The intracellular domains of Notch1 and Notch2 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 contribute to different outcomes in some cell and disease contexts. To understand the basis for these differences, we examined in detail mice in which the Notch intracellular domains (N1ICD and N2ICD) were swapped. Our data indicate 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 underlie many 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 to 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 intracellular domain swap. Reinterpretation of clinical findings 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.

KEY WORDS: Notch, Domain swap, Endothelium, Heart, Marginal zone B cells, Carcinogenesis

INTRODUCTION

The Notch signaling pathway is active in all metazoa, with a single receptor present in Drosophila, two in Caenorhabditis 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, functions of Notch1 and Notch2 in renal development (Cheng et al., 2007). The importance of the ECD in driving the differences between the two receptors. Instead, we found no support for the hypothesis that NICD composition was a factor, 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 Notch1 ICD (N1ICD; 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 Notch1 locus (in Pax3-Cre, N2ICD; 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


Received 17 April 2015; Accepted 2 June 2015
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 in which 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^{12/12}). Because delta-like 1 (Dll1) haploinsufficiency affects metabolic pathways and growth (Rubio-Aliaga et al., 2009, 2007), we wondered whether the monotone composition of NICD in N21 and N12 homozygote animals would result 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 (Fig. 1E,F).

Some organs in which 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 in which 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; 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 littermates (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 wild-type mice (Fig. 2D). A more detailed analysis of DN subtypes confirmed a normal transition through the Notch1-dependent DN1-DN3 gate (Fig. 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 N112/12 mice, it is possible that N1ICD could induce ectopic T-cell development from common lymphoid progenitors (CLPs) in the bone marrow (BM) of N212/21 mice, where normally only B cells develop and Notch2 is expressed in a Jag1-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 promotion of early T-cell differentiation (data not shown). To extend the analysis and examine the impact of specific ligands, we used an in vitro system to titrate Notch signal strength as described previously (Dallas et al., 2005; Varum-Finney et al., 2011). No significant differences were seen in the ability of wild-type and N212/21 hematopoietic stem cells (HSCs) to generate SK+CD11b+ progenitors or CD25+ (DN2 cells) at any ligand concentration (supplementary material Fig. S1). Combined, these analyses established that ICD1 was equivalent to ICD2 for inducing self-renewal of SK+ cells and inhibiting myeloid

**Fig. 2.** Swap of the intracellular domains of Notch1 and Notch2 did not have a significant effect on the development of major organs in which either Notch1 (inner ear, T cells) or Notch2 (lung, liver and eye) have dominant roles. (A–C) The inner ear of N112/12 homozygotes (B–B″) has slightly more abnormal regions and extra hair cells compared with their wild-type littermates (A,C), but this is not statistically significant (C). Hair cells are stained with FITC-conjugated phallolidin. OHC, outer hair cells; IHC, inner hair cells. Arrowheads point to single extra hair cells and brackets indicate multiple extra hair cells. (D,E) N112/12 homozygote animals have similar distributions of CD4+, CD8+, CD4+CD8+, CD4−CD8− cells (D) and CD25+, CD44+, CD25+CD44+, CD25−CD44− cells (E). (F) qRT-PCR analysis of different lineage marker expression in newborn lung of N1+/−, N2+/21 and N1+/−, N221/21 show no differences. NS, not statistically significant. CC10, marker 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+/−, N221/21 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. Error bars represent s.d.
Disruption of 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 define further 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; supplementary material Fig. S2). By contrast, Notch2 was only detected in supra-basal cells (Fig. 3; supplementary material Fig. S2). This distribution suggested that 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 whether NICD composition has any unique function in the skin, we examined the levels of the cytokine TSLP and performed a 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. 4A,B) 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. 4A). We noted, however, that in this sensitized background the N21 protein was not as potent as N1ICD and a very weak hair phenotype (supplementary material Fig. S3, compare the K14 staining pattern among Msx2cre; N2f/f, Msx2cre; N112/f; N21/21 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 Notch2 (Varum-Finney et al., 2011). Thus, amino acids unique to N1ICD were not selected to favor cooperative interactions with T cell-specific inducers.

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 aminoterminal 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 N112/12 homozygotes with that of N1ICD released from N1 alleles in wild type (N1+/+) 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 supplementary material Fig. S4D). We noted differences in the reactive amount of the V1744 antigen in these tissues between wild-type and N112/12 mice, with the epidermis containing the least relative amount of V1744 antigen in N112/12 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 (supplementary material Fig. S4). The identity of the band containing the NICD was confirmed by its sensitivity to DAPT, a γ-secretase inhibitor (supplementary material Fig. S4A). Next, the total amounts of NICD produced from N1, N12 and N1V1744G (Huppert et al., 2000) were evaluated in the absence or presence of the proteasome inhibitor Lactacystin (Lac). In the absence of Lac, the amount of NICD accumulated in N12-expressing cells was half of that present in HEK293 expressing N1, independent of plasmid concentration (supplementary material Fig. S4B,C). The addition of the inhibitor Lac significantly increased the overall amount of myc-tagged N1ICD in HEK293 cells expressing wild-type N1, N12 or N1V1744G, a mutation producing a degradable NICD species that, when homozygous, led 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 ~75% of N1ICD levels (supplementary material Fig. S4C), suggesting that both the cleavage of N12 and the stability of N2ICD released from such an allele is reduced compared with the wild-type N1. Unfortunately, owing to the lack of an epitope-specific antibody recognizing cleaved Notch2, we could not perform similar experiments for N1ICD in N21 cells.

**Fig. 3.** The expression pattern of Notch paralogs in the epidermis of E13.5 and postnatal day (P) 9 wild-type mice. K14 (Keratin 14) marks the basal cell layer and ITG (Integrin subunit j1) marks the basal membrane of the basal cell layer.
Further analysis revealed that these embryos were recovered at expected frequency before embryonic day (E) 10, 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 N12 allele provides less NICD than does N1, but more NICD than does N1V1744G, one would expect survival of N112/V1744G embryos to be better than N1V1744G/V1744G, which die at E10.5 (Huppert et al., 2000). Indeed, these embryos fared better, with a third (23/79) of N112/V1744G pups surviving to birth (but only 2/23 surviving at weaning). In utero, a high fraction of N112/V1744G embryos displayed vascular phenotypes, several surviving past E15.5 but failing to thrive shortly thereafter (Table 1, data not shown).

These data could indicate that N12 is a weak allele of Notch1, which, though stronger than N1V1744G, 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 result in ectopic activation of deleterious genes). To differentiate between these two hypotheses, we isolated ToPro3−, CD31+, CD45− endothelial cells by fluorescence-activated cell sorting (FACS) from E9.5 embryos of the genotypes N1+/+, N1+/12, N1+/V1744G, N112/V1744G, N1+/− and N112/−, purified total RNA and performed RNA sequencing (RNA-Seq) to determine the gene expression profile from each of the genotypes (see Materials and methods). We reasoned that if the latter hypothesis is true, the transcriptome of endothelial cells containing N2ICD would cluster together and that N1ICD and N2ICD ‘signatures’ would be present...
in the dataset. Alternatively, all the alleles would be clustered according to 'strength' of signal and would 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+/+ , N112/12 and N112/V1744G clustered near each other, whereas 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 data to identify if there were any changes in stalk-tip gene expression present in our samples. Using the tip-cell marker VegfR3 (Kdr − Mouse Genome Informatics) as an anchor, we identified 155 genes trending with a tip-cell signature [including VegfR3, Unc5b, – Mouse Genome Informatics], VegfR3, Unc5b, – Mouse Genome Informatics), VegfR3, Unc5b, – Mouse Genome Informatics), 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 might be due to reduced N2ICD levels and not composition. Interestingly, when we clustered genes that also display dosage sensitivity in human induced pluripotent stem cell (iPSC)-derived endothelial cells (Theodoris et al., 2015), no clear pattern emerged (Fig. 7C).

To confirm that the N12 allele is hypomorphic, we investigated whether 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−/− mice (Table 2; Fig. 6B). Surviving N112/V1744G and N112−/− mice displayed various cardiac phenotypes that included, with variable penetrance, pulmonary valve stenosis, ventricular septal defects (VSDs) 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+/− F1 females for ten generations, at which time we bred N1+/− pairs. Strikingly, only one N112−/− pup survived in 114 live births (74 were N1−/− and 39 were N1−/+). By contrast, N112/12 embryos constituted ~25% of the litters examined at E9.5, E14.5 and E18.5. All five E18.5 N112/12 embryos from this background that we examined histologically had VSDs (Fig. 6D). These results are consistent with N12 being a hypomorphic allele, most likely as a result of dosage sensitivity, which can be modified by the strain background.

Development and homeostasis of marginal zone B (MZB) cells 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 determine where Notch2 is activated and ascertain 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 for Notch2 but experience Notch2 activation and YFP expression is inherited by their descendants. We used flow cytometry to examine YFP expression in T1 (CD23 − , CD21 − ), T2 (CD23 + , CD21 − ),

Table 1. The N12 allele fails to rescue an N1 null and weakly improves survival and vascular phenotypes of the lethal N1V1744G allele

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total (litters) for N112/12 and N112−/−</th>
<th>N112/12 (Total)</th>
<th>N112−/− (Total)</th>
<th>Total (litters) for N112/V1744G and N112−/−</th>
<th>N112/V1744G (Total)</th>
<th>N112−/− (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5</td>
<td>47 (7)</td>
<td>24</td>
<td>23</td>
<td>24 (4)</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>E10.5</td>
<td>64 (7)</td>
<td>37</td>
<td>27</td>
<td>6 (1)</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>E11.5</td>
<td>9 (1)</td>
<td>6</td>
<td>3</td>
<td>6 (1)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>E12.5</td>
<td>8 (2)</td>
<td>5</td>
<td>3</td>
<td>10 (1)</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>E13.5</td>
<td>4 (1)</td>
<td>3</td>
<td>1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>E15.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>11 (1)</td>
<td>5</td>
<td>6 (2 necrotic, 1 absorbed)</td>
</tr>
<tr>
<td>E16.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6 (1)</td>
<td>3</td>
<td>3 (2 necrotic, 1 absorbed)</td>
</tr>
<tr>
<td>E18.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7 (2)</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>P0</td>
<td>21 (6)</td>
<td>21</td>
<td>0</td>
<td>79 (10)</td>
<td>56 (2 dead)</td>
<td>23 (3 dead)</td>
</tr>
<tr>
<td>P21</td>
<td>21 (6)</td>
<td>21</td>
<td>0</td>
<td>25 (4)</td>
<td>23</td>
<td>2 (1 dead)</td>
</tr>
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ND, not determined.
IgM+), follicular B cell (FoB) (CD23+, CD21−, IgM), marginal zone progenitor (MZP; CD23+, CD21+, IgM+) and MZB (CD23−, CD21+, IgM+) populations (Fig. 8B,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 determine whether NICD composition made a contribution, we analyzed MZB cell numbers in the spleens of wild-type, N221/21, N2+/21, N2+/− and N221/− mice (Fig. 8B,B′). Although variable, N221/21 spleens contained as few MZB cells as N2 heterozygotes (∼20% of wild type; Fig. 8C,D) and N221/− contained even fewer. A similar reduction in MZP cells was also detected (Fig. 8C).

To ascertain whether the defects we detected reflected the composition of N1ICD, we quantified the 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 or MZP cells. Instead, we observed an approximately threefold increase in MZP cells and a ∼2.5-fold increase in MZB cells (supplementary material Fig. S5), similar to the effect of N2ICD (Hampel et al., 2011). These findings further strengthen the notion that the NICDs 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 for 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 underlie many 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, such as 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 γ-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 owing to the tissue-specific composition of γ-secretase (Jorissen and De Strooper, 2010). This led us to examine the effect of swapping alleles across a null allele in tissues known for dosage sensitivity. The N1/2 allele was unable to support endothelial
development across a null, but in trans-heterozygote combination with another weak Notch1 allele (N1V1744G) 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 N112 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 Vegfr3. A hierarchical clustering analysis was performed which showed that the alleles Notch1+/+, N112/12 and N112 allele share a similar expression signature distinct from the cells expressing Notch1+/+, Notch1+/12 and Notch1+/+. Tip cell characteristics segregate with a reduced Notch signaling environment. (C) Hierarchical clustering analysis of mouse endothelial genes that were differentially regulated in iPSC-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.

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 N2ICD allele behaved as a weak Notch2 allele during MZB cell differentiation. This observation can fit any one of three models. First, apoptosis eliminates MZPs 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 comes from the rapid turnover kinetics within the MZ (Arnon et al., 2013; Simonetti et al., 2013) and 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::CreLO B cells show labeling in the FoB cell compartment, consistent with either model 2 (shunting of cells with lower Notch2 activity to FoB) or model 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::CreLO activation (Liu et al., 2015).

Importantly, the strain background modified dosage effects in endothelial and cardiac tissue. N112/12 mice are viable and display no overt phenotype on a mixed background (Fig. 1). However, moving the N112 allele into the congenic B6 background increased the penetrance and severity of VSDs in N112/12 animals to such a degree that only one of 114 live births survived to adulthood. This genetic enhancement of weak Notch1 alleles might 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 VSDs 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.

MATERIALS AND METHODS

Mice

The generation and genotyping of the N12 and N21 mice is described by Liu et al. (2013), N1V1744G (Huppert et al., 2000), Notch1 null (N1−/−; Conlon et al., 1995), Notch2 null (N2−/−; BayGenomics gene trap lines LST103), N112 (Notch1tm2Rko; Yang et al., 2004), N2ICD (Kiernan et al., 2005), Msx2-Cre (Pan et al., 2004) and RosaN2ICD (Murtaugh et al., 2003) mice 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 Hospital Medical Center (CCHMC). Washington
University and CCHMC Animal Studies Committees approved all experimental procedures.

**Immunohistochemistry**
All immunohistochemistry procedures, including Hematoxylin and Eosin (H&E) staining, were performed as described by Liu et al. (2011). Briefly, tissues were dissected from euthanized animals, fixed with 4% paraformaldehyde in 1× PBS at 4°C overnight (newborn hearts were fixed for around one week) with constant agitation and then washed thoroughly with 1× PBS. 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 95%, 70%, 50% and 30% ethanol. Heat-mediated antigen retrieval was achieved by autoclaving sections at 121°C for 20 min in 10 mM sodium citrate (pH 6.0). For frozen sections, tissues were soaked in 30% sucrose in 1× PBS overnight and embedded in Tissue-Tek OCT Table 2. Cardiac phenotypes associated with N12 and Notch21 alleles

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of mice analyzed</th>
<th>Number of mice with cardiac phenotype</th>
<th>Heart histology at P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1+/+ (B6/CD1)</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>N1+/12 (B6/CD1)</td>
<td>3</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>N112/12 (B6/CD1)</td>
<td>4</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>N112/12 (B6*)</td>
<td>1</td>
<td>?</td>
<td>ND</td>
</tr>
<tr>
<td>N112/GV (B6/CD1)</td>
<td>2</td>
<td>1</td>
<td>5 (embryos)</td>
</tr>
<tr>
<td>N221/21 (B6/CD1)</td>
<td>4</td>
<td>0</td>
<td>7 (VSD)</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 one pup out of 114 live births was N12/12. Thus far, 100% of E14.5 and E18.5 N112/12 embryos in the B6 background have VSDs. ND, not determined.

Fig. 8. N221 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,B′) Strategies to quantify the different populations of spleen B cells with flow cytometry. (C) The spleen of N221/21 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, N221/21, N2+/-, and N221/-, suggesting the absence of qualitative difference between N2ICD and N1ICD. (E,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 a possible identity conversion among the three populations of cells depending on the strength of Notch2 activity (model in F). Error bars represent s.d. *P<0.05.
compound. Antigen retrieval was achieved by permeabilizing sections at room temperature in 1× PBS with 0.1% Triton X-100 for 20 min. The following primary antibodies were used: rabbit anti-keratin 19 (ab52625, Abcam; 1:100) on liver paraffin sections; rabbit anti-Notch1 (3608, Cell Signaling; 1:200), rabbit anti-Notch2 (5732, Cell Signaling; 1:400), rabbit anti-Notch3 (gift from Dr Urban Lendahl, Karolinska Institute, Stockholm, Sweden; 1:200), chick anti-K14, -K1 and -filaggrin (generous gifts from Dr Julie Segre, National Human Genome Research Institute, NIH, Bethesda, MD, USA; 1:400, 1:400 and 1:200, respectively) and rat anti-β4 integrin (553745, BD Biosciences; 1:100) on frozen skin sections. The primary antibodies were 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 ImmunoResearch). Inner ears were dissected under a stereomicroscope, fixed and stained with FITC-conjugated phalloidin (R&D Systems). All immunofluorescence images were captured with an 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 by decapitation. 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 µm thickness. A pediatric cardiologist inspected all the sections to assess the phenotype of 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 by Liu et al. (2013). The following primary antibodies were used: rabbit monoclonal anti-VÎ744 antibody (4147, Cell Signaling; 1:1000); mouse anti-6Xmyc tag antibody (9E10 ascites, homemade; 1:2000); mouse anti-β-actin antibody (AC-15, Sigma; 1:10,000).

**Flow cytometry analysis**

Flow cytometry was performed as previously described (Liu et al., 2013). Briefly, the spleen and the thymus were mechanically disrupted and filtered using a nylon mesh. Bone marrow cells were flushed from the femur and tibia bones. Red blood cells in the samples were lysed with 1× lysis buffer (0.826% NH₄Cl, 0.1% KHCO₃, 0.002% EDTA) by pipetting up and down for 45 s. The samples were pelleted at 2000 rpm (400 g) for 5 min, resuspended in 1× FACS staining buffer (3% BSA in 1× PBS), incubated with proper antibodies on ice for 30 min, washed and filtered through 40 µm nylon mesh. Bone marrow cells were flushed from the femur and tibia bones. Red blood cells in the samples were lysed with 1× lysis buffer (0.826% NH₄Cl, 0.1% KHCO₃, 0.002% EDTA) by pipetting up and down for 45 s. The samples were pelleted at 2000 rpm (400 g) for 5 min, resuspended in 1× FACS staining buffer (3% BSA in 1× PBS), incubated with proper antibodies on ice for 30 min, washed and filtered through 40 µm membrane for flow cytometry analysis. The following antibodies were used: 1:100: CD44, CD25, CD21, CD23, IgM, Sca-1, c-Kit (all from Jackson ImmunoResearch). Inner ears were dissected under a stereomicroscope, fixed and stained with FITC-conjugated phalloidin (R&D Systems). Inner ears were dissected under a stereomicroscope, fixed and stained with FITC-conjugated phalloidin (R&D Systems). All immunofluorescence images were captured with an ApoTome microscope (Zeiss) and bright-field images taken with a Z3 microscope (Zeiss). Images were further processed with Adobe Photoshop CS2 and Canvas X.

**FACS of embryonic endothelial cells**

For FACS analyses, 9.5-day-old embryos were dissected, washed in 1× PBS and digested in 1 ml pre-warmed Williams E medium containing 1 mg Collagenase (Sigma) at 37°C with vigorous shaking for 30 min; at the same time, genotyping PCR was performed with yolk sac. Digested embryo samples were further lysed with 1× Red Blood Cell lysis buffer (0.826% NH₄Cl, 0.1% KHCO₃; note that EDTA is omitted to avoid activating Notch), pelleted, resuspended in 1× FACS staining buffer (3% BSA in 1× PBS), then stained with FITC-conjugated CD31 (102506, Biolegend; 1:100; PECAM) and PerCP-Cy5.5-conjugated CD45 (103131, Biocyt3) on ice for 30 min, washed and resuspended in 1× FACS staining buffer containing 2 μM To-Pro-3 iodide (T3605, Life Technologies) 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 FACS on an ARIA Cell Sorter (BD Biosciences), based on positive Sca-1 (Ly6a – Mouse Genome Informatics), Kit and CD150 (Slamf1 – Mouse Genome Informatics) expression but negative CD48 expression.

**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. (2008) in their ENRAGE (http://woldlab.caltech.edu/maseq/) RNA-Seq analysis pipeline. Per-spot sequence reads were aligned allowing up to two mismatches and ten multiple mappings to both genome and transcriptome targets. We used Bowtie (http://bowtie.bio.sourceforge.net/index.shtml) and Tophat (https://ccb.jhu.edu/software/tophat/index.shtml) for genome and transcriptome alignments. Analysis of the data was conducted using Genespring 12.6 and the RNA-Seq data was RPMK normalized and filtered on expression, removing those that failed to have a minimum of five RPMK in at least two samples. An ANOVA statistical test was applied to find differentially expressed genes (P<0.05). The data have been deposited in the Gene Expression Omnibus database (GSE69276).

**Quantitative RT-PCR analysis**

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

**Chemical skin carcinogenesis study**

This was performed as described by Demehri et al. (2009) and Nicolas et al. (2003). Briefly, 15 animals for each genotype were treated once on the dorsal skin after shaving with 200 µl aceticone containing 25 µg 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 (Drew Scientific) after fresh blood was diluted in an equal volume of 10 mM EDTA in 1× PBS.

**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 axes 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.

Statistics

Student’s t-test was employed to determine the P-value unless otherwise specified.

Acknowledgements

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; and 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.

Competing interests

The authors declare no competing or financial interests.

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.V.-F. and I.B.; HL105857 to P.Y.J.; This work was supported by the National Institutes of Health [RO1G55479 to Z.L., F.M.M. carried out the lung cell marker study. P.Y.J. analyzed congenital heart defects; and Mouse Cardiovascular Phenotyping Core for performing

Funding

This work was supported by the National Institutes of Health [RO1G55479 to Z.L., C.Z., A.Z., M.M. and R.K.; U01 HL100395 to B.V.-F. and I.B.; HL105857 to P.Y.J.; the William K. Schubert Chair for Pediatric Research [R.K. and E.B.]; an Established Investigator Award from the American Heart Association [P.Y.J.]; and the Lawrence J. & Florence A. DeGeorge Charitable Trust [P.Y.J.]. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.125492/-/DC1

References


