# Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1

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**Background**: On the basis of experiments suggesting that Notch and Delta have a role in axonal development in *Drosophila* neurons, we studied the ability of components of the Notch signaling pathway to modulate neurite formation in mammalian neuroblastoma cells *in vitro*.

**Results**: We observed that N2a neuroblastoma cells expressing an activated form of Notch, Notch1$^{IC}$, produced shorter neurites compared with controls, whereas N2a cell lines expressing a dominant-negative Notch1 or a dominant-negative Delta1 construct extended longer neurites with a greater number of primary neurites. We then compared the effects on neurites of contacting Delta1 on another cell and of overexpression of Delta1 in the neurite-extending cell itself. We found that N2a cells co-cultured with Delta1-expressing quail cells produced fewer and shorter neuritic processes. On the other hand, high levels of Delta1 expressed in the N2a cells themselves stimulated neurite extension, increased numbers of primary neurites and induced expression of Jagged1 and Notch1.

**Conclusions**: These studies show that Notch signals can antagonize neurite outgrowth and that repressing endogenous Notch signals enhances neurite outgrowth in neuroblastoma cells. Notch signals therefore act as regulators of neuritic extension in neuroblastoma cells. The response of neuritic processes to Delta1 expressed in the neurite was opposite to that to Delta1 contacted on another cell, however. These results suggest a model in which developing neurons determine their extent of process outgrowth on the basis of the opposing influences on Notch signals of ligands contacted on another cell and ligands expressed in the same cell.

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## Background

Notch and its ligands (Delta, Jagged/Serrate, Lag-2) have important roles in the development of organisms from worms to humans (reviewed in [1]). In the development of the nervous system, Notch–ligand interactions elicit a restrictive signal that inhibits the generation of neuroblasts from ectodermal precursors. In the vertebrate central nervous system (CNS), Notch signals regulate the earliest steps of neurogenesis, the generation of primary neurons [2–5]. Notch signals restrict the number of cells that assume a neural fate by repressing the expression of basic helix–loop–helix transcription factors such as neurogenin (Ngn1) [6] and NeuroD [7], which are essential for neuronal differentiation.

In addition to its role in cell specification, Notch signaling has a role in the maturation of young neurons. Studies in *Drosophila* show that Notch and Delta are required for the survival and pathfinding of axons in the intersegmental nerve [8,9]. In addition, Kuzbanian, a protease required for the maturation of Notch and/or Delta proteins [10–13] is necessary for axonal pathfinding and extension [10]. Cleavage of Delta by this protease generates soluble fragments that elicit neurite retraction in cortical neurons [13]. In addition, Notch and the oncogene product Abl are expressed in axons and growth cones, and interact synergistically to regulate axon pathfinding and extension [14].

In vertebrates, several lines of evidence suggest a role for Notch signals in the maturation and maintenance of neurons following their commitment to a neuronal fate. In the murine CNS, the mammalian Notch isoform Notch1 is expressed on neural precursors and newly postmitotic neurons [15–17]. Notch1 expression persists throughout the lifetime of most mammalian neurons ([18–20], and D.R.F. and J.S.N., unpublished observations). These studies imply a functional role for Notch proteins in young and mature mammalian neurons that has not been evaluated. This conclusion is also supported by the evidence that Notch signals require functional presenilin proteins in *Caenorhabditis elegans* [21] and *Drosophila* [22,23]. Mutated presenilin genes are causally involved in most cases of familial Alzheimer's disease, a disorder in which mature neurons degenerate (reviewed in [24]). The relationship of Notch

and presenilins suggests that Notch signals might be important in the etiology of this neurodegenerative disorder.

In order to explore the role of Notch signals in late events of neurogenesis, we have studied the effect of gain-of-function and loss-of-function Notch1 and Delta1 constructs in neurite development, and compared the effect of expression of Delta1 *in cis* (that is, in the same cell) and *in trans* (that is, in another cell) on neurite generation. Our results clarify how communication through the Notch receptor controls neurite extension and complexity.

## Results

The effect of Notch signals on cell-fate decisions in neural precursors limited our ability to study the effects of Notch on late events in neurogenesis in P19 cells [25] or in neural precursors. We therefore used N2a neuroblastoma cells as model committed neurons. N2a cells elaborate neurites and growth cones and are easily transfected and selected for stable lines. This enables studies of the effects of a gene when expressed within a cell extending neurites. Neuroblastoma cells may be co-cultured with other cell lines expressing genes of interest to examine the effect when the gene is expressed *in trans* to the cell extending neurites. N2a cells also express low levels of the Notch pathway genes Notch1 and Jagged1 (see below).

### Activated Notch1 and dominant-negative Delta1 produce opposing effects on neurite production

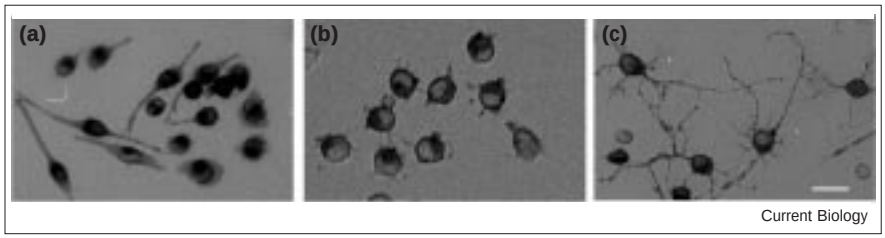
Previous studies had shown that Notch signals affect neuronal processes, but the exact effects of increasing or decreasing those signals were not clear. Therefore, dividing N2a neuroblasts were infected with pseudotyped bicistronic IRES–alkaline phosphatase (IAP) retroviral vectors (see Materials and methods) expressing Notch1$^{IC}$, the intracellular domain of Notch1, which is constitutively active [25], or Delta1$^{EC}$, a Delta1 lacking its cytoplasmic domain that behaves as a dominant-negative for the Notch pathway [2,26], or a control IAP retrovirus. Cells were grown in reduced serum at low plating density (1,300 cells/cm$^2$) to elicit neurite extension [27] and infected cells were identified following fixation and histochemical staining for alkaline phosphatase (AP). Notch1$^{IC}$-expressing N2a cells were able to extend neurites, but these processes were shorter than the neurites of control infected cells (Figure 1a,b). In contrast, cells infected with Delta1$^{EC}$ virus produced longer neurites, more neurites per cell, and a greater neuritic complexity than control infected cells (Figure 1c).

Measurement of the neurites from these experiments revealed that the mean neurite length per cell was significantly reduced in Notch1$^{IC}$-infected cells (Figure 2a). After Notch1$^{IC}$ infection, the distribution of neurite lengths revealed that the proportion of cells having short neurites ($\leq 30$ μm) was markedly enhanced and those having very long neurites ($\geq 90$ μm) was reduced compared with control infected cells (Figure 2b). The distribution of neurite lengths of cells infected with Delta1$^{EC}$ was also significantly different from controls (Figure 2b), but in an opposite direction. The most striking differences were the diminished fraction of cells with short neurites ($\leq 30$ μm) and the enhanced fraction of cells with very long neurites ($\geq 120$ μm) found as a consequence of Delta1$^{EC}$ expression (Figure 2b). A marginally greater mean neurite length per cell was observed after Delta1$^{EC}$ retroviral infection compared with controls (Figure 2a). In addition to its effect on neurite length, Delta1$^{EC}$ expression also drove N2a cells to elaborate a greater number of primary neurites than control cells (Figure 2c). Notch1$^{IC}$-infected cells also included fewer cells with two or more neurites per cell compared with controls (data not shown). The apparent increase in the complexity of neuritic arborizations in cells expressing Delta1$^{EC}$ was also reflected in a significantly increased number of branches compared with control cells (Figure 2d). These results reveal that increasing Notch signals in a neural cell reduces neurite length, whereas reducing Notch signals using Delta1$^{EC}$, a dominant-negative signal for the Notch pathway, promotes the elaboration of more complex dendrite-like arborizations.

### Neurite extension in stable neuroblastoma lines expressing Delta1$^{EC}$ and Notch1$^{EC}$

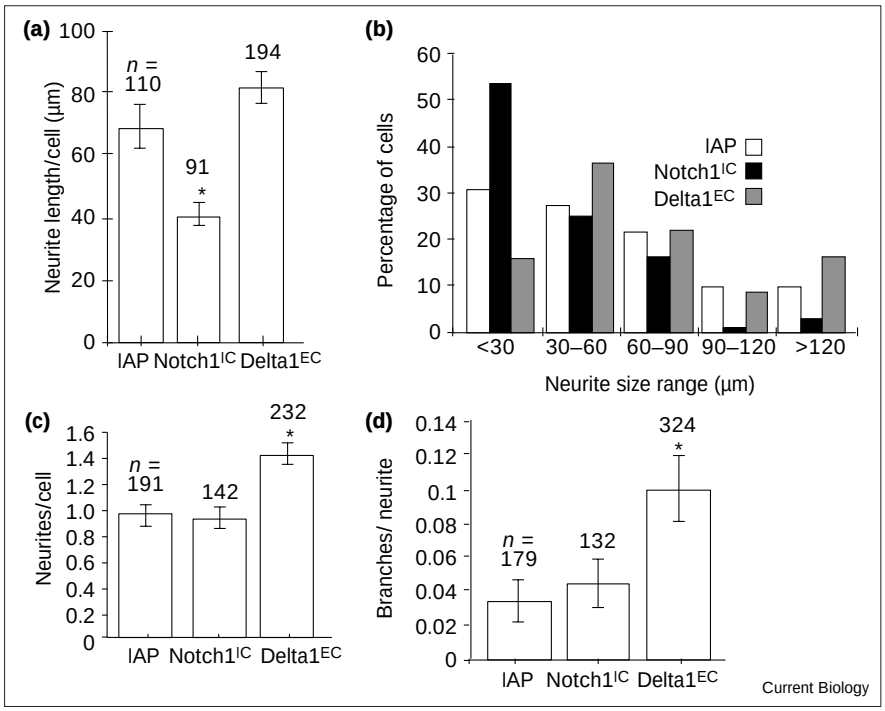
For comparison, we generated stable N2a lines expressing Notch1$^{EC}$, a truncated form of mouse Notch1 in which the intracellular domain is deleted, and N2a lines expressing Delta1$^{EC}$. Notch1$^{EC}$ is predicted to function as a dominant

**Figure 1**




Effect of retroviral Notch1$^{IC}$ and Delta1$^{EC}$ on neuritogenesis in neuroblastoma cells. N2a cells were infected with (a) control pseudotyped retrovirus (IAP), (b) Notch1$^{IC}$-IAP or (c) Delta1$^{EC}$-IAP. Cells were cultured in low serum to induce neurites, then fixed and stained for alkaline phosphatase (AP). (a) Cells infected with control IAP virus show a mixture of cells with short neurites, bipolar unbranched processes, and branched processes. (b) Cells infected with Notch1$^{IC}$-IAP often lacked long processes. (c) Cells infected with Delta1$^{EC}$-IAP frequently showed multiple neurites per cell and extensive branching. The scale bar represents 25 μm.

**Figure 2**




Analysis of neurite length, neurite number and neurite branching in N2a cells infected with control virus (IAP), Notch1$^{IC}$ or Delta1$^{EC}$ retroviruses. (a) Mean neurite length per cell was determined for cells having one or more neurites; *n* = number of cells scored. Analysis of variance (ANOVA) gave a significant difference ($p < 0.0001$). (b) Distribution of neurite lengths per cell. The distributions were significantly different between Notch1$^{IC}$ and controls and between Delta1$^{EC}$ and controls, as judged by a $\chi^2$ test (Notch1$^{IC}$AP vs IAP, $p = 0.002$ and Delta1$^{EC}$ vs IAP, $p = 0.023$). (c) Mean number of primary neurites per cell is shown for infected cells (*n* = total cells scored). ANOVA gives $p < 0.0001$. (d) Mean branches per primary neurite (*n* = total primary neurites scored). ANOVA gives $p = 0.012$. For (a,c,d) the asterisk designates samples different from control by a Student–Neuman–Keuls test at $p < 0.05$.

inhibitor of Notch signaling [28,29]. Lines expressing high levels of Notch1$^{EC}$ and Delta1$^{EC}$ generated longer neurites than control lines (Figure 3a,c), similarly to cells infected with retroviral Delta1$^{EC}$. Additionally, a significantly greater number of neurites per cell was found in lines expressing Notch1$^{EC}$ compared with controls (Figure 3d). Consistent with the retroviral experiments (Figures 1,2), these data reveal that expression of proteins that reduce Notch signals in neuroblastoma cells results in longer, more complex neurites.

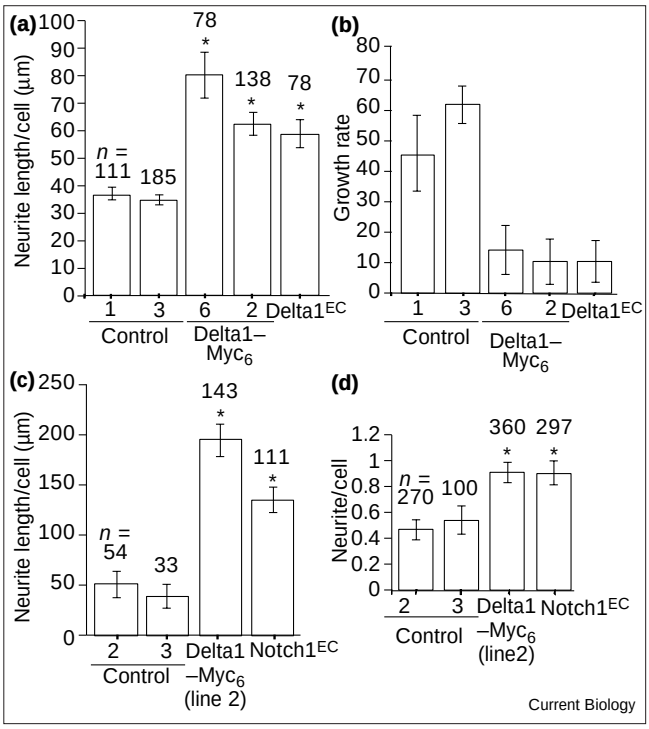
To evaluate whether the cell lines were elaborating neuritic structures or elongated filopodia, we determined whether the processes contained polymerized tubulin, an indicator of neurite maturation. We found that the processes elicited by Delta1$^{EC}$, Notch1$^{EC}$ and Delta1–Myc$_6$ (see below) were visible with TuJ1, an antibody to type III neuron-specific β-tubulin, following a wash with detergent in a microtubule stabilizing buffer (Figure 4). The detergent-resistant, TuJ1-positive processes were also longer in the Notch1$^{EC}$, Delta1$^{EC}$ and Delta1–Myc$_6$ cell lines than in the control lines (data not shown).

#### Non-autonomous regulation of neurite length by Delta1

Numerous studies suggest that Delta proteins expressed on one cell can activate Notch signaling in another cell in direct contact. We therefore evaluated the ability of Delta1 expressed on quail cells to affect neurite development in neuroblastoma cells expressing Notch1. Two lines were tested, an N2a cell line expressing a full-length Notch1 tagged with the Myc epitope (Notch1–Myc [25]) and a vector-transformed N2a line (line 2). These lines were plated on confluent lawns of quail QT6 fibroblasts or of QT6 cells expressing Delta1 (Delta1-QT6), and the co-cultures were kept under reduced serum conditions for 48 hours to elicit neurite outgrowth. The N2a cells and their processes were identified by an antibody to neuron-specific β-tubulin, which is not expressed in QT6 fibroblasts (Figure 5a,b, and data not shown). QT6 cells (and N2a cells) were visualized by rhodamine–phalloidin or phase-contrast microscopy (data not shown) to ensure that the lawn of quail cells was confluent (Figure 5c,d). Neuroblastoma cells expressing Notch1–Myc (Figure 5a,c) or control N2a cells (data not shown) elaborated longer bipolar morphologies when grown on control QT6 cells than when grown on Delta1-QT6 cells (Figure 5b,d, and data not shown). However, the reduction of neurites produced by Delta1-QT6 cells was accompanied by an increase in branches (Figure 5d) and lamellipodia (data not shown).

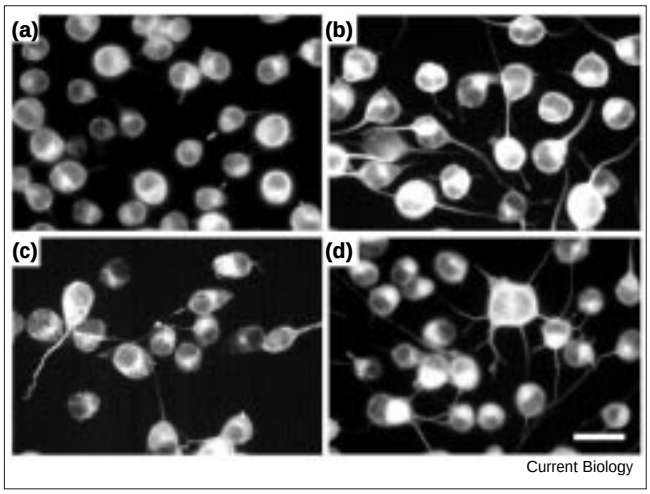
Measurements of TuJ1-stained neurites from these experiments showed that growth on Delta1-QT6 cells elicited a significantly reduced proportion of Notch1–Myc or control N2a cells with long neurites (> 30 μm) compared with the same lines grown on control QT6 cells (Figure 6a). Both the Notch1–Myc and N2a lines responded quantitatively to Delta1 expressed *in trans* with a shortened distribution

**Figure 3**




Analysis of neurite length and cell proliferation in Delta1–$Myc_6$, $Delta1^{EC}$, $Notch1^{EC}$ and control N2a cells. Cell lines were plated at equal densities and grown in low-serum medium. Photographs of live cells were analyzed. (a) Neurite lengths per cell were measured from control lines, Delta1–$Myc_6$ lines, and $Delta1^{EC}$ cells. ANOVA gives $p < 0.0001$. (b) Growth rate was determined from cells in three 20× fields at days 2 and 4 and presented as a growth rate (cells per field per day). (c) Neurite length per cell was measured in a separate experiment as above for control lines, Delta1–$Myc_6$ line 2, $Notch1^{EC}$ cells; $n$ = number of cells scored. ANOVA gives $p < 0.0001$. In this experiment, a longer preincubation with serum was used (32 h), giving longer neurites. (d) The number of neurites per cell was measured for control lines, Delta1–$Myc_6$ line 2, $Notch1^{EC}$ cells. ANOVA gives $p = 0.0001$. For (a,c,d), the asterisk designates samples different from control mean by a Student-Neuman-Keuls test at $p < 0.05$.

**Figure 4**



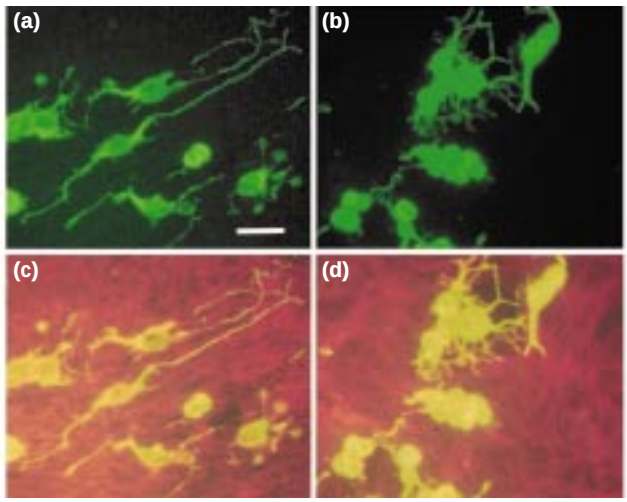

Microtubule-filled processes in stable neuroblastoma cell lines expressing Delta1–$Myc_6$, $Delta1^{EC}$, and $Notch1^{EC}$. Stable N2a cell lines were washed with microtubule-stabilizing buffer with detergent to remove soluble tubulin, fixed and then stained with antibody to β-tubulin (TuJ1). (a) Control line; (b) $Notch1^{EC}$ line; (c) $Delta1^{EC}$ line; (d) Delta1–$Myc_6$ line 2. A control cell line contains short neurites whereas cells expressing $Notch1^{EC}$, $Delta1^{EC}$, and Delta1–$Myc_6$ contain longer, more complex neurites that include insoluble tubulin. The scale bar represents 25 μm.

of neurites (Figure 6b). Additionally, the mean number of neurites per cell was significantly reduced in both the Notch1–Myc and N2a lines as a consequence of contact with Delta1 cells (Figure 6c). However, the mean number of branches per major neurite was affected by contact with Delta1 cells only in the Notch1–Myc line (Figure 6d). Thus, Delta1 expressed *in trans* to neuroblasts reduced neurite length and the number of primary neurites. This result is consistent with elicitation of an increased amount of Notch signal in a neuroblastoma cell after contact with a Notch ligand *in trans*. This interpretation is supported by our observation that the effect on neurite length of Delta1 *in trans* resembled the effect of the ligand-independent activator $Notch1^{IC}$ (compare Figures 6b and 2b) and was opposite to the effects of the dominant-negative Notch1 and Delta1 constructs (Figures 2,3). Secondary branching, however, did not respond in a consistent manner to changes in Notch signals.

### Neurite outgrowth in neuroblastoma lines expressing Delta1

To compare the effect of Delta1 expressed *in cis* on neuritogenesis in stable cell lines, we generated N2a cell lines that constitutively express a full-length Myc-tagged Delta1 (Delta1–$Myc_6$) (Figure 7a). Immunostaining for Delta in serum-starved N2a cells expressing either Delta1–$Myc_6$ or $Delta1^{EC}$ revealed staining on neurites and growth cones (Figure 7b). Immunoreactivity spanned the membrane-bounded cell compartments and the plasmalemma of the perikaryon. Several Delta1–$Myc_6$-expressing N2a lines were evaluated for their expression of Delta1 gene products (Figure 7c). The full-length molecule has an apparent molecular weight of 117 kDa, larger than the predicted size of Delta1–$Myc_6$ (91 kDa), possibly due to glycosylation or some other post-translational modification. In addition, we observed small immunoreactive polypeptides of 38 and 40 kDa in cell extracts. From their sizes and the location of the Myc epitope, fragments of these structures may have been generated by a cleavage somewhere between the seventh epidermal growth factor (EGF) repeat and the membrane-spanning domain. A potential N-linked glycosylation site in Delta1 is located within this region, making it difficult to predict the size of fragments. A similar cleavage was observed for *Drosophila* Delta [30] and may be produced by the ectoprotease

**Figure 5**




Effect of Delta1-expressing quail cells on neurites in Notch1-expressing neuroblastoma cells. Notch1–Myc N2a cells were plated on (a,c) confluent control quail cells (line QT6) or (b,d) QT6-Delta1 cells. After differentiation in low serum for two days, cells were stained with anti-β-tubulin (TuJ1) and visualized with anti-mouse FITC-labeled antibody with or without phalloidin–rhodamine to visualize the N2a cells or all cells. Identical fields with (a,b) TuJ1 staining alone or (c,d) TuJ1 and phalloidin staining are shown. The scale bar in (a) represents 50 μm.

Kuzbanian [13]. These data imply that Delta1 is expressed on growing neuritic processes and may be secreted in overexpressing N2a cells.

The N2a cell lines expressing Delta1–$Myc_6$ were studied for their capacity to proliferate and generate neurites following serum reduction (Figure 3). Significantly longer neurites were apparent in cell lines expressing the highest levels of Delta1–$Myc_6$ compared with control lines (Figure 3a,c). Lines expressing lower levels of Delta1 (line 5) showed insignificant alterations in neurite length and proliferation (data not shown). Therefore, Delta1 expressed *in cis* at high levels resembled the dominant-negative $Delta1^{EC}$ and $Notch1^{EC}$ in its enhancement of neurite outgrowth. These data imply that Delta proteins expressed at high levels act as cell-autonomous inhibitors of Notch signaling. A similar phenomenon was previously observed in *Drosophila* [31,32].

Neuroblastoma lines expressing Delta1–$Myc_6$ and $Delta1^{EC}$ also showed a markedly slower rate of proliferation (Figure 3b). The differences in proliferation rates could have led to an increase in cell density in control lines relative to Delta1–$Myc_6$ lines which might have affected neurite length [33]. Nonetheless, the initial sparse plating (1,300 cells/$cm^2$) resulted in cells that were well separated after 2 days growth in all the lines tested (see Figures 1,4).

The observation that Delta1–$Myc_6$ was cleaved (Figure 7) raised the possibility that its effect on N2a cell neurites was mediated by Delta polypeptides secreted by sister cells, rather than by a cell-autonomous effect in the cell expressing the Delta1–$Myc_6$. However, conditioned medium from Delta1–$Myc_6$ cells had no effect on neuritogenesis or proliferation of control cells even when the medium was concentrated (data not shown). Additionally, contact with Delta1 on another cell surface was unlikely in these experiments because the cell lines expressing Delta1–$Myc_6$ were plated sparsely. Even cells with no discernible cell–cell contacts showed enhanced neurite outgrowth. Delta1–$Myc_6$ cells were also tested under conditions when medium was replaced frequently with control cell conditioned medium to reduce the possibility of an effect of secreted Delta1. Even in these conditions, N2a lines expressing Delta1–$Myc_6$ produced markedly longer neurites than did control lines (data not shown). These experiments suggest that the neurite-lengthening effect of Delta1–$Myc_6$ expressed at high levels in N2a cells is likely to be cell-autonomous or autocrine.

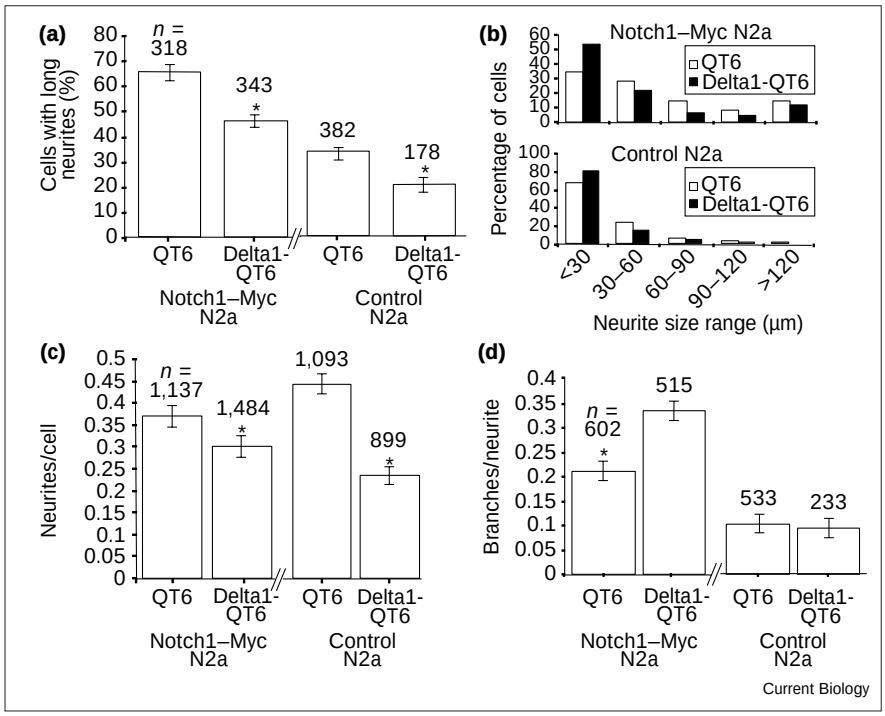
### Regulation of Jagged1 and Notch1 expression by Delta1

The expression of Notch proteins and their ligands is thought to be governed by a cross-regulatory loop such that Notch signals depress the rate of expression of Notch ligands [34]. Therefore we evaluated the effects of Delta1–$Myc_6$ expression on levels of the ligand Jagged1 as another test of whether Delta1 expression *in cis* was influencing the Notch pathway. *Jagged1* mRNA was expressed at low concentrations in control neuroblastoma cells (Figure 8a). In lines expressing the highest Delta1–$Myc_6$ levels, however, a marked induction of a 4.2 kilobase (kb) *Jagged1* transcript was apparent. This induction of *Jagged1* was visible even when mRNA was normalized to levels of actin (Figure 8e). Endogenous *Delta1* transcripts in the N2a control lines (Figure 8b) were not visible, while the *Delta1–$Myc_6$* mRNA was strongly expressed in lines 1 and 2, and at reduced levels in line 3 (Figure 8b). Additionally, Notch1 protein was induced in the Delta1–$Myc_6$-expressing lines (Figure 8f). The levels of *Notch1* correlated with the levels of *Delta1–$Myc_6$*. These data demonstrate an increased expression of *Jagged1* mRNA and Notch1 protein as a consequence of high levels of *Delta1* expression.

## Discussion

In this study we make three main observations regarding the Notch signaling pathway and its regulation of neurite development. First, we find that an activated Notch1 reduces neurite length and primary neurite number, whereas both dominant-negative Notch1 and a dominant-negative Delta1 stimulate neurite extension and increase primary neurite number. Second, neuroblastoma cells grown in contact with cells expressing Delta1 show reduced neurite length and primary neurite numbers, indicating that ligand-induced Notch signals

**Figure 6**



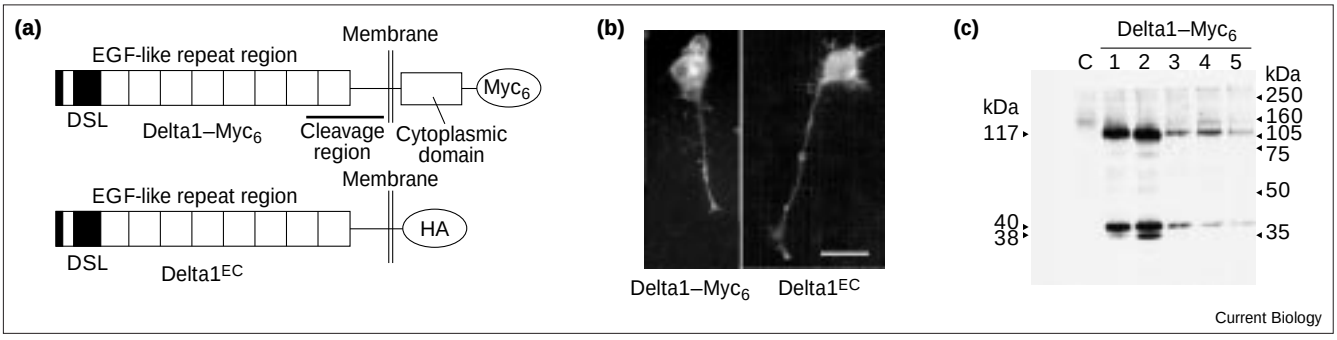

Analysis of neurite outgrowth of Notch1–Myc or control N2a cells cocultured with Delta1-expressing or control quail cells after staining for β-tubulin. (a) The percentage of cells with neurites ≥ 30 μm was reduced in the presence of the Delta1-expressing cells. Notch1–Myc N2a cells and control N2a cells with neurites were scored and compared using a binomial test weighted for the number of fields counted; *n* = number of N2a cells. For Notch1–Myc cells, $p < 0.000006$; for control N2a cells, $p < 0.0027$. (b) The distribution of neurite lengths per cell for cells analyzed in (a). Notch1–Myc neurites, $p < 0.0001$ and control N2a neurites, $p < 0.037$. (c) The number of neurites per cell for cells analyzed in (a); *n* = number of neurites. Means were compared with a two-tailed Student's *t* test, giving $p = 0.039$ for Notch1–Myc neurites per cell and $p = 0.035$ for control N2a neurites per cell. (d) The number of branches per primary neurite was assessed for Notch1–Myc N2a cells grown with control cells or Delta1-QT6 neurites and for N2a cells grown with control or Delta1 cells. Means were compared with a two-tailed Student's *t* test and showed $p = 0.002$ for Notch1–Myc cells and $p = 0.97$ for control N2a cells.

regulate neurite outgrowth. Third, like the dominant-negative forms of Notch1 and Delta1, high levels of Delta1 expressed within a neuroblastoma cell enhance neurite outgrowth and elicit induction of Jagged1 and Notch1 expression in the cell. These results show how Notch signals modulate neurite maturation in a model system, and reveal how Delta1 constructs act as inhibitors of neurite outgrowth when expressed *in trans*, or as stimulators of neuritic development when expressed at high levels within the cell extending neurites (Figure 9).

### Regulation of neurite development by Notch signals

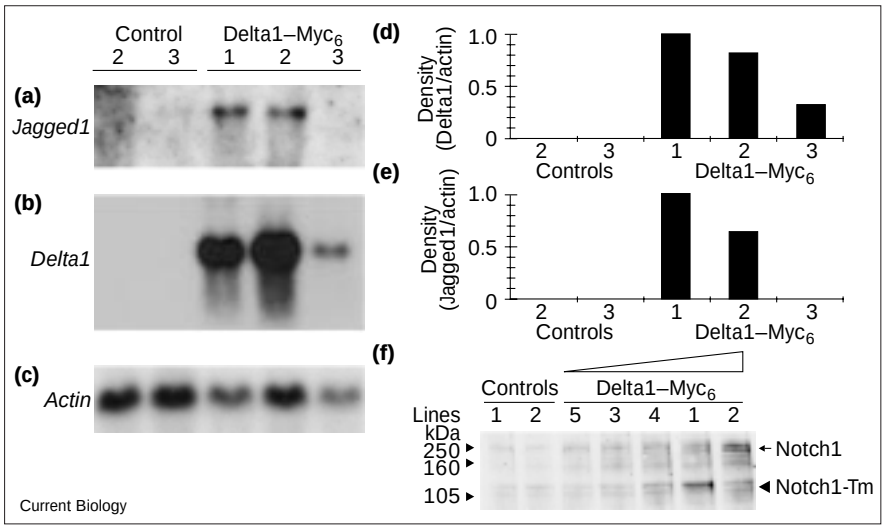
Using neuroblastoma cells, we provide evidence that stimulation and inhibition of Notch signals have essentially opposite effects on neurite elongation. An activated

**Figure 7**




Expression and cleavage of Delta1–$Myc_6$ in N2a cell lines. (a) Structure of Delta1–$Myc_6$ (top) and $Delta1^{EC}$ (bottom) constructs. The region of the putative extracellular cleavage site is indicated. Delta1–$Myc_6$ contains six tandem Myc epitopes at its carboxyl terminus. DSL, Delta–Serrate–lag2 domain. (b) Anti-Myc immunostaining of a differentiated cell line expressing Delta1–$Myc_6$ (line 1; left) and anti-HA staining of a cell infected with $Delta1^{EC}$ (right). Cells were grown on laminin-coated glass slides. The scale bar represents 50 μm. (c) Western blot of extracts from stable N2a cell lines expressing Delta1–$Myc_6$ (lanes 1–5) or from a vector (pCDNA3) control line (C) probed with anti-Myc antibody 9E10. Full-length Delta1–$Myc_6$ is 117 kDa and apparent proteolytic fragments are 40 kDa and 38 kDa. Delta1–$Myc_6$ line 6 expressed levels equivalent to line 4 (data not shown).

**Figure 8**




*Jagged1* and Notch1 expression in Delta1–$Myc_6$ N2a cell lines. (a) A northern blot probed for *Jagged1* reveals a 4.2 kb transcript in Delta1–$Myc_6$ lines 1 and 2. (b) A parallel blot probed for *Delta1* reveals the *Delta1–$Myc_6$* transcript (2.3 kb). (c) A parallel blot probed for γ-actin. (d) The relative abundance of *Delta1–$Myc_6$* mRNA in lines 1, 2, 3 or controls (lines 2 and 3) normalized by the γ-actin level was determined by densitometry. (e) The relative abundance of *Jagged1* mRNA normalized by the γ-actin level. (f) Anti-Notch1 (C20, intracellular domain) immunoblot of Delta1–$Myc_6$ expressing and control N2a lines. Delta1-expressing lines were arranged in their relative order of Delta1 expression according to Figure 7. The sizes of the full-length and cleaved Notch1 (Notch1-Tm) are shown.

Notch1, Notch1$^{IC}$, delivered via a retrovirus, reduced neurite length and the number of primary neurites (Figures 1,2). We were unable to obtain stable cell lines that expressed high levels of the Notch1$^{IC}$ construct, however, raising the possibility that high levels of Notch signals may be somewhat toxic or anti-proliferative in neuroblastoma cells. As another means of activating Notch, we studied the effect on neuroblastoma cells of a Notch ligand expressed *in trans* [35,36], and found that neuroblastoma cells elaborated shorter neurites with fewer primary branches when grown in contact with Delta1-expressing cells (Figures 5,6). Whereas both Notch1-transfected and control lines responded to Delta1 *in trans*, the Notch1-transfected line had consistently longer neurites than the control line (Figure 6b). Although we cannot explain this phenomenon at present, it is noteworthy that cell lines expressing Delta1–$Myc_6$ also had high endogenous Notch1 levels and long neurites (Figure 8f). Nonetheless, our experiments using ligand-independent and ligand-dependent means of activating Notch demonstrate that increased Notch signals produce a reduction in neurite length and number in neuroblastoma cells (Figure 9b).

These experiments also suggest that endogenous Notch signals regulate neurite outgrowth in neuroblastoma cells. Dominant-negative forms of Notch1 or Delta1 [2,26,28,29,37] produced an enhancement of both neurite length and primary neurite number (Figures 2,3). The dominant-negative Notch1$^{EC}$ is thought to interfere with ligand activation of endogenous Notch molecules by binding the available ligand. The mechanism for Delta$^{EC}$ constructs is unclear, but its expression *in cis* makes cells deaf to Notch signals [38]. Therefore, the effects of these two constructs when expressed *in cis* provide indirect evidence that endogenous Notch1 (Figure 3d) and a ligand — possibly Jagged1 (Figure 8a) — are interacting in neuroblastoma cells to control neurite elongation (Figure 9a). This suggests, but does not prove, that endogenous Notch signaling regulates neurite outgrowth (Figure 9a). How Jagged1 influences neurite outgrowth and Notch signals has not yet been evaluated, but a recent study suggests that Delta1 and Jagged1 function similarly [33]. It is also possible that ligand-independent activation of Notch occurred and was inhibited by the dominant-negatives used in our experiments.

Work in *Drosophila* embryos has indicated that the elaboration of axons and their proper tracking to an ultimate destination is partly regulated by Notch–Delta interactions [8–10,14]. When temperature-sensitive mutants of Notch or Delta were subjected to a restrictive temperature following cell-fate determination, axons degenerated or lost contact with their pathway along segmental tracheal cells [8]. As Notch molecules were found on neuronal processes in intersegmental nerves [39,40] and on growth cones in neuronal cultures [14], and Delta was expressed on the tracheal cells [41], Giniger *et al.* [8] proposed a model in which Delta present along an axonal migration pathway was activating Notch molecules expressed on a growing axon. The specific effects of Notch signals on the growing neuritic processes could not be discerned from these experiments, however, because of the complexity of the *in vivo* experiments and the reliance upon loss-of-function mutations only. Moreover, in the experiments, Delta and Notch expression may have been present on both the neuritic process and the pathway, leaving unanswered the question as to where the Delta–Notch interaction occurred. The present studies agree with recent studies in mammalian neurons showing that Delta1 or Jagged1 acting *in trans* resemble

contact-mediated or soluble chemorepulsive factors [13,33] and that activated Notch molecules inhibit neurite outgrowth [42]. Importantly, the present studies uniquely show that high levels of Delta1 expressed *in cis* have an opposite effect on neurites to ligand contacted *in trans*, suggesting major differences between Notch ligands and chemorepulsive cues.

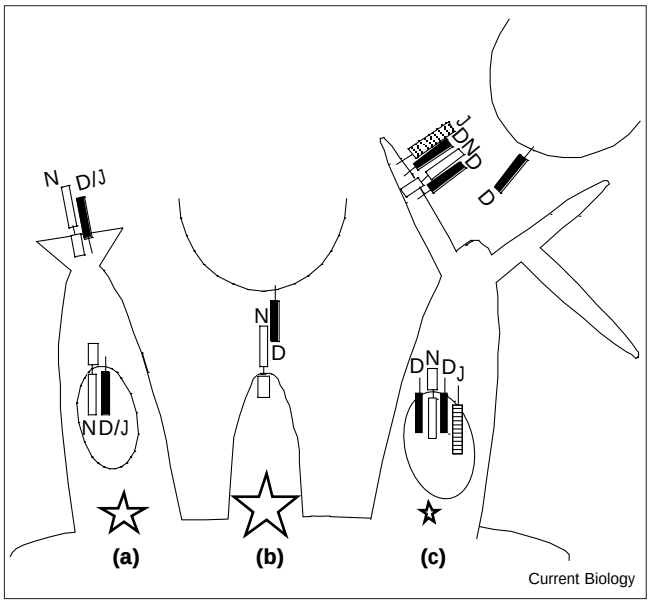
### Autonomous and non-autonomous Delta1 in neurite development

Although Delta1 is commonly thought to activate the Notch pathway, we provide evidence that a Delta1 protein expressed at high levels *in cis* resulted in enhanced neurite outgrowth in neuroblastoma cells (Figure 3). One interpretation of these results is that the Delta1 molecule expressed *in cis* had a dominant-negative effect on Notch signals, similarly to the dominant-negative Notch1 and Delta1 constructs. Previous work in *Drosophila* has indicated that high levels of the Notch ligands Delta and Serrate can produce cell-autonomous inhibition of Notch signaling ([26,31,32] and references therein). The mechanism of this inhibition is not understood, but homotypic ligand dimerization [43] resulting in ligand sequestration has been proposed. The induced expression of Jagged1 observed here (Figure 8a) might also have been a consequence of the inhibitory effects of high Delta1 expression on Notch signaling in these cells, consistent with experiments in *Drosophila* wing discs showing that ectopic Delta activates Serrate expression [44–46]. In the wing disc, however, the induction of Serrate was partially mimicked by activated Notch [46] implying that an enhancement of Notch signals was mediating the induction of Serrate, rather than an inhibition of Notch signals as we propose here. Additionally, high levels of Delta1 in N2a cells induced Notch1 (Figure 8f), a result somewhat at odds with the predicted regulation of Notch gene expression. Thus, the action of Delta1 *in cis* is inconsistent with some studies of Notch signaling and the cross-regulatory loop governing Notch pathway gene expression [34]. Further studies of Delta1 and Jagged1 overexpression are required to evaluate their individual contributions to neurite outgrowth, ligand and Notch induction and Notch signaling. An alternative explanation for our results is that the Delta1–$Myc_6$ construct in our experiments artefactually acted as an antagonist of Notch signals because of the Myc epitopes at its carboxyl terminus.

### Mechanism of the effect of Notch on neurite development

How do Notch signals regulate neuritic growth? In most cases the effects of Notch are mediated by the CSL proteins (CBF, SuH, Lag1), which regulate gene expression in concert with the intracellular domain of Notch [1]. It is conceivable that our results were a consequence of nuclear Notch signals that influenced the state of maturation of neuroblastoma cells by repressing neuronal determination genes [6,7]. The induction of Jagged1 and Notch1 expression by Delta1–$Myc_6$ expression weighs in favor of this hypothesis. Further experiments are needed to distinguish between an effect of Notch that is local and selectively affects neuritic branches and a global effect upon the differentiation state of the cell.

**Figure 9**




Hypothetical model for *cis* and *trans* effects of Delta1 on neurites. (a) Endogenous Notch–ligand interactions regulate the outgrowth of neuritic processes without interaction from a ligand on another cell. N, Notch1; D, Delta1; J, Jagged1. (b) Ligands expressed by a cell along the migration pathway repress the outgrowth of neurites expressing Notch1. (c) High levels of Delta1 inhibit the reception of ligands along the pathway, induce Jagged1 and Notch1, and enhance neurite outgrowth. The relative size of the star indicates the proposed quantity of Notch signaling.

Several recent studies imply that Notch signals may modulate the cytoskeleton through local signal transduction pathways that do not require activation of nuclear gene expression. For example, Giniger [14] has shown that Notch interacts synergistically with the Abl tyrosine kinase to regulate the pathfinding of specific axons. The binding of Notch to disabled, a protein known to interact with Abl may explain how Notch communicates with Abl. Sanpodo is another possible mediator of Notch's regulation of neurite development through its cytoskeletal interactions [47,48]. Although it is not clear how Notch elicits its effects upon neurites, it is appealing to hypothesize that it regulates neurite development through local modulators of the cytoskeleton such as Sanpodo or Abl rather than through a nuclear effect. The latter would require activated Notch fragments to travel potentially great distances from growth cones to the nucleus. Alternatively, nuclear and local effects of Notch signaling may be integrated to regulate neurite development.

Our observations suggest that neurite development may be modulated *in vivo* by a competition between Notch ligands expressed on an opposing cell and ligands expressed on the neurite-extending cell (Figure 9c). The resulting Notch signal perceived by a particular neurite, or possibly the entire cell, would then guide neurite extension. A similar mechanism was proposed by Heitzler and Simpson [49] as a means of modulating and measuring the levels of available receptor and ligand between cells transmitting and receiving a lateral inhibitory signal. In this model, the summed effect of receptors stimulated by ligands *in trans* and receptors inhibited by ligands *in cis* would determine the quantity of Notch signal and, consequently, the extent of neurite outgrowth.

## Materials and methods

### *Reagents*

Delta1 [50] was provided by Domingos Henrique and modified by PCR with Vent polymerase (NEB) using the primers 5′-CTCAGTGAGAGGCATATGGAG-3′ (upstream) and 5′-AGTAGAATTCTTTAAATCGATGCACCTCAGTCGCTATAACACA-3′ (downstream) and cloned into the *Nde*I–*Eco*RI site of Delta1 in Bluescript SK+. Modified Delta1 was subcloned with *Bam*HI–*Eco*RI into pCDNA3 (Invitrogen). A $Myc_6$ tag was added in-frame from the CS2 vector (from Dave Turner) cloned into the *Eco*RI and *Cla*I sites of modified Delta1 in pCDNA3. Jagged1 probes were a gift from Genentech. To construct $Delta1^{EC}$, Delta1 was deleted from the *Mun*I to a *Hin*dIII site in the polylinker and a 3′ oligonucleotide encoding a hemagglutinin (HA) tag was inserted (5′-CAATTGGCTACCCATACGATGTTCCAGATTACGCTTAAGGCGCGCCTTAATTAAAAGCTT-3′). The fragment was subcloned with *Bam*HI and *Xho*I into pCDNA3. $Notch1^{IC}$ (also known as mNotchIC) and Notch1–Myc were as described in [25]. $Notch1^{EC}$ bears a deletion from the *Sau*I (amino acid 1,761) site in Notch1 and a single Myc tag was inserted at the carboxyl terminus.

### *Cell lines and transfection*

N2a cells (American Type Culture Collection) were transfected with plasmids using the calcium-phosphate method. Clonal cell lines were selected in 800 μg/ml G418 and then grown in 400 μg/ml. Quail cells (QT6) expressing Delta1 or control were a kind gift of Olivier Pourquié [36].

### *Retroviral infection of N2a cells*

Replication incompetent retroviral vectors expressing $Notch1^{IC}$, $Delta1^{EC}$ or control were generated from the LIA vector [51], here termed IAP for IRES–alkaline-phosphatase. These vectors direct expression of human placental alkaline phosphatase (AP) under the control of an internal ribosomal entry site (IRES) positioned 3′ to an inserted gene. VSV-G pseudotyped retrovirus preparations were made by transient transfection [52] and concentrated by centrifugation. N2a cells ($1 \times 10^4$) were infected for 18 h, and then plated in low-serum medium to generate neurites as described below. Infected cells were identified by staining for AP as described [53]. The co-expression of the $Notch1^{IC}$ and $Delta1^{EC}$ proteins was verified using anti–Myc or anti-HA immunohistochemistry (not shown).

### *Determination of cell proliferation and neurite length*

N2a cells were plated at 1,300 cells/$cm^2$ on untreated tissue culture plates or Permanox slides (Nalgene) and grown for 24–32 h. To elicit differentiation, cells were grown with 0.2% fetal bovine serum in Eagle's medium [27] and photographed after two days. Measurements of neurites were made from photomicrographs. For retroviral infection experiments, only AP-stained cells were analyzed and for cell lines live cells were photographed under phase-contrast microscopy. NIH Image software was used to determine the length of neurites. Neurites were measured as mean length per cell using multiple fields with similar numbers of cells per field. The rate of cell proliferation was determined as [(mean number of cells per field in three random fields at 4 days of growth ) – (mean number of cells at 2 days of growth)/2]. To study the effect of Delta1 expressed *in trans* on neurite development, neuroblastoma cells were co-cultured with QT6 or Delta1-QT6 cells. First, $1.5 \times 10^4$ quail cells were plated on four-well Permanox slides (Fisher). After 2 h, 5000 N2a Notch1–Myc cells were added to the quail cells and differentiated for 2 days as described above. Cells were fixed and stained with anti neuron-specific β-tubulin antibody (TuJ1) and FITC-labeled anti-mouse antibody, and fluorescence photomicrographs were analyzed for neurite lengths and number as described.

### *Immunoblots, cell staining and northern blots*

Extracts were prepared by lysing cells in sample buffer plus protease inhibitors. Proteins were separated on 10% SDS–PAGE, transferred, and immunoblots were performed as described using an anti-Notch1 antibody (C20, Santa Cruz Biotechnologies). Anti-Myc monoclonal 9E10 antibody was used to detect the Delta1–$Myc_6$ fusion protein and anti-HA antibody (12CA5) was used to detect $Delta1^{EC}$. Northern blots were run and blotted as described [54] with 10 μg of total RNA per lane. For analysis of cocultures with quail cells, cultures were fixed and stained with TuJ1 antibody and visualized with an FITC-labeled anti-mouse antibody. The quail cells were visualized with phase-contrast microscopy or rhodamine–phalloidin. The stable cell lines were tested for microtubules by washing with microtubule-stabilizing buffer (130 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA pH 6.9) and then rinsing with the same buffer including 0.2% Triton X-100.

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