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Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE

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The four highly conserved Notch receptors receive short-range signals that control many biological processes during development and in adult vertebrate tissues. The involvement of Notch1 signaling in tissue self-renewal is less clear, however. We developed a novel genetic approach N_1IP-CRE (Notch1 Intramembrane Proteolysis) to follow, at high resolution, the descendents of cells experiencing Notch1 activation in the mouse. By combining N_1IP-CRE with loss-of-function analysis, Notch activation patterns were correlated with function during development, self-renewal and malignancy in selected tissues. Identification of many known functions of Notch1 throughout development validated the utility of this approach. Importantly, novel roles for Notch1 signaling were identified in heart, vasculature, retina and in the stem cell compartments of self-renewing epithelia. We find that the probability of Notch1 activation in different tissues does not always indicate a requirement for this receptor and that gradients of Notch1 activation are evident within one organ. These findings highlight an underappreciated layer of complexity of Notch signaling in vivo. Moreover, NIP-CRE represents a general strategy applicable for monitoring proteolysis-dependent signaling in vivo.

KEY WORDS: Notch, Regulated intramembrane proteolysis (RIP), Cre recombinase, Fate mapping, Stem cells, Mouse

INTRODUCTION

Notch signaling controls spatial patterning and cell fate decisions throughout the animal kingdom (Artavanis-Tsakonas et al., 1999). The Notch genes encode large, single transmembrane receptors. Interaction between Notch receptors and ligands results in a conformational change followed by two proteolytic steps. First, the ectodomain is shed by an Adam metalloprotease. Next, a presenilin-dependent enzyme called γ-secretase cleaves the receptor within its transmembrane domain. The freed intracellular domain enters the nucleus where it interacts with the transcriptional repressor Rbp-j (Rbpsuh – Mouse Genome Informatics) to mediate transcriptional activation of target genes [the 'canonical' pathway (for a review, see Mumm and Kopan, 2000)]. Although the possibility of proteolysis-independent Notch activity remains, the majority of Notch-mediated signals in metazoans depends on proteolysis (Fortini, 2002; Huppert et al., 2000; Schroeter et al., 1998), where Notch signaling regulates the balance between self-renewal and commitment in ectodermal (Yoon and Gaiano, 2005), mesodermal (Radtke et al., 2004b) and endodermal (Schonhoff et al., 2004) lineages. At present, however, we lack a comprehensive, high-resolution view of Notch1 proteolysis/activation patterns during embryogenesis or in adult vertebrate tissues.

Methods that reveal Notch pathway activity in an unbiased manner rely on the use of antibodies specific for cleaved Notch1 (α-VLLS) proteins (Cheng et al., 2003; Tokunaga et al., 2004) or the use of Notch-responsive reporter mice (Duncan et al., 2005; Ohtsuka et al., 2006; Souilhol et al., 2006). These have been informative, but have several limitations: (1) the artificial nature of reporter transgenes may leave some Notch activity unreported (Ohtsuka et al., 2006; Souilhol et al., 2006); (2) because much Notch activity is mediated by the same DNA-binding protein (Rbp-j), target-based reporters are not receptor-specific, a crucial deficiency if different Notch receptors perform distinct functions; (3) existing reporters only provide a snapshot of pathway activity; (4) target-based reporters may respond to input from other signaling pathways (Ohtsuka et al., 2006); and (5) each target-based reporter reflects only part of the Notch transcriptome (Ong et al., 2006).

Here, we present a novel Cre recombinase approach (NIP-CRE), exploiting the requirement for receptor proteolysis to visualize cellular lineages experiencing Notch1 proteolysis. We provide evidence that this correlates with Notch1 activation. This approach should be widely applicable to the remaining Notch receptors and any biological process involving proteolysis of tethered, non-nuclear proteins.

MATERIALS AND METHODS

Generation of N1:cre mice

Homologous recombination was used to replace the Notch1 (N1) intracellular domain with Cre recombinase using the same strategy used to generate processing-deficient Notch1 mice (Huppert et al., 2000). SKB-L, a genomic clone encompassing mouse *Notch1* exons 26-32, was used to generate the N1::cre targeting vector. PCR-mediated cloning removed the start ATG, added a *Sgr*A1 site and a C-terminal 6×Myc epitope tag and SV40 polyadenylation sequences from pCS2-6MT (a gift from David Turner, University of Michigan, Ann Arbor, MI) to *nls*Cre (pMC1Cre, a gift from K. Rajewsky, Harvard University, Boston, MA). This fragment was

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sequence-verified, excised with *Sgr*A1 and *Bam*H1 and cloned directly into SKB-LG immediately downstream of Val1744 at Arg1752. Subsequently, a Neo selection cassette flanked by Flp recombinase target (FRT) sites (a gift from Jeff Milbrandt, Washington University, Saint Louis, MO) was inserted. Cre insertion resulted in deletion of mouse *Notch1* exons 29, 30 and 31 from the targeting vector. The targeting vector was linearized with *Bgl*II and *Bam*H1.

RW4 ES cells were cultured and electroporated with 25 μg linearized and gel-purified N1::cre targeting vector essentially as described (http://escore.im.wustl.edu/). G418-resistant colonies were expanded and ES cell DNA was isolated and analyzed using an external probe (5′-probe) to monitor homologous recombination using Southern blotting as described (Vooijs et al., 1998). ES cells were expanded, karyotyped and injected into blastocysts. Mice that transmitted the *N1::cre* allele through the germ line were crossed with Flp-deleter mice (Rodriguez et al., 2000) to remove the selection cassette, as monitored by neo-specific PCR. N1::cre mice were subsequently typed on tail DNA by PCR using oligonucleotides N1C-F 5′-GCTTCTGTCCGTTTGCCG-3′ and N1C-R 5′-CGGCAAACG-GACAGAAGC-3′, yielding an expected 412 bp fragment. To delete the *R26R* allele in the germ line of mice, R26R mice were crossed with CMV-Cre deleter mice. Double transgenic offspring were backcrossed to C57Bl/6 and tissues were analyzed from Cre^{-} $R26R^{+}$ mice or from double-transgenic offspring. Recombination at the R26R locus in DNA from peripheral blood was performed as described and analyzed by TAE gel electrophoresis (Akagi et al., 1997). N1::cre mice on a C57Bl6/J background were crossed with R26R/+ reporter mice (Soriano, 1999) to obtain embryos or tissues for histological analysis.

Northern blotting

Total RNA was isolated from snap-frozen E13.5 embryos split in head (H) and body (B) using a modified acid-guanidinium-thiocyanate-phenol-chloroform method. RNA (15-20 μg) was separated on MOPS-formaldehyde gels, blotted onto Zeta-probe (Bio-Rad) nylon membranes and hybridized at 68°C in high-SDS buffer (Clontech). A *cre* ORF probe and a mouse *Notch1* probe (mN1-b) encompassing the Notch1 extracellular domain were used for expression analysis of N1::cre alleles.

Cell-based analysis of N1::cre proteolysis

A constitutively active N1ΔE::nlsCre6MT construct was generated from the *N1::cre* targeting vector by subcloning a *Sgr*A1-*Mlu*1 fragment containing *nls*Cre into pCS2/N1ΔE6MT to replace NICD with Cre, creating an identical fusion to that expected to occur upon ligand-mediated proteolytic cleavage of N1::cre in vivo. HEK293 cells and presenilin 1/2-deficient fibroblasts (De Strooper et al., 1999) were cultured in DMEM, 10% FCS and transfected in 12-well plates (0.2-2×10^5 cells) with 100 ng pCS2/N1ΔE::cre6MT by calcium phosphate or Fugene (Roche), respectively. Forty-eight hours after transfection, total cell lysates were prepared in Laemmli SDS-lysis buffer and analyzed by PAGE. γ-Secretase inhibitor (DAPT in DMSO, 1 μM, Sigma) treatment of HEK293 cells was for 16 hours prior to lysis. PS1/2-dKO cells were also co-transfected with wild-type presenilin 1 (*Psen1* in pCDNA3.1). Nitrocellulose membranes were blocked with 5% non-fat dried milk in PBST (0.1% Tween20) and incubated overnight at 4°C with anti-cleaved-Notch1 antibody (1:1000, Val1744, Cell Signaling), supernatant from anti-MYC hybridoma (clone #9E10) or with polyclonal anti-Cre antiserum (1:1000, Novagen). Membranes were developed using ECL reagents (Pierce).

Tissue analysis

X-Gal staining

Embryos or tissues were dissected in ice-cold PBS-$MgCl_2$ (2 mM), fixed at 4°C in 4% paraformaldehyde (PFA) and processed for X-Gal staining as described (Hogan, 1994). Stained embryos or tissues were fixed and embedded in paraffin. For cryosections, following fixation tissues were equilibrated overnight at 4°C in 30% sucrose in PBS-$MgCl_2$, rinsed in PBS and embedded in OCT compound (Miles Scientific). Sections (5 μm-15 μm) were processed for X-Gal staining as above.

In situ hybridization

Dissected intestines were immediately fixed in 4% neutral buffered formalin overnight at 4°C and further processed for paraffin embedding. RNA in situ hybridization on sections was performed as described (Gregorieff et al., 2005; van Es et al., 2005b).

Immunohistochemistry

For α-VLLS staining (Val1744, Cell Signaling), 4% PFA-fixed tissues were embedded in paraffin, dewaxed, blocked with 3% H_2O_2 in methanol and antigens retrieved in citrate buffer (pH 6.0). Sections were blocked in 3% BSA in PBS and incubated with primary rabbit polyclonal antibody Val1744 (2 days at 4°C followed by overnight incubation at ambient temperature). After washing, antibodies were visualized with DAB (Powervision, DAKO). Sections were counterstained with Neutral Red or Hematoxylin.

RESULTS

To generate a genetic sensor of Notch1 proteolysis in vivo we replaced the mouse Notch1 intracellular domain (NICD1), immediately downstream of the transmembrane domain, with the site-specific recombinase Cre (Fig. 1A and see Fig. S1A in the supplementary material), such that the Cre activity is now governed by ligand-induced proteolysis of the Notch1 transmembrane domain tether. In Cre-reporter strains such Rosa26R (R26R) (Soriano, 1999), Notch activation is visualized by β-galactosidase expression (Fig. 1B,D,E). Because Notch1 proteolysis releases Cre that leads to a cell- heritable expression of *lac*Z, Notch1 signaling in actively cycling stem/progenitor cells will mark all their descendents producing a ‘clone’, whereas Notch1 activation in transit amplifying or differentiating cells will result in small clones (2-4 cells) or in salt-and-pepper patterns of individually labeled cells (Fig. 1C).

To test the fidelity of this system, we conducted cell-based transfection experiments with truncated Notch1-Cre fusion proteins (N1ΔE::cre), which are ligand-independent constitutive substrates of γ-secretase (Kopan et al., 1996). Immunoblotting with a cleavage-specific Notch1 antibody (αVLLS) confirmed that cleavage of N1ΔE::cre by γ-secretase occurred at the identical amino acid position as in wild-type Notch1 (Val1744). Moreover, Cre recombinase itself was not a substrate for proteolysis; release of Cre from N1ΔE::cre required presenilin activity (see Fig. S2 in the supplementary material). This indicated that the Notch1-Cre fusion protein behaves similarly to wild-type Notch1.

Using gene targeting in mouse embryonic stem cells we engineered a *Notch1-cre* fusion allele (*N1::cre*; Fig. 1A and see Fig. S1A,B in the supplementary material). To minimize concerns that this allele might act as a dominant-negative modifier of Notch signaling (Huppert et al., 2005), *N1::cre* mRNA was engineered to be less stable than the wild-type *Notch1* allele by including the exogenous late SV40 polyadenylation signal (see Fig. S1C in the supplementary material). Two independent *N1::cre* mouse lines were derived from gene-targeted ES cells that were healthy and fertile, indicating the absence of dominant-negative effects as a consequence of competition for ligands. Furthermore, homozygous *N1::cre* embryos die at E9.5, confirming that this is a null *Notch1* allele (Conlon et al., 1995) (not shown).

$N1::cre^{tg/+};R26R^{tg/+}$ (henceforth, *N1::cre*) embryos and adult tissues displayed remarkably consistent patterns of *lac*Z activation, indicating non-random proteolysis patterns of N1::cre (Fig. 1, compare D with E), validating this approach. Here, we report a survey of all three germ layers, identifying novel aspects of Notch1 signaling by comparing the clonal patterns of Notch1 activity with

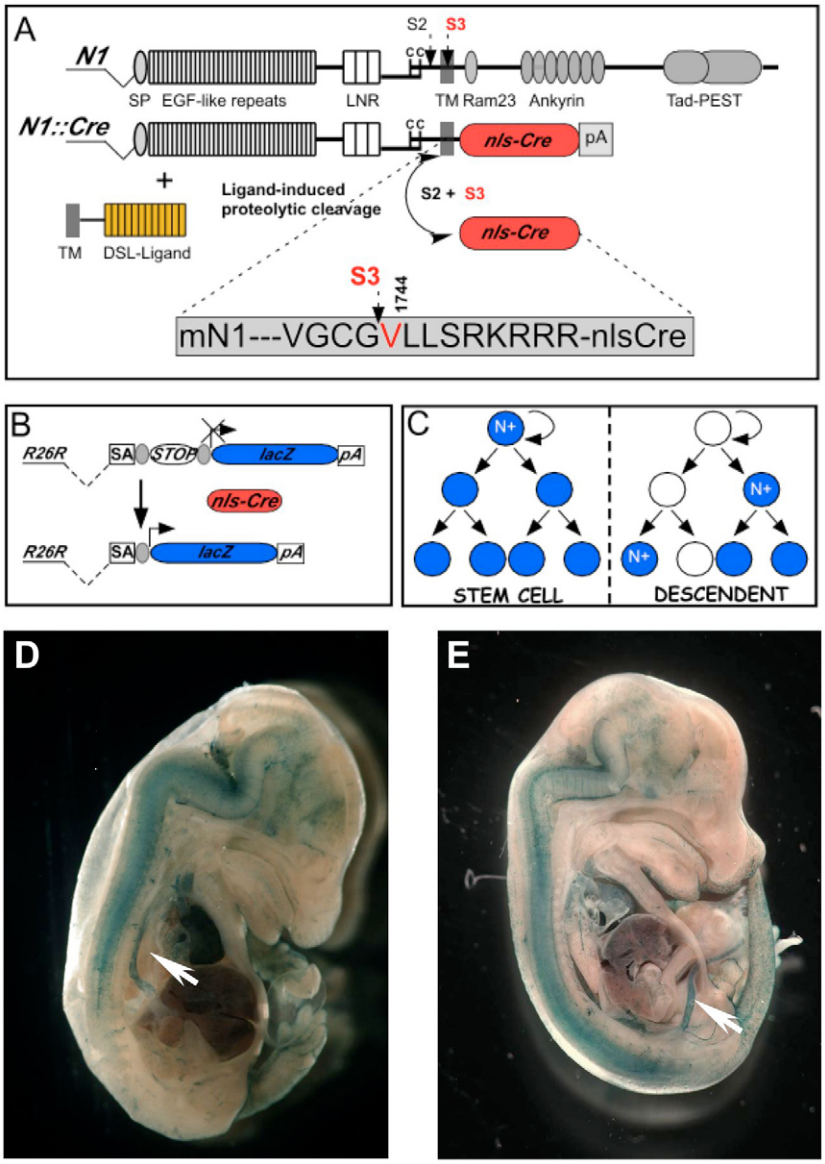

Fig. 1. Strategy of Cre-mediated lineage tracing of Notch1 activity. (**A**) Schematic depicting the mouse Notch1 protein. Indicated are the extracellular domain containing a signal peptide (SP) and 36 EGF-like repeats, the three Lin-Notch repeats (LNR), the transmembrane domain (TM) and the Ram domain, seven ankyrin repeats and the PEST and transcriptional activation domain (Tad-PEST). S2 and S3 indicate Adam metalloprotease-dependent cleavage and the γ-secretase dependent cleavage at Val1744, respectively. Using gene-targeting, Cre recombinase was inserted immediately downstream of Val1744 at Arg1752. Interaction of N1::cre receptors in vivo with Notch DSL ligands results in S2 and S3 proteolytic cleavages and release of Cre recombinase from the plasma membrane. (**B**) Cre recombinase can irreversibly activate the ubiquitously expressed R26R reporter and permanently mark cells with *lac*Z expression in vivo. (**C**) If N1::cre is activated in a stem cell, all surviving descendents appear blue; when Notch1 is activated in progenitors or differentiated cells, a mixture of blue and white cells will appear in any given tissue. (**D**,**E**) Sagittal view of whole-mount X-Gal staining of E14.5 N1::cre;R26R embryos showing identical patterns of widespread labeling of several tissues. Black arrows indicate strong thymic staining and white arrows indicate dorsal aorta (D) and umbilical artery (E). Magnification: 10×.

the behavior of ubiquitous *lacZ*-expressing *Notch1*-deficient Gt(ROSA)26Sor ES cells ($N1^{+/+}$:$N1^{\Delta 1/\Delta 1}$;*Rosa*26-*lacZ*$^{tg/+}$) in chimeric mice (for details, see Hadland et al., 2004).

Notch1 activation in the ectoderm: epidermis and appendages

The epidermis and the hair follicles are maintained via activation of two separate populations of multipotent adult stem cells. Notch1 has been suggested to play a key role in asymmetric epidermal stem cell division, in promoting epidermal differentiation and in maintaining hair follicle architecture (Blanpain and Fuchs, 2006). Whether Notch1 signaling is involved in epidermal stem cell renewal is less well defined. Starting as early as E11.5, N1::cre-marked cell populations were observed in the apical ectodermal ridge (AER) of fore- and hind-limbs, where Notch1 is required (Pan et al., 2005). At E12.5, *lacZ* staining became more prominent in the AER (Fig. 2A), expanding laterally between the dorsal and ventral surfaces of the epidermis (Fig. 2C); this is reminiscent of Notch activity in NAS mice that contain a transgenic Notch pathway reporter composed of multimerized Rbp-j-binding sites driving *lacZ* (Souilhol et al., 2006). Later in development, *lacZ* staining was detected exclusively in supra-basal epidermal cells (Fig. 2C). Transit amplifying cells, which reside in the basal layer and undergo several rounds of cell division, were expected to have experienced Notch1 activation (Lowell et al., 2000), but no *lacZ* expression was detected. As epidermal morphogenesis proceeded (E14.5 onwards), *lacZ*-labeled cells remained confined to the supra-basal layer (Fig. 2D) and their abundance increased until virtually all supra-basal cells were *lacZ*-positive by E16.5 (Fig. 2E). Immunostaining with α-VLLS antibodies (Fig. 2B) identified ongoing Notch proteolysis and activation only in supra-basal cells during these stages (Pan et al., 2004; Pan et al., 2005), consistent with a role for Notch1 in promoting differentiation (Okuyama et al., 2004). Strikingly, the adult epidermis demonstrated an almost complete absence of *N1::cre*-marked clones (Fig. 2F); this provides independent confirmation that the epidermal stem cells did not experience Notch1 activation. Infrequently, labeled subpopulations of cells were detected within adult hair follicles localized to the bulge region (Fig. 2F), where both epidermal and melanocyte stem cells reside (Moriyama et al., 2006). Control experiments demonstrated that the

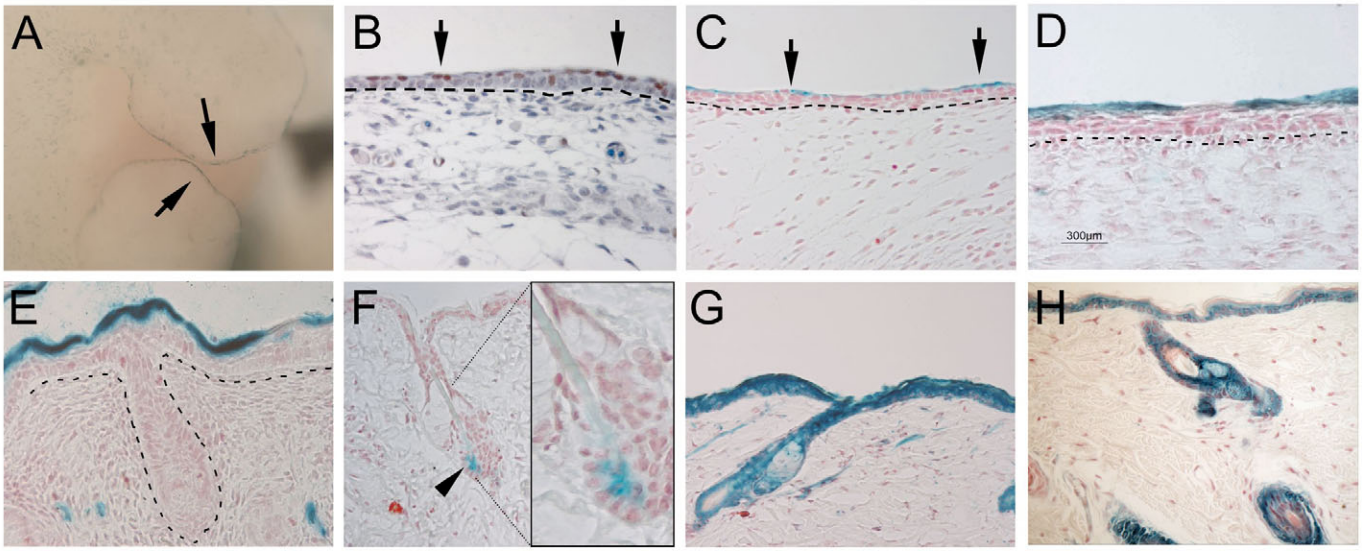

Fig. 2. Fate of Notch1-activated cells in the epidermal lineages. (**A**,**C**) At E12.5, *lac*Z is first activated in the AER of the fore- and hind limb buds (A, arrows) and the epidermis between the dorsal and ventral surface (C, arrows). (**B**) Nuclear α-VLLS staining demonstrating Notch1 activation in suprabasal nuclei of the epidermis at E12.5 (arrows). (**D**,**E**) *N1::cre* marks only supra-basal cells in the epidermis at E14.5 (D) and at E16.5 (E), where the majority of supra-basal cells in the skin are labeled, but most of the follicular epidermis remains negative. (**F**) In adult skin, staining was mostly absent in the epidermis; *lac*Z-labeled cells were present only in the differentiated cells of the hair follicle and the bulge (arrowhead, and high magnification inset). (**G**) Germ-line-deleted *R26R* mice label all keratinocytes. (**H**) Notch1 is not required for skin development because $N1^{-/-}$; *Rosa*26-*lac*$Z^{tg/+}$ cells efficiently contribute to all epidermal structures in adult mice. Dashed line (B-E) delineates epidermis and dermis. Magnification: A, 40×; B,C,D,E, 20×; F,G,H, 10×.

lack of cell labeling was not due to a lack of R26R reporter activity or to poor recombination frequency at the *R26R* locus in this tissue (Fig. 2G) (Vooijs et al., 2001). Cre-induced epidermal-specific deletion of *Notch1* does not affect cell fate selection or differentiation of epidermal progenitors until after weaning (Nicolas et al., 2003; Pan et al., 2004). In agreement, we observed that *Notch*1-deficient ES cells contributed to the epidermis and to the hair follicle throughout life, without any apparent developmental defects (Fig. 2H). Our analysis argues that Notch1 activation does not play a role in the control of 'stemness' within the epidermal stem cell niche in mice, but may promote differentiation in their descendents. Furthermore, it demonstrates that the frequency of Notch activation in an organ is dynamic, changing as animals age.

Notch1 activation in neurons

Deletion of *Notch1* or of the downstream effector *Hes* genes leads to precocious neuronal differentiation, but may also directly promote glial cell fate (Gaiano and Fishell, 2002). In the retina, Notch signaling regulates cell cycle exit, apoptosis and differentiation of neurons (Silver and Rebay, 2005), but whether it is also involved in maintaining the earliest retinal progenitors is less clear (Jadhav et al., 2006). Genetic marking techniques have shown that all neurons and Müller glia are derived from a common multipotent progenitor (Turner and Cepko, 1987). In *N1::cre* mice, scattered *lacZ* labeling of the retinal neuroepithelium was first observed at E14.5, coincident with the presence of NICD1 (Fig. 3A and not shown). In adults, robust cell labeling was observed throughout all retinal layers in both eyes (Fig. 3B,C). These data are in agreement with the dynamic expression patterns of the Notch-regulated *Hes* genes in the developing neuroepithelium and in the adult retina (Ohtsuka et al., 2006), and are consistent with a role for Notch1 activation in the earliest retinal progenitors identified by random clonal analysis (Turner and Cepko, 1987).

Notch1 has been shown to promote differentiation of V2 interneurons at the expense of motor neurons in the embryonic neural tube (Yang et al., 2006), and is essential for proper neuron and glial formation within the neural tube (Lutolf et al., 2002). At E11.5, α-VLLS staining identified a pool of progenitors with ongoing Notch1 signaling in the ventral part of the hindbrain neural tube (Fig. 3E), partially overlapping with N1::cre labeling at E12.5 (Fig. 3F). By contrast, Notch1-deficient cells displayed a bias against contribution to ventral progenitors (multiple sections from three embryos were examined). The absence of *Notch1*-deficient cells in this small series could suggest the existence of lateral interactions with wild-type cells (Fig. 3G,H), similar to that reported for Notch2 in the roof plate (Kadokawa and Marunouchi, 2002). Although this possibility will need to be followed up in a larger sample, it raises the interesting possibility that Notch1 activation might contribute to both the specification of motor neuron progenitors and, later, to their differentiation.

In the postnatal cerebellum, glial cells and granular neurons were frequently labeled, whereas the Purkinje cells were rarely labeled (multiple embryos examined with *lac*Z and GFP reporters; Fig. 3D and see Fig. S5L in the supplementary material). This is in contrast to NAS mice in which Purkinje cells were specifically labeled (Souilhol et al., 2006), which is likely to reflect the activity of other Notch receptors. This observation demonstrates the utility of NIP-CRE: Notch1 has been shown to be essential for Purkinje cell differentiation (Lutolf et al., 2002) and NIP-CRE suggests this role is non-cell-autonomous. Although this possibility is still under investigation, these observations suggest that Notch1 activation in vertebrates can occur in neuroblasts (retina) or in specific populations of differentiating neurons (granular neurons, interneurons) where Notch1 selects a specific differentiation program.

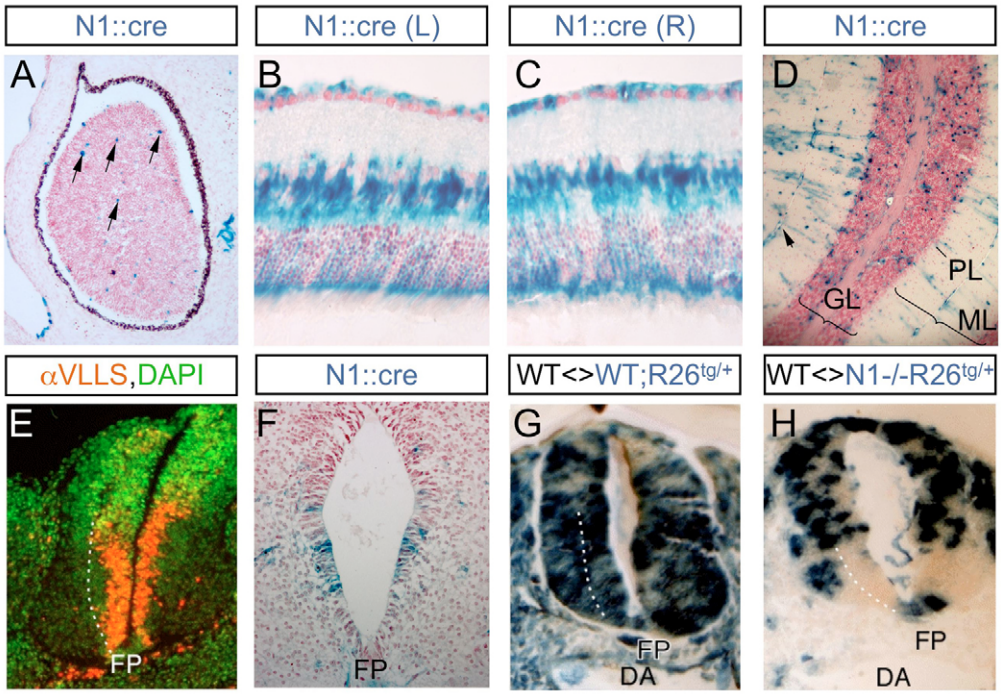

Fig. 3. Fate of Notch1-activated cells in neuronal lineages. (**A**) *N1::cre* labels retinal progenitors (arrows) at E14.5. (**B**,**C**) In the adult retina, clones derived from retinal progenitors that experienced Notch1 activation contain all retinal cell types in both eyes. (B) Left eye, L; (C) Right eye, R. (**D**) Adult cerebellum of N1::cre mice showing labeling of molecular layer (ML) and granular layer (GL) but little in the Purkinje layer (PL); note vascular staining (arrowhead) throughout. (**E**) α-VLLS staining (red) identifies Notch1 signaling in ventral neural tube progenitors at E11.5. (**F**) N1::cre marks similar progenitors at E12.5 in the ventral neural tube. (**G**,**H**) Wild-type ES cells (G) efficiently contribute to all regions of the neural tube whereas Notch1-deficient, R26 *lac*Z-tagged cells (H) are excluded from ventral region. Dotted line in E,G,H marks the presumptive border between neuronal progenitors and committed or differentiated motor neurons. Counter stain: Neutral Red (A-D,F), DAPI (E), Haematoxylin (G,H). FP, floor plate; DA, dorsal aorta. Magnification: A, 10×; B-H, 20×.

Notch1 activation in the mesoderm: vasculature

The vascular system is the first organ to function in vertebrates and comprises arteries and veins that are distinct on an anatomical, functional and molecular level. Notch receptors and ligands are expressed in all endothelial lineages and are implicated in several inherited syndromes with vascular involvement (Gridley, 2003). *Notch1*-deficient mice fail to form a proper vasculature, which results in lethality by E9.5 (Huppert et al., 2000; Krebs et al., 2000).

As early as E12.5, endothelial cells within the dorsal aorta became labeled (Fig. 4A) and this pattern was expanded during embryogenesis until most arterial endothelium was marked. By contrast, venous endothelium was negative (Fig. 4A). At E14.5, endothelium in the aorta intercostal arteries was labeled (Fig. 4C). Likewise, umbilical arteries were labeled (Fig. 4E), whereas the umbilical veins and maternal vasculature were negative (Fig. 4E, E16.5). Staining for NICD1 confirmed that Notch1 signaling is active in arterial endothelial cells at these stages (Fig. 4D). Unlike the epidermis, staining of arterial endothelial cells persisted throughout adulthood (Fig. 4G and see Fig. S5H in the supplementary material), indicating that a Notch1 signal was either continuously active in these cells during tissue renewal, or that all arterial endothelial cells were derived from primitive arterial endothelial precursors that experienced Notch1 proteolysis. Interestingly, the venous endothelium of adult mice contained labeled cells, suggesting that these cells were only exposed to Notch1 signaling postnatally (Fig. 4H). Notch1 and Notch4 act redundantly in arterial vasculature (Krebs et al., 2000). To determine if *Notch1* is required for arterial identity, we analyzed the vasculature in *Notch1*-deficient chimeric mice. *Notch1*-deficient cells rarely contributed to arterial vasculature in the yolk sac labeled by *N1::cre* (Fig. 4, compare E with F). Furthermore, *Notch1*-deficient cells showed a strong bias against any contribution to the arterial endothelium in the dorsal aorta (DA) (Fig. 4A and data not shown; the entire aorta was examined in serial section in two embryos and sample sections examined from four additional $N1^{-/-}$ embryos). In the same embryos, *Notch1*-deficient cells efficiently contributed to the endothelium of the posterior cardinal vein (PCV; Fig. 4B), a pattern complementary to the *Notch1* fate map. Control $N1^{+/+}$;*R26-lac*$Z^{tg/+}$ cells showed no bias and contributed to yolk sac, DA and PCV (not shown). It is important to note that NAS transgenic reporters fail to identify Notch activity in yolk sac (Souilhol et al., 2006). Our combined clonal and functional analysis argues for a non-redundant, cell-autonomous requirement for Notch1 in establishing arterial identity.

Notch1 activation in the Heart

The earliest indication of β-galactosidase activity in *N1::cre* mice was detected in endocardial cells of the outflow tract and left and right ventricles at E10.5 (Fig. 5A,B). As heart development proceeds, a single row of marked endocardial cells lined the future aortic valves (AV), as well as the outflow tract of the left ventricle and the brachiocephalic artery at E14.5 (Fig. 1D,E; Fig. 5C,D). This pattern was maintained and expanded during embryonic development, and at E16.5 the majority of endocardial cells in the embryonic heart were labeled. Notably, the X-Gal-marked endocardial cells lining the valves and myocardium were receiving

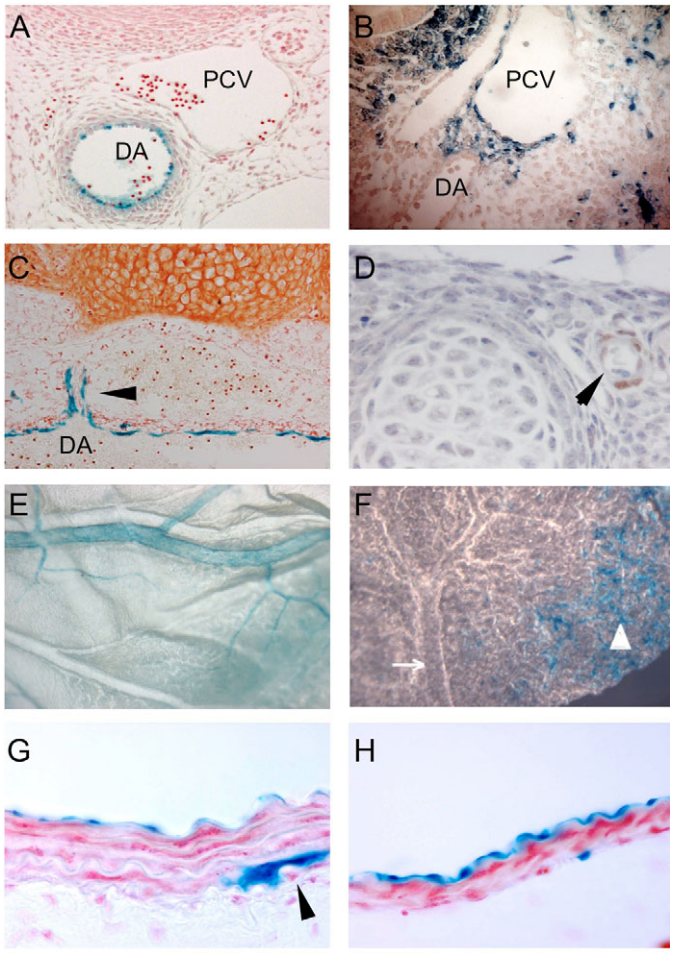

Fig. 4. Fate of Notch1-activated cells in the vasculature. (**A**) N1::cre activity marks the endothelial lining of the dorsal aorta (DA) but not of adjacent posterior cardinal vein (PCV) at E12.5. (**B**) By contrast, *Notch1*-deficient cells populate the PCV but do not contribute to the DA. (**C**,**D**) At E14.5, complete labeling of the endothelial lining of the aorta and intrasomitic arteries is observed (C, arrowhead) consistent with NICD presence as detected with α-VLLS staining (D, arrowhead) of endothelial cells. (**E**) Whole-mount X-Gal staining of yolk sac of E16.5 N1::cre embryo showing labeling of umbilical arteries, whereas maternal vasculature and veins are not labeled. (**F**) Complete lack of contribution from *Notch1*-deficient, R26 *lac*Z-marked cells to the yolk sac vasculature (arrow). A normal contribution is seen to the capillary plexus (arrowhead). (**G**,**H**) N1 activity in adult vasculature labels endothelial cells and a few smooth muscle cells (arrowhead) in the arteries (G) and veins (H). Magnification: A,B,C,F, 20×; D,E,G,H, 40×.

a Notch1 signal at E14.5, as shown by α-VLLS staining (Fig. 5E,F). By contrast, cardiomyocytes were neither labeled by *N1::cre* nor by α-VLLS. In adults, endocardial staining persists in addition to complete labeling of the endothelial vasculature (including veins and coronary arteries; Fig. 4G,H; Fig. 5H). Likewise, virtually complete labeling of all heart valves (mitral, tricuspid, pulmonic and aortic) was observed, indicating that Notch1 activation occurred in their progenitors (Fig. 5G,H and not shown). In chimeric hearts, *Notch1*-deficient cells readily contributed to the cardiomyocyte lineage where *N1::cre* is inactive, indicating that Notch1 is not essential in this lineage (Fig. 5, compare I with J). As with the embryonic dorsal aorta, preliminary chimera analysis suggested a cell-autonomous requirement for Notch1 in the endothelial linings of the coronary and in the endocardium as Notch1-deficient cells were excluded from these cell types. (Fig. 5J). The complementary patterns of *N1::cre* activity and Notch1 function highlight the predictive power of this approach to identify the derivatives of cells with a developmental requirement for Notch1. The demonstration of a developmental role for Notch1 in valve development offers hope that the mouse can serve as a model for human *NOTCH1* haploinsufficiency, which has recently been associated with aortic valve disease (Garg et al., 2005).

Notch1 activation in the endoderm: intestine

All four Notch receptors and several Notch ligands are expressed in the embryonic and adult gut (van Es et al., 2005b). Conditional disruption of the common downstream effector of all four mammalian Notch proteins (Rbp-j) in adult intestine leads to massive differentiation of proliferative crypt cells into postmitotic goblet cells (van Es et al., 2005b), reminiscent of *Hes*1 deletion (Jensen et al., 2000). Currently, it is not known which of the four Notch receptors are crucial in suppressing secretory differentiation of crypt progenitors. Here, we address the role of Notch1 in this process. *Notch1* mRNA expression was confined to the proliferative crypt compartment of the small intestine of adult mice (see Fig. S5K in the supplementary material) (van Es et al., 2005b) overlapping with NICD1 staining (Fig. 6I).

Whereas during embryonic gut development no labeling of epithelial cells cell was observed (Fig. 6A), the adult small intestine of *N1::cre* mice displayed significant labeling along the cephalocaudal axis (see Fig. S3 in the supplementary material). Most proximal segments (duodenum) displayed a high frequency of *N1::cre* activation (Fig. 6B-G), whereas more distal segments (e.g. the ileum, not shown) contained only a few labeled crypt-villus structures. A similar labeling pattern was detected in the colonic epithelium of *N1::cre* mice, albeit to a lesser extent (not shown). The reduction in *N1::cre* activation distally was not a consequence of lack of *R26R* activity in these cells (see Fig. S3 in the supplementary material, and data not shown). Double immunohistochemical staining of X-Gal-stained tissues from *N1::cre* mice for differentiation markers indicated that all four epithelial types (goblet cells, Paneth cells, enteroendocrine cells and enterocytes) were *lac*Z-marked (Fig. 6D-G). Lineage tracing identified uniformly labeled monoclonal crypts feeding labeled cells into adjacent chimeric (polyclonal) villi (e.g. Fig. 6C). Within blue crypts, no unlabeled cells were detected, strongly suggesting that Notch1 activation occurred in a stem cell. Interestingly, we also observed scattered X-Gal-labeled cells in villi (Fig. 6H). Goblet cells were also positive for α-VLLS immunoreactivity (Fig. 6I and see Fig. S5L in the supplementary material), suggesting that, in addition to its possible function in stem cells, Notch1 signaling may also contribute to goblet cell differentiation (Zecchini et al., 2005), which is reminiscent of the role reported for Wnt-β-catenin-TCF signaling (van Es et al., 2005a).

To determine if Notch1 activation was essential for stem cell maintenance, we analyzed the contribution of *Notch1*-deficient cells to adult chimeric small intestine. Surprisingly, Notch1 was not essential to the maintenance of intestinal crypt progenitors, despite its robust expression and activation (Fig. 6L,M). We did, however, observe a significant increase in the acquisition of the secretory cell fates at the expense of enterocytes throughout the cephalocaudal axis in the absence of Notch1 (Fig. 6N,O). These effects were mild compared with those seen in *Rbp-j*-deficient animals (van Es et al., 2005b), indicating that regulation of the enterocyte-secretory fate-switch requires Notch1, but that Notch1 acts redundantly with another Notch receptor in stem cell maintenance in the crypt epithelium. The graded activity of N1::cre observed along the

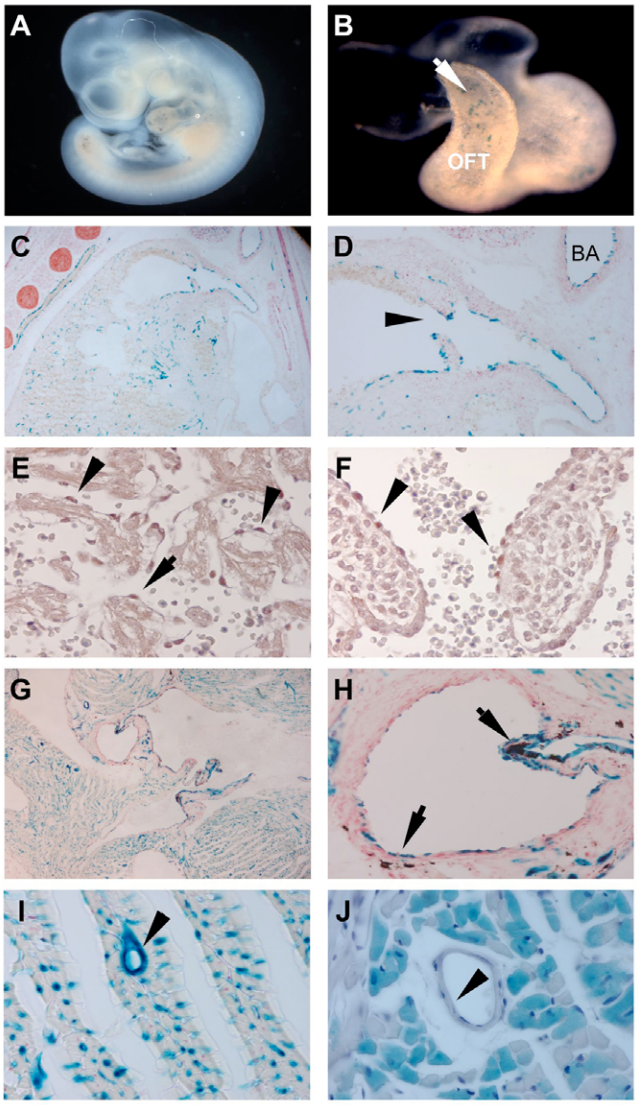

Fig. 5. Fate of Notch1-activated cells in the endocardial lineages. (**A**,**B**) Labeling of the heart (E10.5) in whole-mount (A) and after dissection (B). *Notch1* activity results in *lac*Z labeling within the outflow tract (OFT, arrow) and the ventricles (not shown). (**C**,**D**) Exclusive endocardial staining of the heart, the outflow tract and the lining of the valves (arrowhead) at E14.5. Myocardial cells are not stained. BA, branchiocephalic artery. (**E**,**F**) *N1::cre*-labeled endocardial cells continuously receive a Notch1 signal (arrowheads) at E14.5, as shown by α-VLLS staining. (**G**,**H**) In the adult heart, most endocardial cells retain label; the endothelial lining of the valve and the arteries (H, arrows), where Notch1 is required, are also labeled. (**I**,**J**) Comparison of the complementary staining patterns of endocardial cells (arrowhead) and cardiomyocytes in (I) *N1::cre* and (J) $N1^{-/-}$;$N1^{+/+}$;R26 *lac*Z chimeric hearts, respectively. Note that Notch1 is not active in cardiomyocyte cells (I) and also not required (J). Magnification: A,16×; B, 50×; C,G, 10×; D,H, 20×; E,F, 40×; I,J 63×.

cephalocaudal axis is in contrast with a constant requirement for Notch1 activity throughout the entire intestine, consistent with the interpretation that *N1::cre* also reports ligand density, thus revealing a higher-order organization not previously appreciated.

Colorectal cancer results from mutational activation of the Wnt pathway, most commonly owing to the loss of the tumor suppressor gene *Apc* (Bienz and Clevers, 2000). Consequently, mice carrying a mutated *Apc* allele ($Apc^{min/+}$) develop intestinal adenomas that require an activated Notch pathway for their survival (van Es et al., 2005b). *Notch1* and downstream *Hes* genes are also expressed and activated in adenomas that spontaneously arise in $Apc^{min/+}$ mice (Fig. 6J) (van Es et al., 2005b). To investigate whether the same target cell population that sustains *Apc* mutation in $Apc^{min/+}$ mice also experienced Notch1 activation, we analyzed $Apc^{min/+}$, *N1::cre* compound mice. We observed *lac*Z expression throughout entire dysplastic atypical foci and adenomas, suggesting that Notch1 activation was an early event during colorectal tumor formation (Fig. 6K). This analysis highlights the utility of *N1::cre* mice in marking cancer stem cells, and will facilitate screening for tumors where Notch1 activation is an early event and where inhibition of Notch signaling may be of therapeutic benefit for the treatment of these cancers.

DISCUSSION

Here we present the groundwork for high resolution in vivo mapping of vertebrate Notch1 activation. Although most if not all Notch1 activity depends on intramembrane proteolysis, which is monitored by our NIP-CRE approach, a different strategy will be needed to report proteolysis-independent functions of Notch1. We observed that the *N1::cre* fate maps are highly reproducible between mice from two independent ES cell lines and, in many tissues, correspond to known expression patterns of Notch target genes and reporter strains (Ohtsuka et al., 2006; Souilhol et al., 2006). This indicates that N1::cre activation patterns are not stochastic, reflecting authentic Notch1 activity. Significantly, in addition to confirming known activation patterns, our genetic labeling method combined with functional analyses identified important roles for Notch1 not appreciated in these transgenic reporters. We correlated *N1::cre* activation with function by comparing our results with the published literature and by following the fate of *lac*Z-marked Notch1-deficient ES cells during development and self-renewal. The survey described here demonstrates that Notch1 is activated in derivatives of all three germ layers and, in each, Notch1 has both redundant and non-redundant functions.

First, and unexpectedly, dependence on Notch1 function does not correlate with the probability of its activation. Limited activation of Notch1 in this particular reporter does not necessarily imply lack of an important function (in the somite, for example; see Fig. S5C in the supplementary material) (see also Huppert et al., 2005). High levels of Notch1 activation correlate well with its essential role in T-cell development and, as we show here, in arterial and endocardial/valve development. However, high levels of activation do not necessarily indicate an essential role (in the intestinal stem cell, for example). The mechanistic basis for this observation is not understood. However, an essential role for canonical Notch signals in intestinal ES cells is demonstrated by the impact of γ-secretase inhibitors or loss of *Rbp-j* (van Es et al., 2005b).

Second, a requirement for Notch1 activity was revealed even when other Notch receptors are present. For example, Notch4 is expressed in the arterial endothelial cells, but only Notch1 is essential (Limbourg et al., 2005). Interestingly, venous endothelial cells also became labeled postnatally. This finding would be consistent with a novel role for Notch1 during maintenance of venous endothelial cells that is distinct from the cell-fate choices mediated by Notch1 during the specification of arterial versus venous identity. Recently, it was demonstrated that vein identity is controlled by the orphan receptor CouptfII (Nr2f2 – Mouse Genome Informatics) repressing Notch signaling (You

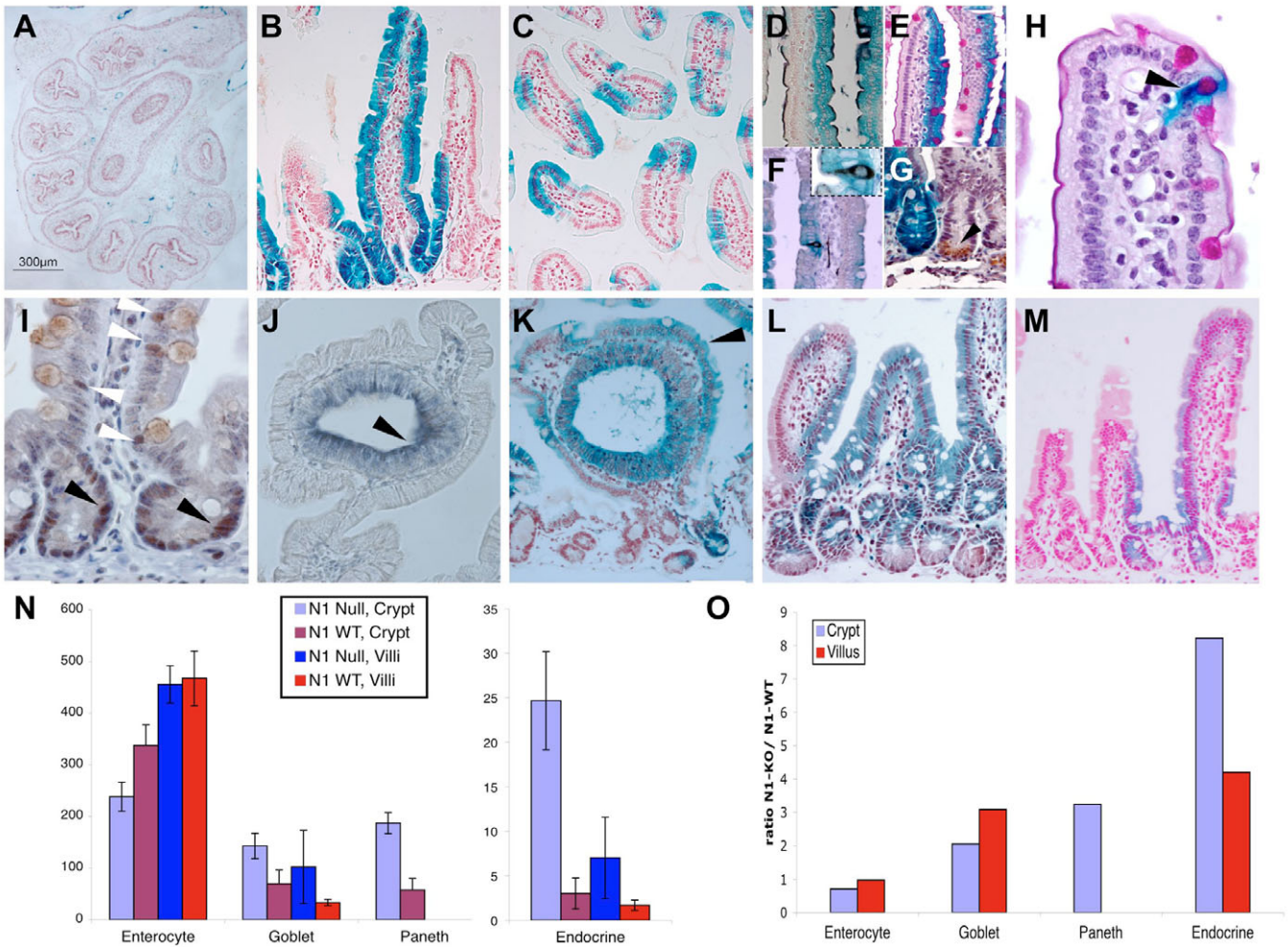

Fig. 6. Fate of Notch1-activated cells in the intestinal lineages. (**A**) N1::cre is not activated in the embryonic gut at E14.5. (**B**,**C**) Abundant activity of N1::cre in the adult duodenum labels the complete crypt-villus axis. Within blue crypts, all cells appear labeled, suggesting monoclonality (B), but polyclonal villi show alternating patterns of Notch1 activity (C). (**D-G**) Immuno identification of N1::cre descendents as enterocytes by alkaline phosphatase activity (D), goblet cells by PAS staining (E), endocrine cells by synaptophysin staining (F, inset), and Paneth cells by lysozyme staining (G, arrowhead). (**H**) Infrequent labeling of single goblet cells (arrowhead) suggests Notch1 signaling may also occur in (committed) differentiated intestinal epithelium. (**I**) α-VLLS staining identifies Notch1 signaling in crypt progenitors (black arrowhead) and in a few scattered goblet cells (white arrowheads) within the villus (see also Fig. S5J in the supplementary material). Notch1 α-VLLS staining is nuclear; the precipitate in the cytoplasm of goblet cells may be an artifact. (**J**) Notch1 is expressed in spontaneous adenomas from $Apc^{min/+}$ mice (arrowhead) and in adenomas from $Apc^{min/+}$:N1::cre mice, indicating Notch1 activation in cells sustaining Apc mutation. (**K**) The normal villus epithelium does not express Notch1 but N1::cre marks this lineage (arrowhead). (**L**) Notch1-deficient ES cells contribute efficiently to the adult intestinal epithelium of chimeric mice; they preferentially differentiate towards the secretory lineages at the expense of enterocytes. Note the significant increase in mucin-producing goblet cells in X-Gal-stained villi and crypts compared with unstained wild-type intestine. (**M**) Control *Notch1* wild-type R26-*lac*Z chimeric intestines show no preference to differentiate towards the secretory lineages. (**N**,**O**) Quantitation of the differentiation defect observed in *Notch1*-deficient intestines by combining immunohistochemical staining for differentiated cell types with X-Gal staining to identify Notch1-deficient cells, expressed as absolute numbers with s.d. (N) and as a ratio of *Notch1*-knockout/*Notch1*-proficient to wild type (O). Note significant increase in all secretory lineages in the absence of Notch1 at the expense of enterocytes. The intestines of mice composed of wild-type R26-*lac*Z cells showed a normal contribution and no defects (not shown). Magnification: A, 4×; B-G,J-M, 20×; H,I, 40×.

et al., 2005). Our results suggest that such repression of Notch1 activity may be relieved after establishing venous endothelial fate.

Third, whereas the notion emerging from expression profiling experiments suggests a common role for Notch1 in maintaining stemness (Ivanova et al., 2002; Ramalho-Santos et al., 2002), a more complex view surfaces from the fate map encompassing the four stem cell compartments surveyed here. Notch1 is highly activated in differentiating keratinocytes during an early developmental window, but the adult epidermis and hair follicles emerge from a progenitor population that did not experience Notch1 activation, do not contain NICD1, and are not labeled with Hes-Gfp or NAS, general reporters of Notch pathway activity. Loss-of-function analysis confirms that epidermal stem cells are not depleted when Notch1 is absent. High levels of Notch1 activation are observed in the intestine but Notch1 is not required for maintenance of this niche, indicating that other Notch receptors may act non-redundantly here, or that Notch1 is redundant with other Notch receptors. By contrast, during endothelial/endocardial development, a strict correlation between Notch1 activation and function was observed (Limbourg et al.,

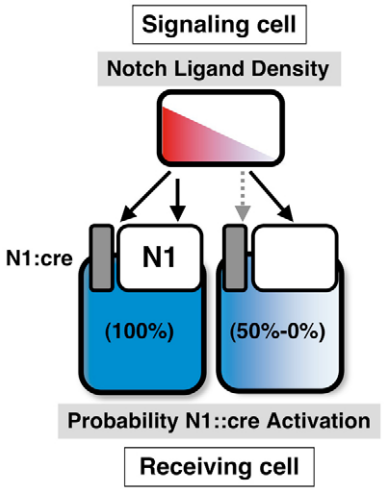

Fig. 7. Notch ligand density and N1::cre genetic mapping. A hypothetical model to explain the relationship between the strength of Notch signaling and fate mapping as a function of ligand concentration. The probability of identifying a marked lineage in tissues within N1::cre mice is correlated with Notch1 function as revealed by loss-of-function phenotypes and the ability to detect activated Notch1 protein by immunostaining (see Table1).

Table 1. Correlation between *N1::cre* fate map, α-VLLS staining and Notch1 loss-of-function in tissues

Tissue	*N1::cre*	LOF	α-VLLS
Intestine	+	Mu	+
Vasculature	+	Mu	+
Endocardium	+	Mu	+
CNS	+	Mu	+
Epidermis	+/–	WT	+
Kidney	+/– –	WT	–
Somites	+/– –	Mu	+/–
Pancreas	+/– –	WT	–

LOF, loss-of-function; Mu, mutant phenotype; WT, wild type.
+, staining; +/–, some staining; +/– –, very infrequent staining; –, no staining.
Note abundant staining throughout the vasculature of kidney but only infrequent staining of proximal tubules and no staining of podocytes where α-VLLS staining is positive but no effect of loss of Notch1 function is seen (see text for details).

2005), despite the presence of Notch4. In the vertebrate nervous system, Notch1 ligands have been suggested to play a key, γ-secretase-dependent role in stem cell survival, but a role for a Notch receptor in this process was not demonstrated (Androutsellis-Theotokis et al., 2006). Whereas N1::cre is active in early progenitor/stem cells within the retina and the ventral neural tube, *N1::cre* activation is not evident in a stem cell contributing to the cortex (data not shown) or to the cerebellum. Likewise, hematopoietic stem cell labeling could not be assessed directly in this reporter strain, but B-cells were derived from progenitors lacking *lac*Z activity (see Fig. S4B,C in the supplementary material). In both cases, either the low probability of Notch1 activation explains the lack of label, or Notch1 does not function in the CNS and in the definitive hematopoietic stem cell (HSC) in the manner suggested. One implication of our finding is that HSCs may emerge from dorsal aorta endothelial cells before they experience high Notch activation, or that definitive HSCs emerge from another, non-endothelial origin. Taken together, our data do not support a global assignment of Notch1 function in stem cells but demonstrate a recurrent function in the differentiating descendents.

Fourth, the lower expression of *N1::cre* as compared with the wild-type *Notch1* allele (see Fig. S1 in the supplementary material) is fortuitous as it permits mosaic analysis. In tissues exposed to high levels of Notch1 ligand, *N1::cre* is easily activated. By contrast, in tissues with low levels of Notch1 ligand, *N1::cre* ineffectively competes with the wild-type allele and only a few cells are marked. We therefore propose that the *N1::cre* map reflects ligand densities as well as recording the consequences of Notch1 activation (Fig. 7). Based on this assumption, the somite, pancreas, hematopoietic/CNS and epidermal/bulge stem cells may all have a low probability of Notch1 activation, perhaps because functional ligand is scarce, whereas arterial endothelial cells, T-cells, endocardial cells, retinal progenitors and the more proximal small intestinal stem cells have a high probability of Notch activation. Our analysis provides in vivo experimental support for the existence of context-dependent thresholds for Notch1 activation and that Notch signaling functions in a dose-dependent manner (Guentchev and McKay, 2006). The molecular bases for these differences are not clear but may reflect the abundance of functional ligand, controlled by ubiquitination and endocytosis (Schweisguth, 2004).

Finally by comparing the timing of *lacZ* activity with the presence of NICD1 one can appreciate that lineage-labeling in N1::cre appears to be delayed by several days. Furthermore, Notch1 activity in some lineages may be left unreported in these mice because of non-uniform reporter expression or because of poor Cre-mediated recombination at the *R26R* locus (Vooijs et al., 2001), although this appears not to be true for the epidermis. Obviously, lineages that undergo apoptosis in response to Notch1 activation require other methods of detection (Yang et al., 2004). Our results in the venous endothelium and the intestine suggest that, similar to the fly peripheral nervous system and the mouse hematopoietic system (Radtke et al., 2004a), Notch1 signaling may be utilized in a recurrent fashion to influence multiple cell fate decisions. Further refinement of the approach presented here, employing hormone-inducible *N1::cre* alleles, will allow the interrogation of consecutive uses of the Notch1 signaling pathway in any given cell type under physiological conditions and in disease processes.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/3/535/DC1

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