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# Visualization of spatiotemporal activation of Notch signaling: Live monitoring and significance in neural development

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**Abstract**

Notch signaling plays various key roles in cell fate determination during CNS development in a context-dependent fashion. However, its precise physiological role and the localization of its target cells remain unclear. To address this issue, we developed a new reporter system for assessing the RBP-J-mediated activation of Notch signaling target genes in living cells and tissues using a fluorescent protein Venus. Our reporter system revealed that Notch signaling is selectively activated in neurosphere-initiating multipotent neural stem cells in vitro and in radial glia in the embryonic forebrain in vivo. Furthermore, the activation of Notch signaling occurs during gliogenesis and is required in the early stage of astroglial development. Consistent with these findings, the persistent activation of Notch signaling inhibits the differentiation of GFAP-positive astrocytes. Thus, the development of our RBP-J-dependent live reporter system, which is activated upon Notch activation, together with a stage-dependent gain-of-function analysis allowed us to gain further insight into the complexity of Notch signaling in mammalian CNS development.


*Keywords:* Notch; Hes1; RBP-J; Neural stem cell; Neural progenitors; Astrocyte; EGFP; Venus; Self-renewal; Gliogenesis

## Introduction

Notch signaling plays a pivotal role in the organogenesis of many developing tissues in both vertebrates and invertebrates and controls cell fates through local cellular interactions; cells expressing Notch ligands communicate with neighboring cells that express Notch receptors (Artavanis-Tsakonas et al., 1999). Following the binding of the Notch receptor extracellular domain of Notch to its ligand, Delta/Serrate/lag-2 (DSL), the Notch receptor intracellular domain (NICD) (Weinmaster et al., 1991) is cleaved by presenilin/γ-secretase (Selkoe and Kopan, 2003). NICD is translocated into the nucleus and assembled into a complex with the DNA binding transcription factor, CSL (CBF1/RBP-J in mammals, suppressor of hairless in *Drosophila* and Lag-1 in *Caenorhabditis elegans*) (Kato et al., 1997), and the co-activator Lag3/Mastermind (Petcherski and Kimble, 2000). This complex then binds to specific *cis*-regulatory DNA sequences via CSL and induces the transcriptional activation of the target genes of the Notch signaling pathway, probably by recruiting p300 and other proteins into the transcriptional activation complex (Wu et al., 2000; Fryer et al., 2002; Wu et al., 2002; Maillard et al., 2003). In the absence of NICD, CSL can recruit repressor complexes to the *cis*-regulatory sequences of Notch target genes. The activation of Notch therefore acts as a switch that reverses the

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transcriptional repression of its target genes (Barolo et al., 2002). In mammals, the basic helix–loop–helix (bHLH) genes *Hes1* and *Hes5* are considered to be primary targets of Notch because Notch activation induces the transcription of *Hes1* and *Hes5* (Kageyama and Nakanishi, 1997). Consistent with this hypothesis, the regulatory regions of these genes contain several RBP-J binding sites (Jarriault et al., 1995).

Several lines of evidence now indicate that Notch1-mediated signaling pathways play crucial roles in mammalian CNS development, including the maintenance of neural stem cell/progenitor states, the inhibition of neuronal cell commitment (Nye et al., 1994; Nakamura et al., 2000), and the promotion of astroglial fates (Gaiano and Fishell, 2002; Grandbarbe et al., 2003). However, the reported findings on the role of Notch signaling in neural development, especially in gliogenesis, remain controversial (Tanigaki et al., 2001; Hitoshi et al., 2002; Grandbarbe et al., 2003). The conflicting conclusions of previous reports can likely be partly attributed to the following facts: (i) functional redundancies in Notch-signal-related molecules and the early embryonic lethality of models carrying mutant forms (Ishibashi et al., 1995; de la Pompa et al., 1997; Ohtsuka et al., 1999) have prevented definitive conclusions in loss-of-function studies, and (ii) gain-of-function studies using an activated form of Notch sometimes have different outcomes, depending on the experimental conditions, because of the context-dependent actions of Notch-signaling.

As a first step towards addressing the complex role of Notch signaling during CNS development, we recently examined the in situ mapping of Notch1 activation using a specific antibody that recognizes the processed form of the intracellular domain of Notch1 after it has been cleaved by the activity of presenilin/γ-secretase (Tokunaga et al., 2004). However, this experimental system requires the cells to be fixed for the immunohistochemical analysis and cannot, therefore, be used to detect Notch1 activation in living tissue or to analyze the fate decision of cells in which Notch signaling had been activated in a prospective fashion.

To overcome these limitations, the initial goal of the present study was to generate a versatile live reporter system to detect the activation of Notch targets that are mediated by RBP-J. To establish such a reporter system, both a *cis*-regulatory element of the Notch target gene and a fluorescent protein are needed. For the *cis*-regulatory element of the Notch target gene, *Hes1* and *Hes5* are the strongest candidates because they are known targets of Notch signaling and they are expressed in the central nervous system. To exclude the possibility that the reporter gene transactivation was regulated by a signaling pathway other than RBP-J-mediated Notch activation, we utilized an RBP-J-dependent regulatory element in the endogenous target and we also employed a mutant promoter lacking the RBP-J recognition motif. We took advantage of the 195-bp promoter region of the *Hes1* gene (one of the endogenous target genes of Notch) that includes two RBP-J binding sites and several other elements known as E and N boxes (Sasai et al., 1992; Takebayashi et al., 1994; Jarriault et al., 1995). Since other *cis*-regulatory element may exist in this region, we also utilized a mutated form of the *Hes1* promoter (*Hes1*-pAmBm), in which two RBP-J binding sites are disrupted, to evaluate RBP-J-dependent *Hes1* promoter transactivation. We also developed a reporter system using an artificial promoter, TP-1, that includes 12 RBP-J binding sites and a minimum promoter (Kato et al., 1997). For live monitoring, a recently reported fluorescent protein Venus (and its destabilized form dVenus) (Nagai et al., 2002; Nagai et al., unpublished results) was utilized; this protein is an enhanced yellow fluorescent protein (EYFP) variant that exhibits fast and efficient maturation, a strong fluorescence intensity, and a tolerance to acidosis and $Cl^-$ exposure (see the first paragraph of the Results section for further details).

In the present study, we clearly showed that our reporter system was sensitive enough to monitor the activated status of Notch signaling in living cells; the present reporter system could also be utilized in studies on the developing CNS as well as studies on the maintenance and differentiation of neural stem cells. Importantly, the new reporter system, in combination with a conventional stage-dependent gain-of-function study, enabled the dynamics of Notch signaling in cell-fate decisions during CNS development to be examined in detail.

## Materials and methods

### *Gene construction of the reporter system*

Venus and dVenus cDNA inserts (Nagai et al., 2002; Nagai et al., unpublished results) were substituted with pEGFP-1 and pEGFP-N1 (Clontech Laboratories) to generate pVenus-1/pdVenus-1 and pVenus/dVenus-N1, respectively. The 195-bp promoter region of *Hes1*p-luciferase (Jarriault et al., 1995) was subcloned into pVenus/dVenus-N1 and pVenus-1/pdVenus-1 to generate Hes1p-Venus/dVenus, respectively. We introduced mutations into both of the two RBP-J binding sites present within the 195-bp *Hes1* promoter region using site-directed mutagenesis to eliminate RBP-J binding activity, based on a strategy used in a previous study (Jarriault et al., 1995), and then constructed a *Hes1*pAmBm-dVenus/Venus variant. TP-1-Venus/dVenus and rBG-Venus/dVenus were generated by inserting the promoter region of TP-1 luciferase (Kato et al., 1997) and the minimal promoter region of TP-1 luciferase into pVenus/dVenus-1. To generate the mouse GFAP promoter-EGFP construct, a 2.5-kb fragment of the mouse GFAP promoter (Miura et al., 1990) was subcloned into pEGFP-1 (Clontech