Second-generation Notch1 activity-trap mouse line (N1IP::Cre<sup>HI</sup>) provides a more comprehensive map of cells experiencing Notch1 activity

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**ABSTRACT**

We have previously described the creation and analysis of a Notch1 activity-trap mouse line, Notch1 intramembrane proteolysis-Cre6MT or N1IP::Cre<sup>LO</sup>, that marked cells experiencing relatively high levels of Notch1 activation. Here, we report and characterize a second line with improved sensitivity (N1IP::Cre<sup>HI</sup>) to mark cells experiencing lower levels of Notch1 activation. This improvement was achieved by increasing transcript stability and by restoring the native carboxy terminus of Cre, resulting in a five- to tenfold increase in Cre activity. The magnitude of this effect probably impacts Cre activity in strains with carboxy-terminal Ert2 fusion. These two trap lines and the related line N1IP::Cre<sup>ERTZ</sup> form a complementary mapping tool kit to identify changes in Notch1 activation patterns in vivo as the consequence of genetic or pharmaceutical intervention, and illustrate the variation in Notch1 signal strength from one tissue to the next and across developmental time.

**KEY WORDS:** Activity, Cre, Neuron, Notch1, Kidney

**INTRODUCTION**

The Notch signaling pathway is an evolutionarily conserved, short-range communication mechanism utilized throughout life in all metazoan species (Artavanis-Tsakonas et al., 1999). The binding of a ligand to a Notch receptor triggers the unfolding of a juxtamembrane negative regulatory region (NRR), which exposes it to cleavage by the ADAM10 protease (a disintegrin and metalloprotease) at an extracellular sequence termed S2 (Groot et al., 2013; Mumm et al., 2000; van Tetering et al., 2009). S2 cleavage leads to the shedding of the extracellular domain and allows γ-secretase to cleave Notch within its transmembrane domain at S3, releasing the intracellular domain of Notch (NICD) from the membrane. Three nuclear localization signals within NICD provide for nuclear translocation, where it complexes with RBPjk, recruits the MAML adaptor and the transcriptional activator p300 (Ep300 – Mouse Genome Informatics) to the promoters of target genes (Kopan and Ilagan, 2009).

Whereas only one receptor (Notch) and two ligands (Delta and Serrate) are present in *Drosophila*, mammals have four receptor paralogs (Notch1–4) and at least five canonical ligands (Dll1, Dll3, Dll4, Jag1 and Jag2). These receptors and ligands exhibit complex expression patterns and play essential roles both during development and in the maintenance of adult tissue homeostasis, which is evident from various Notch-related congenital diseases and tumors in human patients (Koch and Radtke, 2010; Penton et al., 2012) and in mice lacking Notch receptors or ligands in various tissues (Conlon et al., 1995; Demehri et al., 2008; Gale et al., 2004; Hamada et al., 1999).

Several methods have been developed and used to report Notch activity, each with specific strengths and weaknesses [reviewed by Ilagan et al. (2011)]. Taking advantage of the fact that the intracellular domain of Notch (NICD) requires ligand-induced proteolysis to be released from the cell membrane, we have previously described the creation and analysis of a Notch1 activity-trap mouse line, Notch1 intramembrane proteolysis-Cre or N1IP::Cre (Vooijs et al., 2007) (Fig. 1A,E). In this knock-in line, the coding sequence for phage recombinase Cre, which was followed by six myc tags (6MT) and preceded by a nuclear localization signal (NLS), was targeted into one Notch1 allele within exon 28, encoding the transmembrane domain of Notch1 (Fig. 1E). This targeting strategy resulted in the expression of a Notch1-NLSCre6MT fusion protein from this locus, in which the intracellular domain of Notch1 is completely replaced by NLSCre6MT. The binding of the ligand to cells expressing Notch1 and Notch1-NLSCre6MT fusion protein triggers the release of NICD and the NLSCre6MT protein, respectively. Whereas the NICD permits normal development, NLSCre6MT, once inside the nucleus, could catalyze the removal of any floxed allele in the genome. If a reporter is present, Cre activity will ‘trap’ the cell and permanently mark its descendent lineage independent of any further Notch1 activation (Fig. 1A). We have used this line to show that this activity-trap strategy could identify lineages experiencing Notch1 activation (Vooijs et al., 2007).

Despite our success, this NIP-Cre reporter line is relatively insensitive and seemed to predominantly trap cells in which high or repeated activation cycles are known to occur (e.g. endothelium), while missing cells in many tissues known to rely on Notch1 activity [e.g. the hematopoietic system, (Vooijs et al., 2007)]. We speculated that this could be caused by less-efficient transcription from the manipulated allele and reduced activity by the insertion of the 6MT (Vooijs et al., 2007). It was also hypothesized that, in some tissues, sequences required for proper trafficking of Notch to the membrane were lost when NICD was replaced with Cre.

Here, we report that removing the 6MT and adding an additional polyadenylation signal significantly improved sensitivity, allowing us to trap cells experiencing lower Notch activation thresholds. When compared with the original N1IP-Cre reporter (now designated as N1IP::Cre<sup>LowActivity</sup> or N1IP::Cre<sup>LO</sup>), this new line (N1IP::Cre<sup>HighActivity</sup> or N1IP::Cre<sup>HI</sup> allele) traps Notch1 activation in most tissues known to rely on Notch1, and identifies novel cell utilizing Notch1 during their development and/or maintenance, some of which will be reported elsewhere. Importantly, biochemical analysis indicates that this new allele is cleaved as efficiently as...
Notch1, supporting our previous observation that Notch trafficking relies on signals coded in the extracellular domain (Liu et al., 2013). This trap line, N1IP::CreLO and the related sibling N1IP::CreERT2 (Liu et al., 2009; Pellegrinet et al., 2011) line, are powerful new tools for mapping alteration in Notch1 activation in vivo as a consequence of genetic or pharmaceutical intervention.

RESULTS AND DISCUSSION
Biochemical analyses of N1IP::Cre constructs demonstrate that a 6MT C-terminal fusion reduces Cre activity five- to tenfold.

N1IP::Cre mice contain a 6MT fused to the C-terminus of the Cre recombinase protein (Fig. 1A,E). Similar Cre fusions described elsewhere include CreERT2 (Liu et al., 2009; Pellegrinet et al., 2011) line, are powerful new tools for mapping alteration in Notch1 activation in vivo as a consequence of genetic or pharmaceutical intervention.

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Fig. 1. Creation of N1IP::CreHI. (A) Principle of cleavage-dependent Notch reporter. The replacement of Notch intracellular domain with Cre recombinase leads to the release of Cre, but not of NICD, from the cell membrane after ligand-induced S2 and S3 cleavage. Cre will activate reporters, such as Rosa lacZ or Rosa EYFP, which will remain active in all descendants. (B-D) Comparison of the enzymatic activity of Cre and Cre6mt fusion proteins. (B) Structure of the plasmids used in the analysis. (C) Schema of MEF preparation and testing. (D) Comparison of β-gal activity in R26R-MEF cells expressing similar levels of luciferase (note that the data are plotted on a log scale). (E) Comparison of N1IP::CreLO and N1IP::CreHI targeted locus. ANK, ankyrin repeats; LNR, Lin-Notch repeat; TM, transmembrane domain; PEST, proline/glutamic acid/serine/threonine-rich motif; 2pA and 3pA, 2× and 3× polyadenylation signal. (F) Comparison of mRNA levels among Notch1, N1IP::CreLO and N1IP::CreHI in 9.5-day embryos. (G) Comparison of released Cre levels between N1IP::CreLO and N1IP::CreHI in newborn kidneys by western blot using anti-V1744 antibody. (H) Quantification of released Cre relative to the released NICD in the same sample.
recombinase activity within the population, whereas luciferase activity will report relative output from the Cre-expressing plasmids (transfection efficiency), independent of their recombinase activity.

When MEFs were transfected with increasing DNA concentrations, luciferase activity scaled linearly throughout the entire range (from 7.8 to 500 µg, supplementary material Fig. S1), demonstrating that Cre and Cre-6MT were expressed at similar levels. However, β-Gal activity in extracts prepared from transfected cells indicated that NLSCre6MT recombinase activity was significantly diminished relative to Cre at every concentration (supplementary material Fig. S1). Notably, measurable β-Gal activity could be detected at the lowest transfected Cre concentration and continued to increase in a relatively linear fashion, whereas NLSCre6MT activity increased only above 60 ng (supplementary material Fig. S1). To compare Cre activity at similar protein concentrations, we transfected the MEFs with plasmid concentrations yielding identical photon flux and measured β-Gal activity (Fig. 1D). Even at this high plasmid concentration a sevenfold difference was noted between Cre and Cre6MT (Fig. 1D). These results indicate that our original strain, N1IP::Cre mice (Vooijs et al., 2007) and, most likely, the related line N1IP::CreERT2 (Pellegrinet et al., 2011), under-report Notch activity in vivo. This conclusion applies to all CreERT2 lines driven by weak promoters, and could explain the differences in labeling observed when different Cre fusion proteins are expressed from the same locus as in Grisanti et al. (2013).

**The creation of N1IP::CreHI reporter mice**

An unfused activity-trap reporter might be able to trap Notch1 activity at lower thresholds missed with the Cre6MT protein. To generate mice with improved Notch-trap ability, we modified our targeting constructs to create a new line of knock-in reporter mouse, N1IP::creHi (Fig. 1E; supplementary material Fig. S2). First, we removed the construct to create a new line of knock-in reporter mouse, N1IP::creHi mice with improved Notch-trap ability, we modified our targeting constructs to increase the relative abundance of the N1IP::creHi mRNA and to fully restore Cre activity.

To directly compare the mRNA level of N1IP::creHi and N1IP::creLo, we mated Notch1CreHI (N1IP::creHi heterozygote) to Notch1CreLO (N1IP::creLo heterozygote) mice and collected E9.5 Notch1CreHI/CreLO embryos. These animals have no functional NICD and do not survive beyond E10.5. Notch1CreLO embryos served as a control. From these, we purified total RNA for RT-PCR and performed pyrosequencing to compute the ratio of adenine (N1IP::creHi) to cytidine (wild-type Notch1 in Notch1CreHI embryos or N1IP::creLo in Notch1CreLo embryos) at nucleotide 5178 in exon 28. The results show that the addition of the extra polyadenylation signal increases the stability of N1IP::creHi mRNA ~twofold, to a level comparable to that of the endogenous Notch1 [Fig. 1F; (Liu et al., 2011)]. To compare the amount of the Cre protein that is released from the cell membrane after ligand binding from the N1IP::creHi and N1IP::creLo proteins, we performed western blot on wild type, N1IP::creHi heterozygote and N1IP::creLo heterozygote newborn kidneys with an antibody against cleaved Notch1 (V1744), thus normalizing the level of Cre to the NICD produced from the cleaved endogenous Notch1. Consistently, we found a twofold increase in the abundance of cleaved Cre relative to Cre6MT (Fig. 1G,H).

Importantly, because N1IP alleles are cleaved as effectively as the endogenous Notch1 allele in the kidney (Fig. 1G,H), they establish unequivocally that sequences in the intracellular domain of the Cre ORF, thus matching the total number of polyadenylation signals (three) found in the wild-type Notch1 transcript. Finally, we substituted a cytidine for an adenine at position 5178 of exon 28, corresponding to a silent mutation at the third position for L1726 in Notch1 (Fig. 1E; supplementary material Fig. S2). This offers a simple, fast and non-radioactive method to pre-screen embryonic stem cells (ESCs) for homologous recombination with pyrosequencing (Liu et al., 2009), and enabled us to compare the abundance of mRNA produced by each allele (Liu et al., 2013, 2011). We expected these modifications to increase the relative abundance of the N1IP::creHi mRNA and to fully restore Cre activity.
arteries were sparsely labeled in the and phenotypes (Gridley, 2010). By contrast, the inter-segmental arterial endothelial cells, consistent with genetic observations. This indicates that Notch1 is activated earlier than E10.5 in segmental arteries (Fig. 2F, yellow arrows; Fig. 2L, black arrows).

Comparing N1IP::CreHI and N1IP::CreLO in vivo
N1IP::CreHI; RosaR26R/R26R sires were mated with RosaR26R/R26R dams (Soriano, 1999) to trap cells experiencing Notch1 activation. Consistent with the data showing increased Cre protein level and activity, we found that, throughout early embryonic development, N1IP::CreHI embryos were more intensely labeled by whole-mount β-Gal staining than N1IP::CreLO embryos (4°C overnight, Fig. 2). Consistent with what we observed in vitro, N1IP::CreHI-trapped cells experienced Notch1 activation earlier than N1IP::CreLO at all embryonic stages (Fig. 2). Under the same X-gal development conditions, N1IP::CreHI; RosaR26R/R26R embryos contained many labeled cells in the heart (Fig. 2E, asterisk) and the notochord (Fig. 2E, white arrowhead; Fig. 2J, black arrowhead) by E9.5. By contrast, no signal was detected in N1IP::CreLO; RosaR26R/R26R embryos before E10.5, when the only labeled cells were found in the heart [Fig. 2A,B; (Vooijs et al., 2007)]. At E10.5, more cells were labeled in N1IP::CreHI hearts (Fig. 2F, yellow arrows; Fig. 2L, black arrows). This indicates that Notch1 is activated earlier than E10.5 in arterial endothelial cells, consistent with genetic observations and phenotypes (Gridley, 2010). By contrast, the inter-segmental arteries were sparsely labeled in the N1IP::CreLO mice at E11.5 (Fig. 2C). Accordingly, N1IP::CreHI/R26R trans-heterozygotes, in which the activated N1IP::Cre excises the floxed Notch1 allele, suffer embryonic lethality at E10.5 due to the developmental failure of the vascular system in the yolk sac and the embryo (Liu et al., 2011), similar to the result of early endothelial cell-specific conditional knockout of Notch1 (Limbourg et al., 2005) with either one of two available Tie2-Cre strains (Kisanuki et al., 2001; Koni et al., 2001). By contrast, all N1IP::CreLO/R26R trans-heterozygotes survive without discernible phenotype to P50 (Liu et al., 2011). Together, these data suggest that N1IP::CreHI reports the activation of Notch1 in the endothelium with high fidelity, trapping nearly all cells, whereas N1IP::CreLO reported activation only in cells experiencing substantially higher Notch activation levels (Vooijs et al., 2007). Interestingly, removal of ADAM10 with Tie2-Cre (Kisanuki et al., 2001) was not lethal (Zhao et al., 2014), perhaps allowing sufficient activity to persist through an early, crucial developmental window. The use of these lines to elucidate the role of signal strength in endothelial and hematopoietic lineage separation from hemogenic endothelium will be described elsewhere.

At E15.5, staining of sagittal sections showed widespread labeling in the neural tube, brain, retina, major blood vessels, intestine and vertebrae (Fig. 2I), correlating well with the reported role of Notch1 in these tissues. At P0, a majority of the brain is labeled and nerve fibers in the head region can be clearly identified with β-Gal staining (Fig. 2M). Recently, it has been suggested that Notch signaling regulates thyrocyte differentiation (Carre et al., 2011; Ferretti et al., 2008). Accordingly, we observed widespread labeling in the thyroid gland at P0 (Fig. 2M, white arrow).

To assess the behavior of N1IP::CreHI with a different reporter strain, we crossed N1IP::CreHI and N1IP::CreLO with RosaYFP mice (Srinivas et al., 2001). We collected kidneys at post-natal day 28 (p28) and compared the labeling patterns in the kidney cortex. The entire CD31+ vascular tree within glomeruli is labeled in both N1IP::Cre lines (Fig. 3, arrows; see also (Liu et al., 2013)), consistent with the robust activation of Notch1 during the development and maintenance of endothelial cells (Gridley, 2010). Despite the activation of Notch1 in the proximal tubule epithelium during kidney development and its known contribution to the maturation of these cells (Surendran et al., 2010a,b), very little labeling is detected within this compartment in N1IP::CreLO; RosaYFP mice. By contrast, a majority of proximal tubule epithelia are Notch1-lineage positive in N1IP::CreHI; RosaYFP mice (Fig. 3, arrowheads), consistent with its role in the development and in regulation of tubule diameter (Liu et al., 2011).
consistent with the first.

former possibility; the intense labeling of distal tubule is connecting segment (11.18±1.8%) is more consistent with the decline in labeling frequency from the proximal tubules or the result of background proteolytic release of Cre. The was insufficient to divert some cells from assuming a distal fate, might indicate a physiological reality in which signal strength very few connecting segment cells were labeled (Fig. 4). This number of distal tubule cells experienced N1 activation, but detected (Liu et al., 2013). In the Ai3 kidneys, a significant tubules of the kidney, where Notch1 expression is too low to be to determine the false-positive labeling rates in the distal recombination and expression are highly efficient and served labeling patterns in the most sensitive reporter line we have available, Ai3 (Madisen et al., 2010). The Ai3 reporter recombination and expression are highly efficient and served to determine the false-positive labeling rates in the distal tubules of the kidney, where Notch1 expression is too low to be detected (Liu et al., 2013). In the Ai3 kidneys, a significant number of distal tubule cells experienced N1 activation, but very few connecting segment cells were labeled (Fig. 4). This might indicate a physiological reality in which signal strength was insufficient to divert some cells from assuming a distal fate, or the result of background proteolytic release of Cre. The decline in labeling frequency from the proximal tubules (~100%) to the thick ascending limb (62.29±5.2%) to the connecting segment (11.18±1.8%) is more consistent with the former possibility; the intense labeling of distal tubule is consistent with the first.

These data demonstrate that the N1IP::CreLO allele only labels the subset of cells that experience high and/or persistent Notch1 activation. Removing the 6MT increased labeling efficiency in many tissues where Notch1 has a known function. It is worth noting that, although N1IP::CreHI exhibits vastly improved sensitivity over N1IP::CreLO, not all known Notch-mediated decisions are captured. For instance, we did not observe labeling in the somite at E9.5 and E10.5 (Fig. 2E,F,J, K), where Notch1 plays an important role at very low activation levels (Conlon et al., 1995; Huppert et al., 2005; Lewis, 2003).

2013; Surendran et al., 2010a). These data demonstrate that removal of the 6MT and addition of a third polyA site result in higher recombinase Cre activity in vivo, consistent with our in vitro findings.

Although these patterns alleviate the concern that N1IP::CreHI might over-report Notch1 activity, it is possible that a reporter that recombinates at lower Cre concentrations will generate a false-positive signature of Notch1 activity. To evaluate the potential for false positives, we examined the labeling patterns in the most sensitive reporter line we have available, Ai3 (Madisen et al., 2010). The Ai3 reporter recombination and expression are highly efficient and served to determine the false-positive labeling rates in the distal tubules of the kidney, where Notch1 expression is too low to be detected (Liu et al., 2013). In the Ai3 kidneys, a significant number of distal tubule cells experienced N1 activation, but very few connecting segment cells were labeled (Fig. 4). This might indicate a physiological reality in which signal strength was insufficient to divert some cells from assuming a distal fate, or the result of background proteolytic release of Cre. The decline in labeling frequency from the proximal tubules (~100%) to the thick ascending limb (62.29±5.2%) to the connecting segment (11.18±1.8%) is more consistent with the former possibility; the intense labeling of distal tubule is consistent with the first.

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N1IP::CreHI labeling patterns in the reproductive organs

The Notch signaling pathway plays essential roles in mediating the interactions between the stem cells and their niche in various organs, including the reproductive organs in Drosophila (Kitadate and Kobayashi, 2010; Song et al., 2007; Ward et al., 2006) and C. elegans (Hubbard, 2007). Whereas in the Drosophila ovary the Notch receptor is expressed in the niche, the receptor is required in the germ line stem cells in C. elegans. In rodent ovaries, Notch2 is expressed in granulosa cells and the Jag1 ligand is expressed in the germ cells (Johnson et al., 2001; Vanorny et al., 2014; Xu and Gridley, 2013). The ovary of sexually mature virgin N1IP::CreHI, Rosa−/R26R females showed ubiquitous labeling in granulosa cells (Fig. 5F), suggesting that Notch1 acts with Notch2 in the ovary but is not activated in the oocyte (Vanorny et al., 2014). Accordingly, we did not observe germline inheritance of an active reporter in offspring of N1IP::CreHI, Rosa−/R26R females.

The role of Notch in the rodent testis remains controversial. Some reports suggest a possible role for Notch1 in the niche during spermatogenesis (Dirami et al., 2001; Hayashi et al., 2001; Murta et al., 2013), but functional studies could not demonstrate an essential function for Notch1 (Batista et al., 2012; Hasegawa et al., 2012). If N1IP::CreHI is activated in germ cells during spermatogenesis, it will result in germline inheritance of an active reporter, manifest in uniform lacZ expression (stained blue by X-gal) in offspring of N1IP::CreHI, Rosa−/R26R/R26R fathers. However, we only saw three blue embryos out of more than 100 embryos that we stained, suggesting that Notch1 activation in germ cells during spermatogenesis is an infrequent event or too low to be detected by this line. Examination of the adult testis revealed extensive N1IP::CreHI activation within Sertoli cells (as marked by SOX9 and TUBB3; Fig. 5I), consistent with previous studies showing transgenic Notch Reporter GFP (TNR-GFP) activation in Sertoli cells during multiple developmental stages (Garcia et al., 2013; Hasegawa et al., 2012). There was also N1IP::CreHI
activation within the interstitial compartment of the testis, probably within vascular endothelial cells or perivascular cells, both of which express Notch receptors and activate TNR-GFP during adulthood (DeFalco et al., 2013).

By contrast, widespread labeling was detected as early as at E12.5 in the mesonephric tubules, the precursors of the epididymis (Fig. 5A), persisting to the maturing epididymis (Fig. 5B,C). The Wolffian duct in female embryos was labeled before it degenerates into Gartner’s duct (Fig. 5E). Vasculature at the gonad-mesonephros junction also was labeled (Fig. 5E). Sections of newborn and adult epididymis showed that epithelial cells around the lumen of all major segments, including the initial segment, caput, corpus and tails are labeled, albeit at different frequencies (Fig. 5G,H). Anti-K14 and EYFP double-staining of sections from N1IP::CreHI; Rosa+/eYFP mice showed that all labeled cells are K14-negative principal cells (Fig. 5J). Stained particles in the lumen of epididymis (Fig. 5K) were negative for DAPI staining, suggesting that they are not sperm but rather epididymosomes, membranous vesicles that are derived from principal cells of the epididymis (Saez et al., 2003). This is consistent with the idea that Notch1 activation might not occur during spermatogenesis, but instead in the epididymis, which is essential for the maturation of the sperm. However, assignment of functional significance will necessitate further studies.

**N1IP::CreHI labeling pattern in the neural tissue**

Notch activity is dispensable for establishing the neuroepithelium as all Notch pathway mutants complete this process (de la Pompa et al., 1997; Oka et al., 1995). We noted faint labeling in the ventral half of the developing CNS (black arrow, Fig. 2F). To identify the labeled cells we sectioned E10.5 embryos that had been stained for β-Gal activity, and used a stereoscope to photograph the slices (Fig. 6A-H). Two discrete progenitor domains adjacent to the floor plate were strongly labeled in the diencephalon (Fig. 6A), midbrain (Fig. 6B), hindbrain (Fig. 6C,D) and spinal cord (Fig. 6E-H). Labeling of subpopulations of early-
born neurons in the dorsal aspect of the midbrain and hindbrain was also detectable (Fig. 6B-D). This stripe pattern had been observed previously with Dll1 and Jagged1 expression in the chick hindbrain and neural tube (Marklund et al., 2010; Myat et al., 1996). To align Notch activity stripes with its ligand we observed previously with Dll1 and Jagged1 expression in the chick hindbrain and neural tube (Marklund et al., 2010; Myat et al., 1996). To align Notch activity stripes with its ligand we crossed Dll1+/lacZ (Hrabe de Angelis et al., 1997) mice with N1IP::CreHI, RosaEYFP/eYFP mice to generate E10.5 embryos heterozygous for Dll1, Notch1 and Rosa-eYFP. Staining for lacZ (Dll1 expression, Fig. 6I), EYFP (Cre activity) and Jagged1 confirmed that the cells experiencing Notch activation overlapped with the ligand Dll1 (Fig. 6I). To identify the cells that experienced high levels of Notch1 activity we stained a section of the neural tube from N1IP::CreHI; Rosa+/Ai3 mice with Olig2 and Nkx2.2, which confirmed that progenitors in the pV2 and pV3 domains were labeled much more intensely than those in the adjacent pMN and pV1 domains (Fig. 6I). We and others have shown previously that Notch1 signaling was necessary to specify neuronal subtypes, in particular V2 neurons and motor neurons in the spinal chord (Del Barrio et al., 2007; Misra et al., 2014; Yang et al., 2006). It would be interesting to investigate whether V2 neuron specification requires higher Notch1 signal strength than other neuronal cell types.

In the CNS, neural progenitors undergo multiple rounds of asymmetric division. Notch signaling oscillates to keep progenitors cycling between committed progenitor state (Hes target gene expression detected, proneural genes repressed) and pre-neuron [proneural gene expression detected, Hes1 repressed; (Imayoshi et al., 2013; Kobayashi and Kageyama, 2010; Shimojo et al., 2008, 2011)]. After each division, the neuron daughter cell is thought to activate Notch in its sister to reinforce the progenitor fate. A perfect activity trap would label progenitors after the first Notch-dependent decision, trapping all of their subsequent descendants (Fig. 7A). Analysis of cortical layers from N1IP::CreLO; Rosa+/Ai3 (Fig. 7B; supplementary material Fig. S3) shows co-labeling of EYFP (N1IP::CreHI activity) and Foxp1 (a neuronal marker for late-born upper layer neurons: Foxp1 labels neurons in layers II-IVa) in E15.5 cortical neurons (Fig. 7B;B′; supplementary material Fig. S3). EYFP labeling was also detected in FoxP1+ earlier born neurons and in stem/progenitor cells in the ventricular and subventricular zones (VZ/SVZ) (supplementary material Fig. S4; arrows). By contrast, we detected no EYFP labeling in Foxp1+ cortical neurons of N1IP::CreLO; Rosa+/Ai3 embryos (Fig. 7C; supplementary material Fig. S3). In addition, striatal projection neurons were labeled very efficiently in N1IP::CreHI; Rosa+/Ai3 but only marginally in N1IP::CreLO, Rosa+/Ai3 (Fig. 7; supplementary material Fig. S4). These data underscore the differences in activation threshold between Cre and Cre6MT (and, by inference, CreERT2).

The images in supplementary material Fig. S3 suggest that EYFP labeling is denser in upper layer neurons than in lower layer neurons and in progenitors located in the neocortex VZ/SVZ. Because Notch1 signaling is thought to be activated in every asymmetric progenitor division (de la Pompa et al., 1997; Imayoshi et al., 2010), two possible scenarios could fit this pattern. First, a common progenitor pool generates both lower layer (early) and upper layer (late) neurons, and late-stage progenitors receive stronger Notch1 signals. Alternatively, the sensitive trap allele is still not sensitive enough, and significant labeling requires gradual accumulation of Cre protein over time, and thus favors late-stage progenitors that produce upper layer neurons. We cannot rule out a third possibility that separate progenitor pools contribute to lower and upper layer neurons, with stronger signaling through the Notch1 receptor in the latter.

Summary

Here, we demonstrate that Cre activity is compromised by fusion of additional amino acids to the C-terminus. This observation implies that all Ert2 fusion proteins are less active than Cre even when equal nuclear concentrations are achieved, as shown in Grisanti et al. (2013). The comparison between the two N1IP::Cre lines reveals that Notch1 proteolysis, and thus signal strength, vary with time within the same tissue (e.g. early- versus late-born cortical neurons,
or anterior versus posterior stem cells along the intestine). Whether the increase in signal strength is a consequence of unrelated biology or an important requirement for the Notch signaling pathway remains to be determined.

**MATERIALS AND METHODS**

**Preparation of MEF cells**

MEF cells were prepared under sterile conditions using a modified protocol based on Takahashi et al. (2007) and Xu (2005). Briefly, RosaR26R/R26R dams were crossed to a CD1 sire and embryos (RosaR26R/R26R) were collected at E13.5. Head, visceral organs and the urogenital system were removed. The remaining tissues were pooled, washed three times with PBS and minced into ∼2 mm pieces, using a sterile razor blade in a 100 mm diameter dish containing 20 ml of warm Trypsin-EDTA (∼25 cuts per embryo). The cell mixture was pipetted repeatedly with a 25 ml pipette and incubated at 37°C for 15 min. After incubation, 10 ml of fresh Trypsin-EDTA was added, the tissue was further dispersed using a 10 ml pipette and placed at 37°C for an additional 15 min. The final cell mixture was transferred to a 50 ml conical tube, 20 ml of MEF medium (DMEM, 10% FBS, 2 mM glutamine, 1000 U/ml penicillin and 1000 µg/ml streptomycin) was added to total volume of 50 ml, and spun down at 1500 g for 5 min. The supernatant was discarded and the cell pellet remains to be determined.

**Generation of N1IP::CreHI knock-in mice**

In brief, the early SV40 polyadenylation sequence with one extra artificially introduced polyadenylation signal (AATAAA) was cloned in front of the FRT-flanked-neomycin selective cassette. The silent mutation in L1726 was introduced with a site-directed mutagenesis kit (Stratagene) into the SKB-L genomic clone that encompasses mouse Notch1 exons 26-32 (Vooijs et al., 2007). The SgrAI/BglII-digested NLsCre fragment and the Sall/BglII-digested SV40 polyadenylation sequence-FRT-neo cassette were then cloned into SKB-L through the SgrAI/BamHI site to replace the last 105 bases of exon 28 and to delete exons 29 and 30 (please note that BamHI and BglII share a compatible cohesive end, and after ligation the BamHI site is lost) (supplementary material Fig. S2). This plasmid was then sequenced and linearized with EcoRI for ESC electroporation.

**ESC targeting and screening**

We targeted the Notch1 locus using 129X1Sv/J-derived SCC10 ESCs (for further information regarding this line see http://escr.im.wustl.edu/SubMenu_celllines/WTEScell_linesSCC10.html) and selected for G418-resistant ESC colonies. Cells were first pre-screened with pyrosequencing, utilizing the C5178A polymorphism (Liu et al., 2009), and those with a C/A ratio between 40 and 60% at this position were further screened by both long-range PCR and Southern blot to confirm bona fide homologous recombinants [HR, (Liu et al., 2009); supplementary material Fig. S2]. Positive HRs were karyotyped, and one line (ES190) was injected into C57B6 blastocysts. The resulting chimera male mice were mated to wild-type C57B6 female mice. F1 males were mated to FRT deleter mice (Rodriguez et al., 2000) to remove the FRT-flanked neomycin cassette.

**Western blot**

Western blot was performed as described in Liu et al. (2013). Rabbit monoclonal anti-cleaved Notch1 (anti-V1744, Cell Signaling) antibodies were used at 1:1000 dilution.

**X-gal staining**

X-gal staining was performed as described in Vooijs et al. (2007). Briefly, embryos or freshly dissected tissues were fixed in 4% PFA in 1× PBS supplemented with 2 mM MgCl2 on ice for 1-2 h and washed with 1× PBS +2 mM MgCl2. For whole-mount staining, embryos were developed in X-gal staining solution at 4°C overnight; for frozen section staining, tissues were immersed in 30% sucrose in 1× PBS+2 mM MgCl2 overnight and
embedded in optimal cutting medium (OCT, Tissue-Tek) and stained in X-gal staining solution at 37°C overnight or until signal was detected.

**Immunohistochemistry**

Immunohistochemistry was performed as described in Liu et al. (2013). Briefly, frozen sections were blocked with normal donkey serum and then incubated with primary antibodies at 4°C overnight. Following three times washing with 1× PBS, the sections were incubated with fluorescence-conjugated secondary antibodies, washed with 1× PBS and mounted for imaging. The following primary antibodies were used: chicken anti-GFP (1:500; Aves Lab, GFP-1020) or rabbit anti-GFP (1:1000; Invitrogen, A11122), rabbit anti-Foxp1 (1:2000; Abcam, ab16645), mouse anti-Calbindin (1:500; Sigma-Aldrich, C9848), rabbit anti-Tamm-Horsfall protein (THP) (1:250; Biomedical Technologies, BT-590), rabbit anti-Claudin (1:200; Genex, GTX47709), rabbit anti-olig2 (1:500; Millipore, AB9610), goat anti-Nkx2.2 (1:50; Santa Cruz Biotech, SC-15015), chicken anti-Ki67 (1:200; a generous gift from Dr Julie Segre, National Human Genome Research Institute, National Institutes of Health), goat anti-Jag1 (1:100; Santa Cruz Biotech, SC-6011), rabbit anti-β-galactosidase (1:5000; Cappel Labs/MPI Biomedical, 08559761), rat anti-CD31 (1:100; BD Bioscience, 550274), goat anti-aquaporin-2 (1:2000; Santa Cruz Biotech, SC-9982), rabbit anti-SOX9 (1:4000; Millipore, #AB5535) and mouse anti-TUBB3 (TUJ1) (1:1000; Covance, #MMS-435P). Images were acquired with either ApoTome2 (Zeiss) or a Nikon 90i upright wide-field microscope. Images were processed using Nikon Elements software, Photoshop or Canvas.

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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., E.B., S.B., S.C. designed and performed experiments; M.T., Y.G. and R.G. performed experiments.

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Supplementary material

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