The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands

Ena Ladi,1 James T. Nichols,1 Weihong Ge,2,3 Alison Miyamoto,1 Christine Yao,1 Liang-Tung Yang,1 Jim Boulter,2 Yi E. Sun,2,3 Chris Kintner,6 and Gerry Weinmaster1,4,5

1Department of Biological Chemistry, 2Department of Psychiatry and Behavioral Sciences, 3Department of Molecular and Medical Pharmacology, and 4The Molecular Biology Institute, Geffen School of Medicine, and 5Jonsson Comprehensive Cancer Center (JCCC), University of California, Los Angeles (UCLA), Los Angeles, CA 90095
6The Salk Institute for Biological Studies, La Jolla, CA 92037

M utations in the DSL (Delta, Serrate, Lag2) Notch (N) ligand Delta-like (Dll) 3 cause skeletal abnormalities in spondylocostal dysostosis, which is consistent with a critical role for N signaling during somitogenesis. Understanding how Dll3 functions is complicated by reports that DSL ligands both activate and inhibit N signaling. In contrast to other DSL ligands, we show that Dll3 does not activate N signaling in multiple assays. Consistent with these findings, Dll3 does not bind to cells expressing any of the four N receptors, and N1 does not bind Dll3-expressing cells. However, in a cell-autonomous manner, Dll3 suppressed N signaling, as was found for other DSL ligands. Therefore, Dll3 functions not as an activator as previously reported but rather as a dedicated inhibitor of N signaling. As an N antagonist, Dll3 promoted Xenopus laevis neurogenesis and inhibited glial differentiation of mouse neural progenitors. Finally, together with the modulator lunatic fringe, Dll3 altered N signaling levels that were induced by other DSL ligands.

Introduction

Functional studies of Notch (N) pathway genes have implicated this signaling system in the development of almost all structures within the vertebrate body plan. In particular, losses in core components (N1, Delta-like [Dll] 1, Dll3, presenilin-1, kuzbanian, and RBP-J) as well as in targets and modulators (Hes7, Mesp2, and lunatic fringe [LFng]) of the N signaling pathway all perturb the formation and patterning of somites (for review see Weinmaster and Kintner, 2003; Giudicelli and Lewis, 2004). Correct segmentation and patterning of somites is essential for proper axial skeletal formation, and mutations in Dll3 produce vertebral segmentation and rib defects in both spondylocostal dysostosis patients (Bulman et al., 2000; Turnpenny et al., 2003) and the pudgy mouse (Kusumi et al., 1998, 2004). Although it is clear that N signaling regulates somitogenesis, it is not clear which DSL (Delta, Serrate, Lag2) ligand activates N during this process. Of the DSL ligands that are expressed in the presomitic mesoderm (PSM), only Dll3 and Dll1 mutant mice display somitic defects; however, Dll3 and Dll1 mutant phenotypes differ with respect to the expression of somite markers and genes whose rhythmic expression is regulated by N (Dunwoodie et al., 2002; Zhang et al., 2002; Kusumi et al., 2004). Although it is difficult to discern from phenotypes and gene expression patterns alone, these different mutant phenotypes may reflect distinct roles for Dll1 and Dll3 in regulating N signaling during somitogenesis. In fact, the somite defects that are seen in Dll3 mutant mice are more similar to those reported in modulators of N signaling (LFng, Hes7, or Mesp2) rather than in mice lacking the well-characterized activating N ligand Dll1.

Activation of N signaling relies on contact between cells to allow the transmembrane DSL ligand on one cell to bind its receptor on an apposing cell. During its trafficking to the cell surface, N is constitutively processed by a furin-type protease producing a heterodimer that is composed of noncovalently associated extracellular and transmembrane subunits (Logeat et al., 1998). In response to ligand binding, the N heterodimer dissociates to release the extracellular domain from its membrane-bound portion (Sanchez-Irizarry et al., 2004; Weng et al.,...
Results

Dll3 does not activate N signaling

We isolated cDNA clones encoding rat Dll3 (rDll3) and engineered a full-length COOH terminally HA-tagged rDll3 for expression in L cells. An analysis of biotinylated cell surface proteins indicated that rDll3 andDll1 are expressed to similar levels on the surface of expressing cells (Fig. 1 A). To determine which N receptors are activated by rDll3, we used a coculture assay to measure activation of the N downstream effector CSL (Nofziger et al., 1999; Hicks et al., 2000, 2002; Bush et al., 2001). In brief, NIH 3T3 cells were cotransfected with each of the known N receptors (N1–4) and with a CSL reporter construct containing multiple CSL-binding sites that were upstream of a luciferase gene (Hsieh et al., 1996). After coculture withDll1, Jagged-1 (J1), rDll3, or parental L cell lines, quantification of luciferase activity indicated the level of ligand-induced CSL-dependent N signaling. Dll1 and J1 activated CSL in N1-, N2- (Fig. 1 B), N3-, and N4-expressing cells (not depicted). However, rDll3 did not activate signaling from any of the four known N receptors (Fig. 1 B and not depicted) despite equivalent cell surface levels. This lack of activity was not limited to the HA-tagged rDll3 cDNA clone because cells expressing untagged mouse Dll3 (mDll3; Dunwoodie et al., 1997) were also inactive in this assay (Fig. 1 B). Moreover, Dll3 cells did not activate the CSL reporter that was expressed in C2C12 myoblasts, COS, or N2A neuroblastoma cells (unpublished data), indicating that Dll3 cannot activate N signaling in multiple cell types.

Another measure of ligand-induced N signaling is the inhibition of myogenic differentiation of C2C12 myoblasts stably expressing N1 after coculture with cells expressing DSL ligands. In this assay, suppressed expression of the muscle structural gene myosin light chain 2 (MLC2) provides a read-out of N signaling that is induced by ligands (Fig. 1 C; Nofziger et al., 1999; Bush et al., 2001). In contrast toDll1 and J1 cells that strongly suppress the expression of MLC2, neither...
N1 nor N2 C2C12 myoblasts showed diminished MLC2 expression relative to parental L cells when cocultured with Dll3 cells (Fig. 1 C and not depicted). Therefore, Dll3 cells do not suppress C2C12 myogenic differentiation, which is consistent with our findings that Dll3 does not activate an N-responsive reporter construct (Fig. 1 B), providing an additional measure of Dll3’s inability to activate N signaling.

**L Fng does not enable Dll3 to activate N1 signaling**

We have previously reported that the glycosyltransferase LFng enhances Dll1-induced N signaling by using CSL reporter assays (Hicks et al., 2000; Yang et al., 2004). To determine whether LFng modification of N enables Dll3 to function as an activating ligand, NIH 3T3 cells were cotransfected with either alkaline phosphatase–tagged LFng or secreted alkaline phosphatase with N1 and a CSL reporter. Although LFng enhanced Dll1 activation of N1 and suppressed J1 activation of N1 as previously reported, neither Dll3 nor mDll3 activated N1 in the presence or absence of LFng (Fig. 1 D). Moreover, the other fringe family members radical and manic did not facilitate Dll3 activation of N1 or N2 (unpublished data). Together, these data suggest that fringe glycosylation of N does not enable Dll3 to function as an activating ligand.

**Dll3 coexpressed with Dll1 does not perturb Dll1-induced N signaling**

Given the inability of Dll3 to activate N signaling and the fact that Dll1 and Dll3 are coexpressed during development (Dunwoodie et al., 2002; Zhang et al., 2002; Takahashi et al., 2003), we asked whether Dll3 could alter Dll1-induced N signaling. Cells stably expressing both Dll1 and Dll3 were derived from the Dll1 line and were tested for CSL activation by either N1 or N2 (unpublished data). Together, these data suggest that fringe glycosylation of N does not enable Dll3 to function as an activating ligand.

**Dll3 does not bind to N1 in trans**

Because neither rDll3- nor mDll3-expressing cells activated N in either CSL reporter or myogenesis coculture assays, we determined whether Dll3 binds to N1. Based on the structure of a soluble Dll1Fc fusion protein that binds N1 and activates signaling, a Dll3Fc protein was generated by fusing the extracellular domain of Dll3 to Fc to allow clustering by anti-Fc antibodies, which is required for binding and activation (Hicks et al., 2000, 2002; Yang et al., 2004). When comparable amounts of Dll3Fc and Dll1Fc (Fig. 2 A) were assayed for binding to N1 cells, only Dll1Fc binding was detected (Fig. 2 B). Furthermore, although a low level of Dll1Fc binding was detected with vector-transfected cells, which is presumably a result of endogenous N, Dll3Fc did not bind to either vector or N1-transfected 293T cells (Fig. 2 B). Using fluorescent microscopy to monitor binding, Dll3Fc did not bind to any of the known N receptors even though Dll1Fc binding was readily imaged (not depicted). Moreover, the coexpression of LFng with N1 or N2 did not enable Dll3Fc binding (not depicted).

Although in agreement with our coculture data, the lack of detected Dll3Fc binding could result from low expression or misfolding of soluble Dll3Fc. Therefore, we determined whether a soluble N1Fc could bind Dll3-expressing cells. Although N1Fc binds to cells expressing either Dll1 (Fig. 2 C) or J1 (not depicted), N1Fc did not bind to Dll3 cells (Fig. 2 C). The lack of detected Dll3–N1 interactions in these binding assays is consistent with the inability of Dll3 cells to activate N signaling in CSL reporter and myogenesis assays (Fig. 1, B and C).

When compared with other Dll ligands, it is obvious that the Dll3 DSL domain has not been conserved (Fig. 2 D). Given that the DSL domain is required for ligand binding and signaling (Henderson et al., 1997; Shimizu et al., 1999), our data suggest that the divergent Dll3 DSL module does not support binding to N when presented either on the surface of interacting cells or as a soluble protein. Because Dll1Fc binds to N-expressing cells and Dll1 activates N signaling, we reasoned that replacement of the Dll3 NH2-terminal and DSL domains (NT-DSL) with those of Dll1 (D1NT) would allow Dll3 to interact with N. To test this idea, we replaced the Dll3 NT-DSL in Dll3Fc with D1NT to produce a soluble D1NTD3Fc. Although comparable amounts of D1NTD3Fc, Dll1Fc, and Dll3Fc were used (Fig. 2 A), we were unable to detect the binding of D1NTD3Fc to N1-expressing cells. Moreover, when the D3NT sequences were replaced with D1NT in the full-length Dll3 HA-tagged protein (D1NTD3), cells expressing D1NTD3 did not bind N1Fc (Fig. 2 C). Together, our findings suggest that the
DII3 inhibits N signaling cell autonomously

Because DII3 did not bind or activate any of the known N receptors when presented in trans, we determined whether DII3 inhibits N signaling when expressed with N in the same cell (cis or cell autonomously), as previously reported for other DSL family proteins (Henrique et al., 1997; Sakamoto et al., 2002; Itoh et al., 2003). To determine whether DII3 could cell autonomously inhibit N signaling that is induced by other DSL ligands, NIH 3T3 cells transiently expressing either N1 or N2, CSL reporter, and either vector, DII1, or DII3 plasmids were cocultured with DII1, J1, or L1. In these assays, CSL reporter activity was decreased 60% when either DII1 or DII3 were coexpressed with either N1 or N2 (Fig. 3 A). Serrate has also been reported to cell autonomously inhibit N (de Celis and Bray, 2000; Kiyota and Kinoshita, 2004), and J1 that was coexpressed with N1 or N2 also suppressed CSL activation (unpublished data). Therefore, unlike other DSL ligands that both activate and inhibit N signaling, DII3 cannot activate signaling in trans but effectively inhibits ligand-induced N signaling when coexpressed with N.

DII3 cell-autonomous expression does not decrease cell surface N1

To ensure that the loss in N signaling, which is detected when either DII3 or DII1 were coexpressed with N, was not caused by decreased N1 cell surface expression, N1 cell surface levels in the presence of either DII1 or DII3 were determined. Biotinylation of cell surface proteins indicated that the coexpression of either DII1 or DII3 with N1 did not decrease the amount of N1 that was detected in whole cell lysates (WCLs; Fig. 3 C) or the level of N1 that was detected at the cell surface (streptavidin [SAV]; Fig. 3 C). These findings are in agreement with a study on N cell surface expression in cells coexpressing chick Deltal and mouse N1 (Sakamoto et al., 2002). To more accurately quantitate the level of N1 cell surface expression, cells coexpressing either DII1 or DII3 with an NH2-terminal HA-tagged N1 (HA-N1) were stained with AlexaFluor488-conjugated HA antibody and were analyzed by flow cytometry. In agreement with our biotinylation data, the expression of DII3 with N1 did not significantly alter cell surface N1 (Fig. 3 D), suggesting that losses in cell surface N1 cannot account for losses in signaling (Fig. 3 A). Furthermore, biotinylation analysis of DII1 and DII3 indicate that neither DII1 nor DII3 surface expression was altered when coexpressed with N1 (Fig. 3 E). This suggests that the overexpression of ligand and receptor in the same cell does not alter trafficking to the cell surface.

DII3 directly interacts with N1 in coexpressing cells

Our binding studies did not detect interactions between DII3 and N1 (Fig. 2, B and C); however, these experiments measured trans interactions rather than interactions between DII3 and N1 within the same cell. Therefore, to detect cis interactions between DII3 and N1, we determined whether DII3 coimmunoprecipitated with N1. 293T cell lysates from cells coexpressing N1 and either vector, HA-tagged DII1, or DII3 were immunoblotted with an HA antibody Western blotting. In contrast, DII3 stably interacted with N1 antibodies and HA antibody Western blot to detect DII1 or DII3 (12CA5; top) or with N1 antibody (Y3–4; bottom). Middle panel is a HA Western blot of DII1 and DII3 from WCL.
to activate N signaling when tested in X. laevis embryos (Dunwoodie et al., 1997). In X. laevis, the formation of primary neurons can be used as a reliable readout of N signaling. In embryos in which N signaling is increased, the generation of primary neurons is markedly reduced, whereas the expression of N antagonists causes a reciprocal increase in primary neurons (Wettstein et al., 1994). Because Dll3 was previously reported to inhibit neurogenesis, we reinvestigated its activity in relation to that of Dll1. As previously demonstrated, injecting 250 pg Dll1 mRNA at the two-cell stage causes a marked decrease in the number of cells that express the neuronal marker β-tubulin at neural plate stages (Fig. 4, compare A with B; Chitnis et al., 1995; Dunwoodie et al., 1997). This decrease is indicative of activated N signaling (Chitnis et al., 1995) and is similar in nature to that observed when embryos are injected with NICD, XDelta1, or mDll4 (Shutter et al., 2000). In contrast, injecting either 250 pg or 1 ng mDll3 mRNA not only failed to inhibit neurogenesis but, in some cases, produced an increase in the number of β-tubulin–positive neurons (Fig. 4, C and D; and Table 1). Thus, in our hands, Dll3 does not activate N signaling when ectopically expressed during neurogenesis but, instead, behaves as an N inhibitor. We cannot account for the difference between these results and those obtained with equivalent amounts of injected Dll3 mRNA from the same Dll3 clone, which was reported previously (Dunwoodie et al., 1997). One possibility is that a suppression or delay in neuronal differentiation is often an artifact that occurs in RNA injection experiments. Nonetheless, our results indicate that Dll3, over a large concentration range, primarily acts as an inhibitory ligand in the X. laevis assay, which is in line with our findings in mammalian cell culture assays. Importantly, Dll1 prevents neurogenesis at the same concentration (250 pg), whereas Dll3 promotes neurogenesis, highlighting the different activities of these DSL ligands.

**Dll3 antagonism of N signaling regulates neuronal and glial differentiation**

Both Dll1 and Dll3 are expressed in the developing brain (Dunwoodie et al., 1997; Campos et al., 2001), where N signaling is known to regulate the differentiation of progenitors into neurons and glia. We have previously reported that D1Fc inhibits neurogenesis and promotes gliogenesis in mammalian neural stem cells (NSCs; Morrison et al., 2000; Ge et al., 2002). To further demonstrate Dll3 antagonism of N signaling, we transfected cortical NSCs with Dll3 or vector and induced astrogliogenesis with D1Fc as previously described (Ge et al., 2002). In this system, Dll3 reduced expression of the astrocyte markers glial fibrillary acidic protein (GFAP) and S100β, which were measured by the activation of GFAP and S100β reporters (Fig. 5, A and B). Because both GFAP and S100β regulatory regions contain functional CSL-binding sites, they are direct targets of N activation and, thus, serve as readouts of N signaling as well as gliogenesis (Ge et al., 2002; Hermanson et al., 2002). Dll3 not only suppressed D1Fc-induced N signaling that was required to drive the transcription of GFAP and S100β, but it also antagonized signaling that was induced by endogenous ligands (Fig. 5, A and B; Fc treated). Even in the absence of D1Fc, NSCs ectopically expressing Dll3 did not express GFAP, and the number of GFAP-positive cells as well as the level of GFAP expression was decreased by Dll3 transfection (Fig. 5, C and D) or infection with Dll3 adenovirus (Fig. 5 E).

In contrast to Dll1 that is known to block neurogenesis, Dll3 promoted neurogenesis of early (neurogenic) stage NSCs, as measured by increased NeuroD promoter activity (Fig. 5 F) and expression of the neuronal-specific marker β-tubulin (TuJ1; Fig. 5 G). Because N signaling prevents neurogenesis (Lewis, 1996), neural induction in the presence of Dll3 in cortical progenitors is in agreement with Dll3 functioning as a signaling antagonist of N in both our cell coculture (Fig. 3 A) and X. laevis injection assays (Fig. 4, C and D). Together, our findings suggest that Dll3 promotes neurogenesis and inhibits gliogenesis through antagonizing N and uncover a biological role for Dll3 as a negative regulator of cell fate decisions that are influenced by N signaling.

**Table 1. Quantitation of β-tubulin–positive neurons in D1- or mDll3-injected embryos**

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>Increase in neurons</th>
<th>Reduction or complete loss of neurons</th>
<th>No change</th>
<th>Total embryos scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (β-galactosidase)</td>
<td>4</td>
<td>3</td>
<td>48</td>
<td>55</td>
</tr>
<tr>
<td>Dll1 (250 pg)</td>
<td>1</td>
<td>23</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>Dll3 (250 pg)</td>
<td>15</td>
<td>0</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Dll3 (1,000 pg)</td>
<td>15</td>
<td>3</td>
<td>7</td>
<td>25</td>
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Embryos were injected and processed (Chitnis et al., 1995) to determine the relative amounts of β-tubulin that were scored either as an increase, reduction, or complete loss of neurons or as no change.

**L8Fng and Dll3 function cell autonomously to modulate N signaling levels**

Although the loss of either Dll1 or Dll3 leads to defects in somitogenesis, Dll1 mutants display a complete loss of somite polarity markers, which is similar to RBP-J knockout mice, highlighting that Dll1 and RBP-J are core components of the N pathway (for review see Giudicelli and Lewis, 2004). In contrast,
Figure 5. **Dll3 suppresses D1Fc-induced astrocytic differentiation.** Embryonic day 11.5 mouse cortical NSCs cotransfected with reporters for GFAP (A) or S100β (B) and either vector or Dll3 were treated with control Fc or D1Fc to induce astrogliogenesis. (C) NSCs cotransfected with GFP and either vector or Dll3 were cultured with D1Fc for 4 d and stained for GFAP. (D) Quantitation of transfected cells expressing GFAP from six independent experiments. (E) GFAP expression in NSCs infected with control (lane 1) or Dll3 adenovirus (lane 2) as determined by Western blotting. (F) Neuronal stage mouse cortical NSCs cotransfected with NeuroD promoter luciferase construct and vector or Dll3. Promoter activation is plotted as relative luciferase units for six experiments. P < 0.05. *, significant difference between DIll3 and vector; **, significant increase between Fc and D1Fc. Error bars represent SD. AU, arbitrary units. (G) Tubulin (TuJ1) expression in NSCs infected with control (lane 1) or Dll3 adenovirus (lane 2) as determined by Western blotting.

losses in Dll3 produce only disorganization in somite patterning, which is a phenotype that is strikingly similar to gains or losses in the N modulator LFng. These findings suggest that although DIll1 is an activating ligand that is absolutely required for N signaling, DIll3 and LFng may serve to regulate levels of N signaling during somitogenesis. To explore the relationship between LFng and DIll3 in modulating N signaling that is induced by other DSL ligands, we used the CSL reporter assay. When cells transiently expressing N1, CSL reporter, and either DIll3 or vector as well as low (100 ng) or high (500 ng) amounts of LFng DNA were cocultured with DIll1 cells, LFng appeared to counteract the inhibitory effects of DIll3 in a dose-dependent manner (Fig. 6 A). Specifically, increases in transfected LFng increased signaling even in the presence of DIll3, suggesting a dynamic interplay between LFng and DIll3 in modulating the level of N1 signaling. Conversely, LFng enhancement of DIll1-induced N signaling was suppressed by DIll3 in a dose-dependent manner (Fig. 6 B). Together, these experiments illustrate the dynamic nature of DIll1-induced N signaling in response to DIll3 and suggest that, like LFng, DIll3 also modulates N signaling.

Fringe proteins have been reported to override the cis-inhibitory effects of other DSL ligands in flies and chicks (Hukriede et al., 1997; de Celis and Bray, 2000; Sakamoto et al., 2002). It has been proposed that LFng modification of N and/or DSL ligand disrupts cis-inhibitory complexes to allow N activation by adjacent ligand cells. In this regard, two DIll3 EGF-like repeats have broad O-fucosylation consensus sequences, which is a prerequisite for LFng glycosylation (Panin et al., 2002). To determine whether LFng glycosylation of N1 or DIll3 disrupts interactions between DIll3 and N1, communo-precipitation of N1 with DIll3 was measured in the presence of LFng. Irrespective of LFng coexpression, N1 communo-precipitated with DIll3 (Fig. 6 C), and, conversely, DIll3 communoprecipitated with N1 (not depicted), indicating that LFng does not alter cis interactions between DIll3 and N1. Even increasing amounts of LFng did not disrupt DIll3–N1 interactions (unpublished data).

Because LFng did not prevent interactions between DIll3 and N1, it seemed that LFng enhancement of DIll1 signaling in combination with DIll3-inhibitory effects could account for LFng reversing DIll3 cis inhibition. LFng both potentiates DIll1-induced N1 signaling and inhibits signaling by J1 (Hicks et al., 2000; Yang et al., 2004). Therefore, we reasoned that if LFng exclusively modulates trans signaling and has no effect on DIll3–N1 cis interactions, the coexpression of DIll3 and LFng with N1 should result in a greater inhibition of J1-induced N signaling than with either modulator alone. Conversely, if LFng functions directly to prevent cis inhibition by DIll3, then DIll3 coexpression should not further inhibit signaling by J1. The coexpression of either LFng or DIll3 suppressed J1-induced N1 signaling, whereas the coexpression of both LFng and DIll3 produced a stronger block in signaling (Fig. 6 D). This indicates that LFng does not disrupt DIll3 cis inhibition but rather functions to modulate the productivity of N signaling that was induced by trans ligand. LFng coexpression with DIll3 further inhibited N1 signaling that was induced by J1, yet reversed the DIll3 inhibition of signaling in response to DIll1. This suggests that, together, LFng and DIll3 could finely tune the levels of N signaling that are induced by different DSL ligands.

**Discussion**

In contrast to other DSL ligands, the reported activating ligand DIll3 (Dunwoodie et al., 1997) did not bind or activate N when tested in a number of different assays. Despite the inability of DIll3 to induce signaling from any of the known N receptors, we find that DIll3 is a potent antagonist of ligand-induced N signaling when coexpressed with N. Indicative and supportive of DIll3 antagonism of N signaling, we show that DIll3 promotes primary neurogenesis in *X. laevis* embryos and enhances neuronal differentiation of mouse cortical neural progenitors in vitro, whereas glial differentiation is reduced. In combination with LFng, which is a well-known modulator of ligand-induced N signaling, DIll3 regulates the level of signaling, suggesting that it may contribute to the dynamic changes in N signaling that are required in development.

**DIll3 is a highly divergent DSL family member**

DIll3 has 36, 41, 31, and 29% overall amino acid homology to mDIll1, *X. laevis* Delta2, mDIll4, and *Drosophila melanogaster*...
Delta, respectively, identifying Dll3 as the most divergent DSL member (Dunwoodie et al., 1997). Dll3 is also the shortest of the mammalian Dll ligands, with only six EGF-like repeats compared with eight repeats identified for Dll1 and Dll4. A comparison of the Dll3 DSL domain with mDll1, mDll4, X. laevis Dll2, D. melanogaster Delta, and zebrafish Delta-A-D highlights its divergence (Fig. 2 D). Interestingly, although a large number of Delta homologues have been identified in zebrafish, none appear to have the degenerate DSL domain that is characteristic of Dll3. The DSL domain is thought to be important in receptor binding and activation (Henderson et al., 1997; Shimizu et al., 1999). However, a soluble form of J1 containing only the NT-DSL fused to Fc binds poorly to N, and the addition of the first two J1 EGF-like repeats is required to enhance binding (Shimizu et al., 1999). Therefore, it was surprising that replacement of the Dll3 NT-DSL sequences with those of Dll1 in D1NTD3Fc did not promote binding to N1. In fact, if one assumes that Dll1 NT-DSL facilitates interactions, it seems that Dll3 EGF-like repeats antagonize Dll1 NT-DSL binding to N, suggesting that additional differences in Dll3 perturb Dll3–N interactions. In support of this idea, the replacement of NTDSL sequences in full-length Dll3 with those of Dll1 did not promote N1Fc binding to D3 cells. These findings suggest that Dll3 EGF-like repeats do not function as reported for J1 EGF-like repeats, identifying additional differences between Dll3 and other DSL ligands. Interestingly, the second Dll3 EGF-like repeat is in-complete, and missense mutations map to this and other repeats in spondylocostal dysostosis patients (Bulman et al., 2000; Turnpenny et al., 2003), suggesting that these repeats are important for Dll3 function. An analysis of different Dll1–Dll3 chimeric proteins will be required to further investigate the structural differences between Dll1 and Dll3.

In addition to differences in Dll3 extracellular sequences, the intracellular domain is significantly smaller than that of Dll1 and other DSL family members (Dunwoodie et al., 1997). The Delta intracellular domain is required for the activation of N signaling, perhaps reflecting its role in endocytosis that is regulated through ubiquitination (for review see Le Borgne et al., 2005). Although the exact function of Delta ubiquitination in N signaling is not well understood, it is interesting to note that although the Dll1 intracellular domain contains 17 lysine residues, which are potential sites for ubiquitination, there are no lysines in the Dll3 intracellular domain. Given the importance of ubiquitination in Delta-induced N signaling, the lack of lysines in Dll3 is consistent with our findings that Dll3 is unable to activate N signaling.

Finally, the COOH terminus of Dll3 lacks a PDZ-binding motif that is present in other DSL proteins and directs different cellular responses through interactions with PDZ domain–containing proteins (Ascano et al., 2003; Pfister et al., 2003; Six et al., 2003; Wright et al., 2004), identifying additional functional differences for Dll3. Thus, in addition to extracellular changes that prevent N binding in trans, Dll3 appears to have undergone other alterations that do not promote signaling. Because our data suggest that Dll3 functions to inhibit rather than activate N signaling, associations of Dll3 with N pathway genes likely reflect a role for Dll3 as a signaling antagonist rather than as an activating ligand.

**Dll3 as a modulator of cellular differentiation induced by N signaling**

Dll1 and Dll3 have overlapping as well as distinct expression patterns in the developing cortex and spinal cord (Dunwoodie et al., 1997; Kusumi et al., 1998; Campos et al., 2001; Sparrow et al., 2002). Specifically, Dll1 is expressed within the ventricular zone, whereas Dll3 is located more laterally in a population of cells that is thought to be fated for terminal neuronal differentiation. It has been proposed that Dll1 ventricular cells express Dll3 after migrating away from the ventricular zone, and that this sequential expression of Dll1 and Dll3 is linked to progression toward a neuronal fate. Consistent with this idea, we find that ectopic expression of Dll3 in either X. laevis oocytes or neural progenitors blocks N signaling and promotes terminal
neuronal differentiation. However, pudgy and Dll3 knockout mice display only subtle neuroepithelial defects in the lateral ventricles with a low penetrance of ~50%, suggesting that if Dll3 does regulate neural differentiation, its effects must be transient. Nonetheless, our findings that Dll3 regulates neuronal cell fate are consistent with a role for Dll3 as a modulator of N signaling rather than as a core component of the pathway and support a role for Dll3 in altering progenitor cell fate through attenuating N signaling.

**Dll3 is an inhibitor of N signaling**
We have found that Dll3 is not an activating ligand for N but rather functions to cell autonomously inhibit signaling. In support of Dll3 as an N antagonist, findings in mice have also proposed that Dll3 counteracts the activity of Dll1 in regulating N signaling during somite patterning (Takahashi et al., 2003). Consistent with Dll3 functioning as a negative regulator, the expression of an N target gene, N-regulated ankyrin repeat protein (NRARP), is extinguished in nascent somites in mice lacking either N1 or Dll1 but is increased in pudgy mice (Krebs et al., 2001). Perhaps, Dll1-induced N signaling activates NRARP expression, whereas Dll3 antagonizes this signaling, and, thus, a loss of Dll3 leads to an increase in N signaling and a consequent increase in NRARP expression. In contrast, expression of the N target gene Hes5 is either absent or reduced in the PSM of Dll3 mutant embryos (Dunwoodie et al., 2002), which is supportive of Dll3 as an activator of N signaling. However, it is important to note that Dll1 mutant embryos that are defective in N signaling show a reduction or loss of Hes5 in both the PSM and neural tube, whereas Dll3 mutants maintain strong Hes5 expression in the neural tube (Barrantes et al., 1999; Dunwoodie et al., 2002). Because somitogenesis, unlike the developing nervous system, requires cyclic N signaling, losses in Dll3 may adversely affect negative feedback loops that are required to maintain proper levels of N signaling in the PSM in order to affect Hes5 expression. Nonetheless, Dll3 and Dll1 mutant somite phenotypes are clearly different, suggesting that Dll3 and Dll1 have distinct functions in regulating N signaling during somitogenesis (Kusumi et al., 2004). Our findings support this idea, and we suggest that the different activities identified in this study for Dll1 and Dll3 may account for the distinct mutant phenotypes. Finally, our finding that Dll3 has diverged to function solely as an inhibitor of N signaling that is induced by other DSL ligands is reminiscent of reports for other signaling antagonists that are structurally related to their activating ligands but function to inhibit rather than activate signaling (Vinos and Freeman, 2000; Daluiski et al., 2001).

**Materials and methods**
**Cell lines and mammalian expression constructs**
Parental cell lines were obtained from the American Type Culture Collection and were propagated as suggested. Stable C2C12 cell lines expressing N1 and L cell lines expressed Dll1 or J1 have been described previously (Lindsell et al., 1995; Hicks et al., 2000). Stable Dll3-expressing cells were generated by using hypoxanthine and thymine selection as previously described for J1-expressing L cells (Lindsell et al., 1995).

Dll3 was isolated from an embryonic day 13 rat brain cDNA library (GenBank/EMBL/DDB accession no. AF084576) based on homology to mDll3 (cDNA obtained from S. Dunwoodie, University of New South Wales, Sydney, Australia; Dunwoodie et al., 1997) and was tagged with triple tandem repeat of the influenza virus HA epitope. HA-tagged rDll3 and the previously described Dll1 were subcloned into pcDNA3 expression vectors (Turner and Weintraub, 1994). DIIFc was generated by fusing the extracellular domain of DIIF (1–1476 bp) to Fc and subcloning into the pcDNA3 expression vector (Invitrogen). DIIF/D1Fc and DIIF/D3D3 were constructed by replacing the first 651 bp of DIIFc or HA-tagged DIIF3, respectively, with the NH2 terminus and DSI domain of DIIF (1–725 bp). N1Δmyc replaced the COOH-terminal 436 amino acids of full-length rat N1 with six myc epitopes in the pcDNA3-mt vector (Yang et al., 2004). N1Δmyc HA-tagged N1 (HA-N1) was generated by inserting the triple HA epitope immediately downstream of the signal peptide by using a PCR overlap strategy and subcloning into pcDNA4.
cold PBS but lysed in Triton X-100 buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton X-100). WCLs were incubated on ice for 1 h with a mixture of anti-N1 intracellular domain mAbs (PCR12 and 93–4) at 1:200 each. For pull-downs between Lfng-modified N1 and DI3 (Fig. 6 C), the immunoprecipitation protocols were based on methods published by Sakamoto et al. (2002). In brief, 250 ng cell lysate (at a final volume of 10 μl) was incubated in a 0.3-mm dish and were transfected with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions using 0.3 μg N1, DI3, or vector and 1 μg of secreted alkaline phosphatase or Lfng DNA. Cells were lysed in 1% Triton X-100 lysis buffer (100 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, and 1% Triton X-100; Sakamoto et al., 2002). Lysate was incubated with a mAb complex (31F10; Roche Biosciences) and rabbit anti-rat Ig for 30–60 min on ice. Immunoprecipitates were collected on protein A-agarose beads (Invitrogen). Specific proteins were identified after SDS/PAGE, transferred to NitroBind membrane (Osmonics), probed with 1:200 each. For pull-downs between N1 and Di1 using 60 ng of secreted alkaline phosphatase or Lfng DNA, and 0.1 μg N1, Dll3, or vector and vector were incubated for 45 min at 37°C. After binding, the cells were washed with medium and lysed in Triton X-100 buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, and 1% Triton X-100). NSCs were isolated and cultured from embryonic day 11.5 BALB/c embryos, D1Fc, Dll3Fc, D1NTD3Fc, or Fc conditioned medium was generated as previously described (Hicks et al., 2002; Yang et al., 2004). For binding studies, cells were transfected with pHcRed (BD Biosciences and Clontech) and 48 h posttransfection, cells were stained live with pHcRed-expressing cells emitting fluorescence at 660 nm. The pHcRed-expressing cells were preclustered with FITC-conjugated goat anti-human Fc antibodies, and were detected using ECL Plus Western blotting detection system (GE Healthcare) and a scanner (Typhoon9410; GE Healthcare).

**References**


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