.V-F and IB were supported by National Institutes of Health grant U01 HL100395, and PJ is supported by NIH HL105857, an Established Investigator award from the American Heart Association and the Lawrence J. & Florence A DeGeorge Charitable Trust.

**Author Contributions**

Z.L. and R.K. conceived the project and wrote the manuscript; Z.L. performed most of the experiments; E.B. performed RNA-seq analysis and edited the manuscript; B.VF. and I.B. performed HSC *in vitro* culture study; C.Z. and A.Z. helped with inner ear and skin immunostaining analyses; P.Y.J analyzed congenital heart defects; M.M. contributed lung cell marker study.
References


Figure. 1 Swap of the intracellular domains between Notch1 and Notch2 did not affect animal survival and growth on mixed background. (A and B) Domain structure of wild type Notch1, Notch2 (A) and chimeric Notch12, Notch21 (B) protein. EGF repeats, epidermal growth factor repeats; LNR, Lin-Notch repeat; TM, transmembrane domain; RAM, RBPjk association module; ANK, ankyrin repeats; NLS, nuclear localization signal; PEST, proline/glutamic acid-serine/threonine-rich motifs. (C and D) Homozygote N112/12 (C) and N221/21 (D) were born at expected Mendelian ratio. (E and F) Homozygote N112/12 (E) and N221/21 (F) have similar growth rate as their sex matched littermates.
Figure. 2 Swap of the intracellular domain between Notch1 and Notch2 did not have significant effect on the development of major organs in which either Notch1 (inner ear (A-C), T cells (D and E)) or Notch2 (lung (F), liver and eye (G)) have dominant roles. (A-C) The inner ear of N1^{12/12} homozygotes (B-B'') has slightly more abnormal regions and extra hair cells when compared to their wild type littermate (A, C), however, this is not statistically significant (C). Hair cells are stained with FITC-conjugated phalloidin. OHC, out hair cells; IHC, inner hair cells. Arrowheads points to single extra hair cells and parentheses multiple extra hair cells. (D and E). N1^{12/12} homozygote animals have similar distribution of CD4\(+\), CD8\(+\), CD4\(\cdot\)CD8\(+\), CD4\(\cdot\)CD8\(\cdot\) cells (D) and CD25\(+\), CD44\(+\), CD25\(\cdot\)CD44\(+\), CD25\(\cdot\)CD44\(\cdot\) cells (E). (F) qRT-PCR analysis of different lineage marker expression in newborn lung of N1\(^{+/+}\), N2\(^{+/21}\) and N1\(^{+/+}\), N2\(^{21/21}\) show no difference. NS, statistically not significant. CC10, maker for Clara cells; Foxj1, marker for ciliated cells; CGRP, marker for pulmonary neuroendocrine cells. (G) No obvious difference could be detected in the liver of N1\(^{+/+}\), N2\(^{+/21}\) and N1\(^{+/+}\), N2\(^{21/21}\) animals after staining with Keratin 19, a bile duct epithelial cell marker; similarly, no morphological abnormality could be detected in the eye and heart of these animals after H&E staining.
Figure. 3 The expression pattern of Notch paralogs in the epidermis of embryonic 13.5 (E13.5) and postnatal day 9 (P9) wild type mice. K14 (Keratin 14) marks the basal cell layer and ITG (Integrin subunit β1) marks the basal membrane of the basal cell layer.
Figure. 4 No significant functional difference could be observed between N1ICD and N2ICD in maintaining skin barrier function and promoting tumorigenesis. (A and B) The loss of the ICD from Notch1 locus, but not from Notch2 locus, disrupts the normal skin barrier function and leads to increase TSLP production and white blood cell (WBC) count (A) as well as obvious skin phenotype (B); the presence of ICD1 or ICD2 in the Notch1 locus, but not Notch2 locus, restores the normal skin barrier function (A and B). NS, statistically not significant. (C-E) The presence of N1ICD under the control of N2 locus could not prevent animals that lost wild type Notch1 from developing carcinomas after DMBA treatment.
Figure. 5 The levels of cleaved N1ICD in N1^{12/12} animals were reduced to a different degree in different tissues compared to their wild type counterparts. (A) Comparison of cleaved N1ICD level in the kidney, brain and dermis between newborn wild type and N1^{12/12} animals. (B) Comparison of cleaved N1ICD level in the heart between newborn wild type, N1^{+/−} and N1^{12/12} animals.
**Figure. 6** N12 is weaker than N1 in the cardiovascular system. (A) Different from wild type or N1+/− embryos, N112−/− embryos display a range of vascular developmental defect at E10.5, leading to embryonic lethality. (B) Color Doppler analyses reveal pulmonary valve stenosis in N112/12 animals. Blood flow across the pulmonary valve in wild type mice is laminar and of a uniform velocity, whereas the flow is turbulent and accelerated in an N112/12 mouse. Pul. Valv., pulmonary valve; Pul. Art., pulmonary artery. (C) Histological analyses of hearts from newborn N112/VG mice reveal membranous ventricular septal defects (mVSD). AO, aorta; LA, left atrium; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RV, right ventricle. (D) Histological examination of a representative E18.5 heart from congenic C57/Bl6 N112/12 embryos. An arrow indicates the presence of ventricular septal defect (VSD).
Figure. 7 Transcriptome analysis of endothelial cells generated from a Notch allelic series. (A) Hierarchical clustering analysis depicting the segregation of transcripts isolated from endothelial cells sorted from E9.5 embryos harboring different Notch alleles. Note clear separation of two categories: a group consisting of at least one wild type Notch1 allele and a weak Notch allele, and a
group containing a single Notch1 allele or combinations of weak Notch alleles in endothelial cells.

B) Pearson correlation analysis with a cut-off range: [0.8,1.0] was used to identify genes that trend with the tip marker gene Vegf3R. A hierarchical clustering analysis was performed which showed that the alleles Notch1\textsuperscript{+/+}, N1\textsuperscript{12/vg} and N1\textsuperscript{12/-} share a similar expression signature distinct from the cells expressing Notch1\textsuperscript{+/+}, Notch1\textsuperscript{+/12} and Notch1\textsuperscript{+/vg} alleles. Tip cell characteristics segregate with a reduced Notch signaling environment. C) Hierarchical clustering analysis of mouse endothelial genes that were differentially regulated in iPS derived Notch1 heterozygous endothelial cells exposed to shear stress (Theodoris et al., 2015). These genes did not segregate based on Notch signaling strength in mouse embryonic endothelial cells.
Figure. 8 N2^{21'} is weaker than N2 in promoting marginal zone B cell development in the spleen. (A) A model depicting the development of MZB and follicular B cells (Fo) in the spleen. Notch2 promotes the development of MZP and MZB cells but inhibits the development of Fo cells. (B and B') Strategies to quantify the amount of different populations of spleen B cells with flow cytometry. (C) The spleen of N2^{21/21'} mice has reduced number of MZP and MZB cells. (D) The number of MZB cells correlates with the dosage of Notch2 activity in the spleen of wild type, N2^{+/21}, N2^{21/21}, N2^{+-}, N2^{21/2}, suggesting the absence of qualitative difference between N2ICD and N1ICD. (E and F) Fate mapping of cells with reduced Notch2 activity using N2IP::Cre, Rosa GFP mice reveals strong GFP labeling in MZP, MZB and Fo cells, suggesting the possible identity conversion among the three populations of cells depending on the strength of Notch2 activity (model in F).
Table 1. The N12 allele fails to rescue a N1 null and weakly improves survival and vascular phenotypes of the lethal N1^{V1744G} allele.

<table>
<thead>
<tr>
<th>Genotype:</th>
<th>Total Embryos/pups (litters)</th>
<th>Number of embryos or pups in each genotype (phenotype if found dead)</th>
</tr>
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<tr>
<td></td>
<td>N1^{12/12} N1^{+/-}</td>
<td>N1^{12/12} N1^{+/VG}</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E9.5</td>
<td>47 (7)</td>
<td>24 (4)</td>
</tr>
<tr>
<td>E10.5</td>
<td>64 (7)</td>
<td>6 (1)</td>
</tr>
<tr>
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<td>12 (1)</td>
</tr>
<tr>
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<td>N/A</td>
</tr>
<tr>
<td>E13.5</td>
<td>4 (1)</td>
<td>10 (1)</td>
</tr>
<tr>
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</tr>
<tr>
<td>E18.5</td>
<td>ND</td>
<td>7 (2)</td>
</tr>
<tr>
<td>P0</td>
<td>21 (6)</td>
<td>79 (10)</td>
</tr>
<tr>
<td>P21</td>
<td>21 (6)</td>
<td>25 (4)</td>
</tr>
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</table>
Table 2. Cardiac phenotypes associated with N12 and notch 21 alleles.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of Mice Analyzed</th>
<th># of Mice with cardiac Phenotype</th>
<th># of Mice Analyzed</th>
<th># of Mice with cardiac Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1+/+ (B6/CD1)</td>
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<td>0</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>N1+/12 (B6/CD1)</td>
<td>3</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>N112/12 (B6/CD1)</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>3 (VSD)</td>
</tr>
<tr>
<td>N112/12 (B6*)</td>
<td>1</td>
<td>?</td>
<td>5(embryos)</td>
<td>5 (VSD)</td>
</tr>
<tr>
<td>N112/VG (B6/CD1)</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>7 (VSD)</td>
</tr>
<tr>
<td>N221/21 (B6/CD1)</td>
<td>4</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* In the B6 background, N1+/12 parents produced E18.5 embryos with N112/12 genotype at the expected Mendelian distribution but only 1 pup out of 114 live births was N112/12. Thus far, 100% of E14.5 and E18.5 N112/12 embryos in the B6 background have VSD.
**Fig. S1.** Notch1 ICD swapped into Notch2 locus functionally replaces Notch2 ICD during Notch2-dependent ex vivo culture with Notch ligands Delta and Jagged. (A-C) Representative dot plots after 14 day bone marrow (BM) HSC culture on plastic coated with either different densities of immobilized Delta1, Jagged1 (extracellular domain Delta1 or Jagged1 fused to Fc domain of Human IgG) or control Human IgG. Boxes in C indicate DN1 (CD44⁺CD25⁻) and DN2 (CD44⁺CD25⁺) subpopulations. (D) Total number of cells, (E) percent Sca⁺ Kit⁺ CD11b⁻ cells, and (F) percent CD25⁺ cells generated in cultures initiated with 100 FACS isolated HSC after 14 days of culture. Data represent mean +/- SEM from 4 independent experiments. p values were determined with 2-tailed paired Student’s t test. Numbers within dot plots denote percentage of events within the respective gates.
**Fig. S2.** Detailed analysis of the expression pattern of Notch1, Notch2 and Notch3 in the epidermis of wild type postnatal day 9 (P9) pups. Whereas Notch1 and Notch3 are expressed in both basal (marked by K14 antibody staining) and supra basal (marked by filagrin (FLG) antibody staining) cell layers, Notch2 is only expressed in supra basal cells. Arrows in the merged images indicate the basal cell layer, whose basal membrane is marked by the expression of $\beta1$ integrin (ITG$\beta1$).
Fig. S3. The expression of Notch1, Notch2 and Notch3 in various mutant skin at P9. Basal cells are marked with K14 and supra basal cells with K1. In the skin of Msx2cre, N1^{f/f} pups, complete loss of Notch1 is observed in mutant area marked by hyper-proliferation of basal cells. N2ICD is replaced by N1ICD in the skin of Msx2Cre, N1^{f/f}; N2^{21/21} pups, but basal cell hyper proliferation is still evident. In the skin of Msx2Cre, N2^{f/f}, the complete loss of Notch2 does not result in basal cell hyper-proliferation.
Fig. S4. Analysis of cleavage efficiency of the N12 protein and the stability of N12ICD in cultured HEK293 cells. (A-B) HEK293 cells were transfected with various amount of plasmid expressing a c-terminal tagged proteins; pCS2ΔE-Notch1-6xmyc (N1), pCS2ΔE-Notch12-6xmyc (N12) (A, B), or pCS2ΔE-Notch1 V1744G-6xmyc (N1VG) plasmid (B). (A) Uncleaved fragment (NEXT-6myc) and γ-secretase cleaved fragment (NICD-6myc) were detected with anti-Myc antibody. In the presence of γ-secretase inhibitor DAPT, the production of the cleaved fragment is dramatically reduced whereas the uncleaved fragment increases, validating the identity of the NICD. (B) The presence of proteasome inhibitor Lactacystin (Lac) dramatically increased the amount of the NICD. (C) Quantification of relative NICD level shows that in the absence of Lac, the cleaved N1ICD level in cells transfected with pCS2ΔE-
Notch12-6xmyc (N12) is about 50% of that of pCS2ΔE-Notch1-6xmyc (N1). The presence of Lac increases this ratio to ~75%, suggesting that both the stability of the cleaved ICD from N12 and the cleavage efficiency of the N12 chimeric protein are reduced relative to the wild type N1 locus in cultured 293 cells. (D) To examine whether the binding affinity of anti-V1744 antibody is affected by intracellular amino acids, the same lysates from cells transfected with pCS2ΔE-Notch1-6xmyc (N1) and pCS2ΔE-Notch12-6xmyc (N12) were probed with anti-Myc tag and anti-V1744 antibodies, respectively, and the relative signal intensity was calculated. The results show that the affinity of anti-V1744 antibody was unaffected by the intracellular composition.
**Fig. S5.** Rosa\textsuperscript{Notch}; CD19-Cre mice released N1ICD in the B cell lineage and could efficiently drive the differentiation of MZP and MZB cells when overexpressed.