Developmental Cell, Vol. 1, 795–806, December, 2001, Copyright ©2001 by Cell Press

Xenopus Neuralized Is a Ubiquitin Ligase that Interacts with XDelta1 and Regulates Notch Signaling

Gisèle A. Deblandre,[1] Eric C. Lai,[2] and Chris Kintner[1,3]
[1]The Salk Institute for Biological Studies
P.O. Box 85800
La Jolla, California 92186
[2]Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

Notch signaling in *Drosophila* requires a RING finger (RF) protein encoded by *neuralized*. Here we show that the *Xenopus* homolog of *neuralized* (*Xneur*) is expressed where Notch signaling controls cell fate choices in early embryos. Overexpressing XNeur or putative dominant-negative forms in embryos inhibits Notch signaling. As expected for a RF protein, we show that XNeur fulfills the biochemical requirements of ubiquitin ligases. We also show that wild-type XNeur decreases the cell surface level of the Notch ligand, XDelta1, while putative inhibitory forms of XNeur increase it. Finally, we provide evidence that XNeur acts as a ubiquitin ligase for XDelta1 in vitro. We propose that XNeur plays a conserved role in Notch activation by regulating the cell surface levels of the Delta ligands, perhaps directly, via ubiquitination.

Introduction

Many of the components that comprise the Notch signaling pathway in *Drosophila* were originally identified as mutations in six zygotic genes that give a similar neurogenic phenotype in early embryos (Lehmann et al., 1983). *Notch* encodes a large transmembrane receptor, *Delta* encodes a ligand that activates Notch signaling, and the *E(SPL)* genes, which are transcriptionally activated by Notch signaling, encode downstream effectors. A fourth neurogenic gene, *Mastermind*, is implicated in Notch-mediated transcription, potentially as a coactivator (Petcherski and Kimble, 2000). All of these components are evolutionarily conserved, and homologs of these proteins comprise a similar signaling pathway in other invertebrates and vertebrate species (Artavanis-Tsakonas et al., 1999). By contrast, two of the original neurogenic genes, *Big Brain* and *neuralized*, are known to be required for Notch signaling in *Drosophila*, but the biochemical activities of their encoded products within the Notch pathway remain poorly defined. It is also not known whether these genes are evolutionarily conserved in their action. To address these issues, we have analyzed a *Xenopus* homolog of *neuralized* in terms of its biochemical properties and its role in Notch signaling during early embryonic development.

[3]Correspondence: kintner@salk.edu

In *Drosophila*, *neuralized* (*Dneur*) is required for a subset of the developmental events that are known to be regulated by Notch signaling (Dietrich and Campos-Ortega, 1984; Hartenstein et al., 1992; Lai and Rubin, 2001a, 2001b; Yeh et al., 2000). *Dneur* is required, for example, when Notch restricts, by the process of lateral inhibition, the number of neuroblasts (NBs) that form within the neurogenic region of early embryos or the number of sensory organ precursors (SOPs) that form within proneural clusters in developing imaginal discs. Not only do the number of NBs and SOPs increase dramatically in DNeur loss-of-function mutants as seen when Notch signaling is inactivated, but similar neurogenic phenotypes also occur when DNeur is overexpressed (Lai and Rubin, 2001a). Surprisingly, DNeur overexpression also blocks Notch signaling during wing margin or wing vein formation even though the Notch pathway functions normally in these cases in the absence of DNeur. Thus, Notch signaling critically depends on DNeur activity in specific cases but more generally is exquisitely sensitive to DNeur overexpression.

Dneur is expressed at low levels in tissues where it functions, such as the neurogenic region of the *Drosophila* embryo (Boulianne et al., 1991; Price et al., 1993). During lateral inhibition, levels of *Dneur* RNA increase markedly within cells that are singled out by Notch signaling to differentiate. Indeed, this increase in *Dneur* expression has been used to mark the formation of SOPs based on an A101 enhancer trap line where *lacZ* is inserted into the *Dneur* gene (Huang et al., 1991). This observation suggests that DNeur functions in the signaling cell during lateral inhibition, therefore implying a cell-nonautonomous function during Notch signaling. However, genetic mosaic analysis shows that DNeur acts cell autonomously to allow Notch signaling to restrict the number of SOPs that form in the proneural cluster (Lai and Rubin, 2001a; Yeh et al., 2000). Thus, the high expression level of *Dneur* in differentiating cells conflicts with the evidence that it functions in receiving cells to inhibit their differentiation.

DNeur and related vertebrate proteins are characterized by two large repeats, termed Neuralized Homology Repeats (NHRs), and a C_3HC_4 motif at the carboxyl terminus, called a RING finger (RF) domain, which is characterized by conserved C and H residues responsible for binding two Zn^{2+} ions in two complex interweaved finger-like structures (Nakamura et al., 1998; Price et al., 1993). These structural features have not been useful in predicting how these proteins might be involved in Notch signaling, until recently, when the RF domain has been found in a number of proteins that act as ubiquitin ligases (Jackson et al., 2000). These proteins contain domains that bind to target proteins and an RF domain that binds and activates ubiquitin conjugases, thus forming a complex that promotes the transfer of ubiquitin to target proteins. Ubiquitination of proteins can be a signal for degradation by the proteasome, but it can also serve as a modification that alters the activity of proteins in a variety of different ways (Hicke, 2001). Recently, DNeur has been shown to have ubiquitin ligase

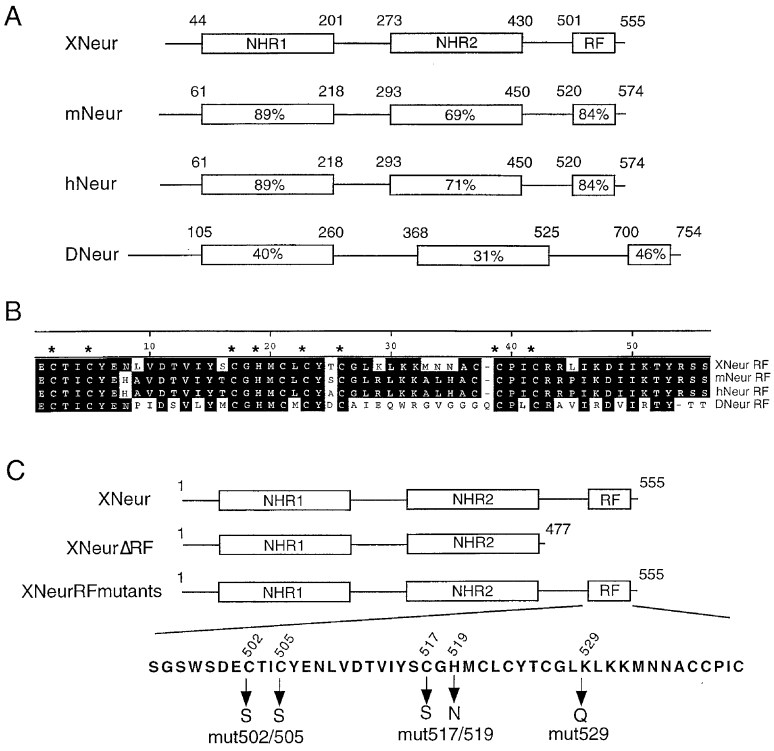

Figure 1. Alignment of Neuralized Proteins

(A) All Neuralized proteins contain two Neuralized Homology Repeats (NHRs) and a C-terminal C_3HC_4 RING finger (RF) domain whose percentage of similarity are given comparatively to XNeur.

(B) The conserved RF domain in Neur proteins. Conserved C and H residues are marked with asterisks.

(C) Constructs and mutations of XNeur used in this study.

activity in vitro, but it remains unclear how this activity relates to its function in the Notch signaling pathway (Yeh et al., 2001).

Here we report the isolation and functional analysis of a *Xenopus* homolog of *neuralized* (*Xneur*). We present its expression pattern in embryos, overexpression phenotypes of wild-type and putative dominant-negative forms of XNeur in *Xenopus* and *Drosophila* embryos, and its biochemical properties as a ubiquitin ligase. Finally, we provide evidence that XNeur interacts with the Notch ligand XDelta1. The results of this analysis indicate that XNeur plays a conserved role in Notch pathway and lead us to propose a model in which XNeur regulates Notch signaling by promoting the loss of XDelta1 at the cell surface, perhaps directly by ubiquitination.

Results

Cloning and Expression Pattern of *Xenopus neuralized*

As described in Experimental Procedures, cDNAs encoding a *Xenopus* homolog of *Drosophila neuralized* (XNeur) were identified via degenerate PCR. The proteins predicted by these cDNA contain the signature domains found in all Neuralized proteins identified so far: the two Neuralized Homology Repeats (NHR) and a C-terminal RF domain (Figure 1A). These subdomains are extremely well conserved among vertebrate species, with XNeur sharing 89% identity in its NHR1 and 84% in its RF domain with the mouse and human counterparts.

When analyzed by whole-mount in situ hybridization (WISH), early *Xenopus* embryos express *Xneur* RNA ubiquitously at low levels. Beginning at neural plate stages, isolated cells with higher levels of *Xneur* RNA are evident in both the developing nervous system and skin (Figures 2A–2D). Based on their number and position, these *Xneur*-expressing cells likely correspond to primary neurons within the neural plate and tube, and to precursors of specialized ciliated cells in the skin (Figures 2C and 2D). The differentiation of both cell types is Notch regulated (Chitnis et al., 1995; Deblandre et al., 1999; Wettstein et al., 1997), suggesting that *Xneur* expression correlates with the process of lateral inhibition. To confirm this interpretation, we examined *Xneur* expression in embryos where Notch signaling is increased or decreased experimentally using RNA injection (Wettstein et al., 1997). As predicted, in embryos in which cell differentiation is blocked with an activated Notch, Notch-ICD, *Xneur* expression is reduced (Figures 2E and 2F). By contrast, in embryos in which cell differentiation is increased with a dominant-negative form of Su(H), Su(H)DBM, the expression of XNeur is markedly expanded (Figures 2G and 2H). These data show that Notch signaling negatively regulates the number of *Xneur*-expressing cells and suggest strongly that *Xneur*, as in *Drosophila*, is expressed at higher levels in differentiating cells as they are selected out by lateral inhibition (Boulianne et al., 1991; Price et al., 1993).

Xneur Overexpression Blocks Lateral Inhibition in *Xenopus* Embryos

In *Drosophila*, developmental events requiring Notch signaling are exquisitely sensitive to *Dneur* overexpression. We therefore asked whether *Xneur* also interacts with the Notch pathway in *Xenopus* embryos using overexpression by RNA injection. When overexpressed in embryos, *Xneur* produces a large increase in the number of ciliated-cell precursors in the skin, as marked by α-tubulin staining, indicating a loss of Notch signaling

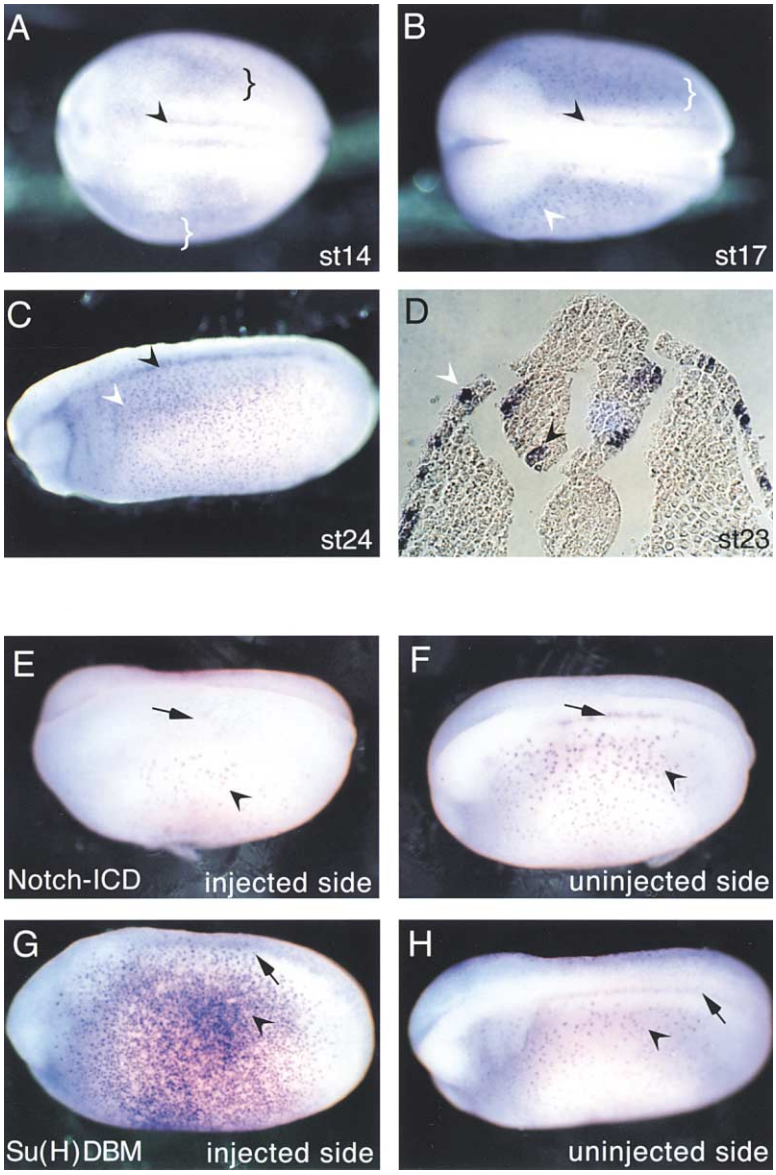

Figure 2. *Xneur* Expression in Early *Xenopus* Embryos

(A–D) WISH analysis of *Xenopus* for *Xneur* RNA expression, shown as dorsal views (A and B), side view (C), or as a transverse section at the level of the spinal cord (D). *Xneur* RNA is expressed within primary neurons in the neural plate ([A–D], black arrowhead) and ciliated cells in the skin ([A–D], white arrowhead). Lower and more diffuse expression of XNeur is detected at stages prior to cell type determination ([A and B], brackets).

(E–H) Embryos injected with RNA encoding Notch-ICD (E and F) or Su(H)DBM (G and H) were stained for *Xneur* RNA by WISH. Arrows and arrowheads indicate primary neurons and ciliated-cell precursors, respectively.

(Figures 3A–3C). This neurogenic phenotype became milder as the levels of *Xneur* RNA injected into embryos were reduced but never reversed into a phenotype indicative of increased Notch signaling. We next determined which regions of XNeur blocked Notch signaling using the ciliated-cell assay. In *Drosophila*, a form of DNeur lacking the RF domain consistently disrupts Notch signaling regardless of the levels of overexpression. A similar form of XNeur (XNeurΔRF) also produced large increases in the number of ciliated-cell precursors (Figures 3D–3F) and in this respect was more potent then wild-type XNeur, both in terms of number of embryos affected (83% versus 73%) and the severity of the phenotype. Forms of XNeur lacking either one of the NHR domains gave no phenotype, indicating that both NHRs are required for activity. Just the RF domain of XNeur produced extremely deleterious effects on the general morphology of the embryos and more specifically on the integrity of the skin, resulting for example in blistering and necrotic tissue (data not shown). Together these results indicate that Notch signaling can be disabled in the developing *Xenopus* skin by overexpressing either wild-type *Xneur* or *XneurΔRF*.

Embryos overexpressing *Xneur* or *XneurΔRF* RNA were also stained with a *N-tubulin* probe to examine the differentiation of primary neurons. Injection of *Xneur* RNA produced a mild increase in the number of primary neurons, indicating a weak loss of Notch signaling. By contrast, *XneurΔRF* RNA produced no effect. Thus, even though *Xneur* is expressed by both primary neurons and ciliated-cell precursors, alterations in the level of XNeur activity is much more effective in blocking Notch signaling in the skin relative to the neural plate.

Xneur Overexpression in *Drosophila* Produces Similar Phenotypes as *Dneur*

In *Drosophila*, overexpressing DNeur leads to opposing changes in Notch signaling, depending on the levels of overexpression. Relatively low levels of overexpression produce phenotypes associated with increased Notch

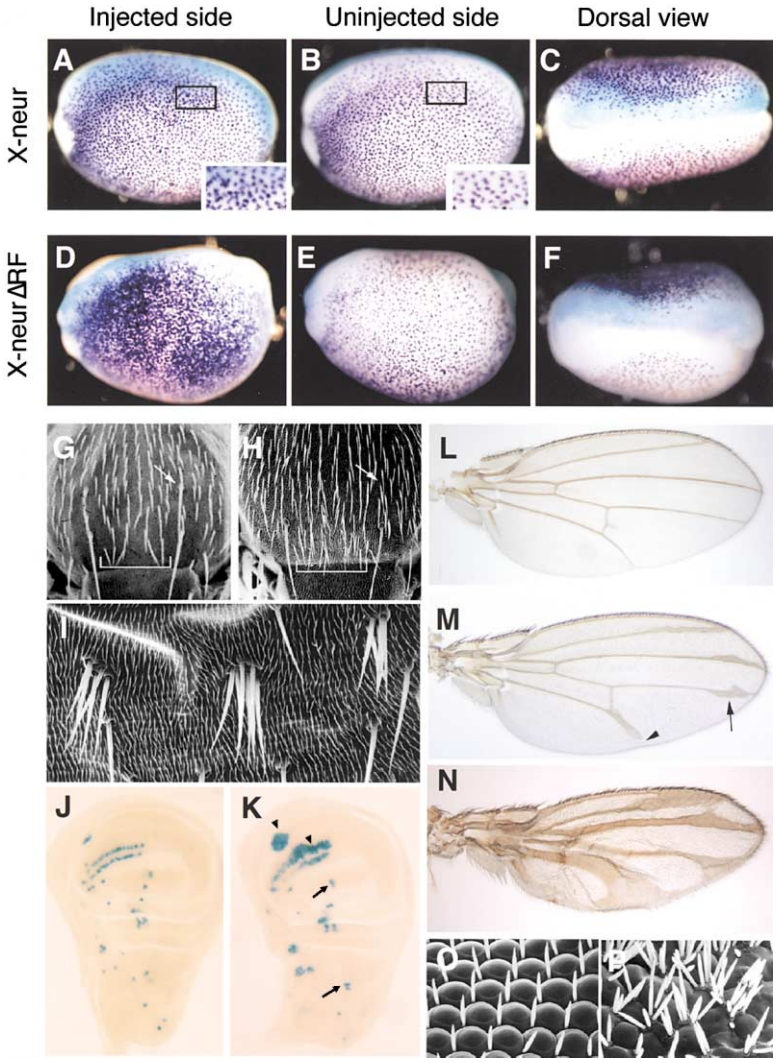

Figure 3. XNeur Functionally Interacts with the Notch Pathway

(A–F) Stage 16 *Xenopus* embryos injected with RNA encoding XNeur (A–C), or XNeurΔRF (D–F) were stained by WISH using an α-tubulin probe (dark purple) to mark ciliated-cell precursors and X-gal to mark the injected side (light blue).

(G–N) SEMs of adult flies of the following genotypes: (G) wild-type, (H) *sca-gal4/UAS-Xneur*, and (I) *sca-gal4/2x UAS-Xneur*. Misexpression of *Xneur* eliminates some notum macrochaete positions ([G and H], arrows) but increases the density of the microchaete field ([G and H], brackets).

(I) Examples of abdominal bristle tufts induced by XNeur.

(J and K) *Xneur* misexpression changes the number of SOPs marked by the A101 enhancer trap in third instar wing imaginal discs; (J) *sca-gal4*; *A101*, (K) *sca-gal4/2x UAS-Xneur*; *A101*. Some sensory organ precursor positions are missing ([K], arrows) but many positions are multiplied ([K], arrowheads).

(L–N) Effect of XNeur misexpression on the adult wing; (L) *bx-Gal4/+*, (M) *VMQ-Gal4/UAS-Xneur*, (N) *bx-Gal4/+*; *2x UAS-Xneur*. (L and M) Lower levels of XNeur induce mild distal vein thickening ([M], arrow) and a minor truncation of vein L5 ([M], arrowhead) while (N) higher levels of XNeur induce massive vein thickening.

(O and P) Effect of XNeur misexpression of the adult eye; (O) *GMR-Gal4/+*, (P) *GMR-Gal4/UAS-Xneur*.

signaling, while relatively high levels of overexpression block Notch signaling (Lai and Rubin, 2001a). By contrast, *Xneur* overexpression is unable to activate Notch signaling in *Xenopus* and consistently leads to a phenotype comparable to a *neur* loss-of-function phenotype in *Drosophila*. To examine this issue further, XNeur was misexpressed in *Drosophila* imaginal disks using the Gal4-UAS system (Brand and Perrimon, 1993). When misexpressed in the wing imaginal disc, XNeur increased bristle density in adults (Figures 3G–3I) and the number of SOPs as marked by the A101 enhancer trap within proneural clusters (Figures 3J and 3K). Both phenotypes indicate loss-of-Notch signaling and are similar to the phenotypes seen when two copies of DNeur are misexpressed. Less frequently, adult macrochaete were lost in response to XNeur misexpression (Figures 3G and 3H), with a corresponding loss of SOP positions as marked by activity of the A101 enhancer trap in third instar imaginal discs (Figures 3J and 3K). Thus, XNeur overexpression can produce phenotypes representative of Notch pathway hyperactivity as seen when DNeur was overexpressed at low levels. Similarly, at low levels of misexpression in the wing imaginal dics, *Xneur* caused a mild truncation of wing vein L5, indicative of gain-of-Notch pathway activity and a thickening of the distal portions of all wing veins indicative of loss-of-Notch pathway activity (Figures 3L and 3M). At higher levels of misexpression, XNeur caused a massive thickening of all wing veins, indicating a near-complete failure of lateral inhibition of the vein fate (Figure 3N). Finally, we observed that misexpression of *Xneur* during development of the eye resulted in extremely rough eyes, with defects in ommatidial assembly and strong tufting of interommatidial bristles (Figures 3O and 3P). Therefore, the phenotypes caused by misexpression of *Xneur* and *Dneur* were largely similar in nature, although phenotypes indicative of increased Notch signaling were more prevalent with *Dneur* than with *Xneur* (Lai and Rubin, 2001a). These results support the idea that XNeur and DNeur are functionally similar in terms of their role in Notch signaling.

XNeur Is Unstable, Ubiquitinated, and Degraded by the Proteasome in an RF-Dependent Fashion

To determine whether XNeur functions in the ubiquitin pathway, we first asked whether XNeur is an unstable protein since many ubiquitin ligases are themselves ubiquitinated and targeted for protein degradation. First,

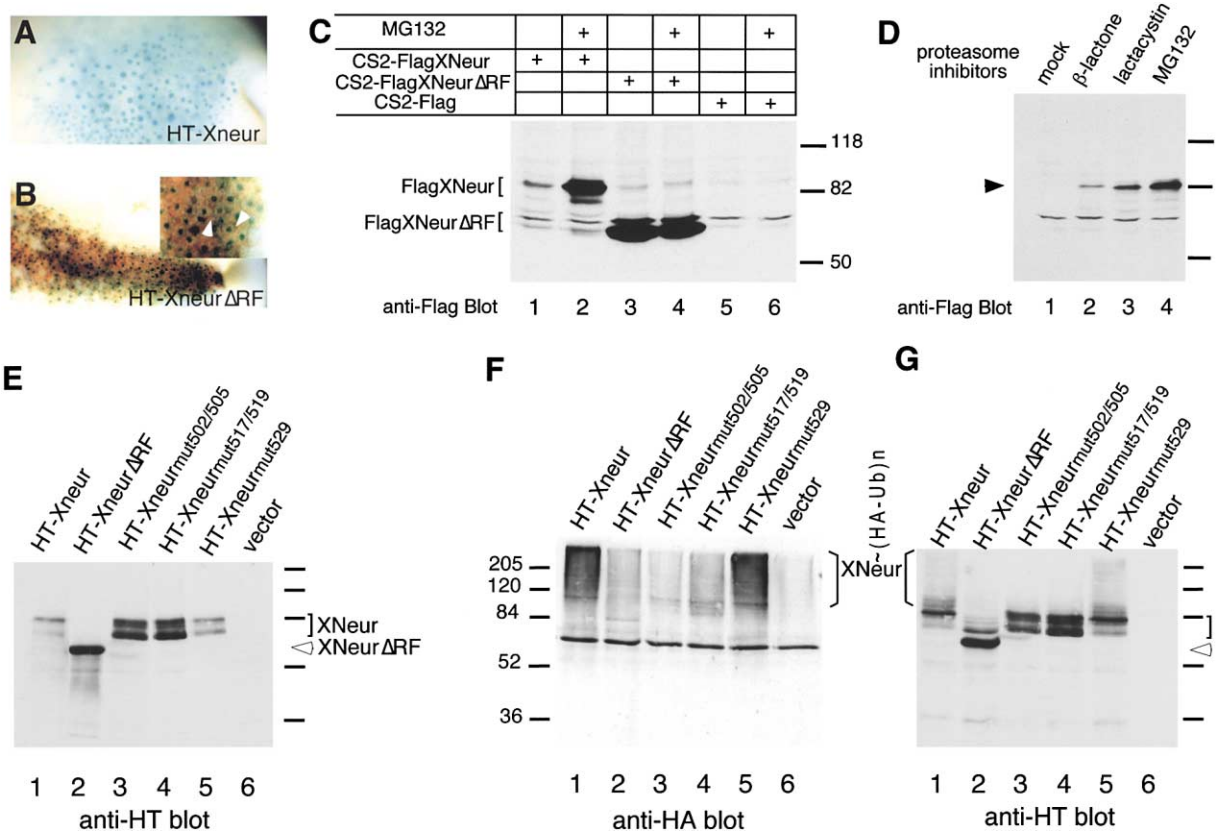

Figure 4. XNeur Is Degraded by the Proteasome and Ubiquitinated in an RF-Dependent Fashion

(A and B) Surfaces of stage 14 embryos injected with RNAs encoding His-tagged XNeur or XNeurΔRF and then processed by HRP immunohistochemistry with anti-RGSHis antibodies. XNeurΔRF accumulates at the plasma membrane (arrowheads in [B] inset). Injection site is marked by X-gal staining of a *NlacZ* tracer (blue nuclei).

(C and D) Western blot of total cell extracts prepared from 293T cells transfected with Flag-tagged XNeur or XNeurΔRF, which were treated for 24 hr with DMSO only ([C], lanes 1, 3, and 5, and [D], lane 1), with 2 μM MG132 ([C], lanes 2, 4, and 6, and [D], lane 4) or with 2 μM *clasto*-lactacystin β-lactone or lactacystine ([D], lanes 2 and 3).

(E) Western blot of His_6-tagged (HT) wild-type or mutant forms of XNeur (see Figure 1C) purified on Ni^{2+} beads from transfected 293T cells.

(F and G) A similar analysis as in (E) was performed except that pCS2-HA-ubiquitin was cotransfected with the various HT-XNeur forms. Blot was probed with anti-HA (F) to detect ubiquitinated proteins and subsequently with anti-RGS-His antibodies (G) to detect XNeur proteins.

proteins levels were examined in embryos injected with synthetic RNA encoding His-tagged forms of either XNeur or XNeurΔRF (HT-XNeur or HT-XNeurΔRF), either by immunohistochemistry or by Western blot analysis. By either analysis, HT-XNeur was undetectable, whereas HT-XNeurΔRF was highly expressed (Figures 4A and 4B, and data not shown), even though both RNAs produced equivalent levels of protein product when translated in vitro (data not shown). Similar results were obtained using other epitope tags, indicating that the relative instability of XNeur is not tag related.

The mechanism responsible for the instability of XNeur was examined in HEK-293T cells using transient transfection assays. As in *Xenopus* embryos, Flag-XNeurΔRF is readily detected after Western analysis of extracts prepared from transfected HEK-293T cells while Flag-XNeur is not (Figure 4C, lanes 1 and 3). Significantly, when transfected cells were treated for 24 hr with the proteasome inhibitor MG132 (2 μM), the levels of Flag-XNeur detected by Western analysis increased dramatically and were now comparable to the levels of Flag-XNeurΔRF that were drug insensitive (Figure 4C, lanes 2 and 4). Flag-XNeur levels were also stabilized following treatment with other, more specific inhibitors of the proteasome: lactacystin or *clasto*-lactacystin β-lactone, the active form of lactacystin (Figure 4D). The role of the RF in XNeur degradation was tested by introducing point mutations, resulting in two forms of XNeur lacking critical residues needed for the RF structure (XNeurmut502/505 and XNeurmut517/519; see Figure 1C) and a third control form of XNeur with a silent mutation (XNeurmut529; Figure 1C). When His-tagged forms of the three mutants were transfected in HEK-293T cells and analyzed by Western blotting, higher levels of the XNeur with RF point mutants and XNeurΔRF were recovered relative to wild-type XNeur or the negative control mutant XNeurmut529 (Figure 4E, compare lanes 2, 3, and 4 with 1 and 5). In conclusion, these results indicate that XNeur is relatively unstable in both *Xenopus* and tissue culture cells and that this instability depends on the integrity of the RF domain and on the proteasome degradation pathway.

The increased degradation of XNeur by the proteasome is a strong indication that XNeur is ubiquitinated. To test this possibility directly, the various His_6-tagged versions of XNeur described above were transiently

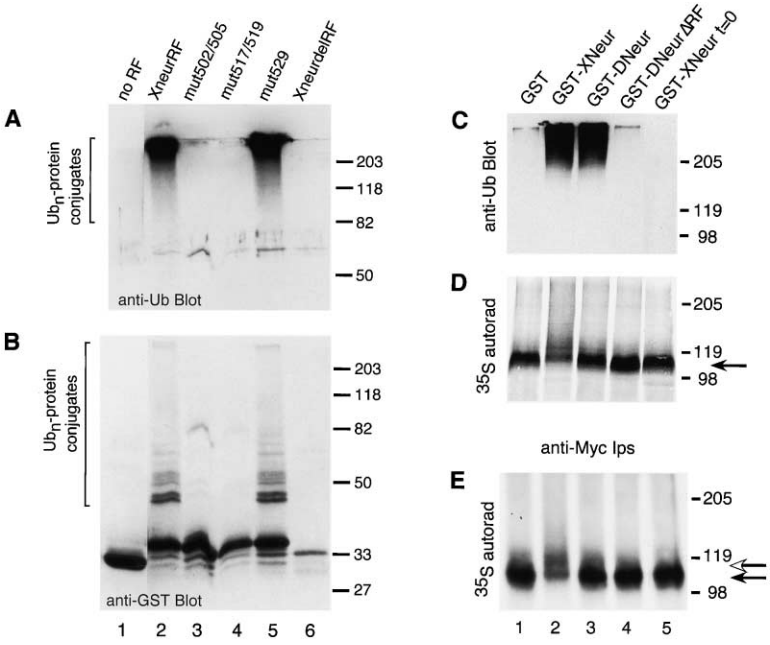

Figure 5. XNeur Promotes Ubiquitination In Vitro

(A and B) In vitro ubiquitination reactions (see Experimental Procedures) containing the indicated GST-RF proteins were analyzed by Western blot and probed with an anti-ubiquitin antibody (A) to detect ubiquitinated proteins or with anti-GST antibodies (B), which reveals a typical ladder pattern expected if increasing amounts of ubiquitin are bound to the GST-RF proteins themselves (each ubiquitin moiety adds 8 kDa).

(C–E) In vitro ubiquitination assays containing ^{35}S-labeled XDelta1-Myc along with GST only (lane 1), or with GST fused to full-length XNeur (lanes 2 and 5), DNeur (lane 3), or DNeurΔRF (lane 4) were analyzed by Western blot. As a control, a reaction containing GST-XNeur was stopped before incubation (lane 5). When probed with anti-ubiquitin antibodies, similar levels of ubiquitinated proteins were detected in the reactions containing DNeur ([C], lane 3) or XNeur ([C], lane 2). When the same Western blot was subjected to autoradiography to detect ^{35}S-labeled XDelta1-Myc, slower migrating material was detected in the reaction sample containing full-length XNeur (lane 2), indicating ubiquitination ofXDelta1-Myc. (E) [^{35}S]XDelta1-Myc was isolated from the in vitro ubiquitination assays by immunoprecipitation with an anti-Myc antibody and detected by autoradiography. Note that [^{35}S]XDelta1-Myc can be detected as a single species migrating around 110 kDa ([D and E], closed arrow), except in the reaction containing XNeur, where a second species, one ubiquitin larger in size, is also detected (open arrow, [E], lane 2).

transfected in HEK-293T cells in the presence of a vector coding for HA-tagged ubiquitin (HA-Ubiq). His-tagged proteins were recovered and analyzed by Western blotting as outlined above. When the membranes were probed with an antibody to the HA tag to assess the levels of ubiquitination, polyubiquitinated proteins were readily detected in the wild-type XNeur sample, as well as in the negative control mutant XNeurmut529 (Figure 4F, lanes 1 and 5), but were much less apparent in the mutant forms of XNeur where the RF domain was deleted or mutated in essential residues (Figure 4F, lanes 2–4). Consistently, protein levels, as assessed by reprobing the blot with anti-HT antibodies, were lower in the samples where polyubiquitination was higher (Figure 4G, lanes 1 and 5). These data indicate that ubiquitin chains are efficiently transferred to XNeur in a manner dependent on the integrity of the RF domain, strongly suggesting that XNeur acts as an ubiquitin ligase.

XNeur RF Domain Has Ubiquitin Ligase Activity In Vitro

We next tested whether the RF domain of XNeur has ubiquitin ligase activity by assaying its ability to promote in vitro ubiquitination in the presence of ubiquitin-activating and -conjugating enzymes (E1 and E2). The RF domain from XNeur or various mutant forms of the RF (Figure 1C) were expressed in bacteria as recombinant GST fusion proteins and purified. These GST proteins were incubated in an in vitro ubiquitination assay containing recombinant human E1 and human Ubc4 (as an E2 ubiquitin conjugase) and analyzed by Western blotting with anti-ubiquitin antibodies. As expected, high molecular weight smears of ubiquitin staining, indicative of polyubiquitination, were observed in the products of the reactions involving GST-RF and GST-RFmut529 (Figure 5A, lanes 2 and 5). Reprobing the same blot with anti-GST antibodies allowed the detection of the same two fusion proteins migrating in a typical ladder pattern corresponding to addition of increasing number of ubiquitin moieties (Figure 5B, lanes 2 and 5). The polyubiquitination activity of XNeur RF was abolished by the three RF mutations (Figure 5, lanes 3, 4, and 6). This result is consistent with the inability of these mutants to be efficiently ubiquitinated in transfection experiments (Figure 4F) and further argues that XNeur is the target of auto-ubiquitination. In addition, these results show that the RF domain of XNeur has the biochemical activity of a ubiquitin ligase.

XDelta1 Is a Putative Substrate for XNeur

As ubiquitin ligases, the Neuralized proteins could conceivably influence Notch signaling by targeting a component in the Notch pathway to ubiquitination. For reasons outlined in the Discussion, we next considered the possibility that XNeur promotes the ubiquitination of the Notch ligand XDelta1. Accordingly, a ^{35}S-labeled, Myc-tagged form of XDelta1 was added to the in vitro ubiquitination assay described above, except that full-length XNeur, DNeur, and DNeurΔRF were used as GST fusion proteins in order to include sequences outside the RF domain that might be involved in substrate recognition. When these in vitro ubiquitination reactions were analyzed by Western blotting, XNeur and DNeur, but not XNeurΔRF or GST alone, generated slow migrating material that stained with anti-Ub antibodies, indicating that these proteins activate ubiquitination in vitro, as described above for XNeurRF (Figure 5C, lanes 2 and 3). When the same blot was analyzed by autoradiography, the [^{35}S]XDelta1-Myc was detected as a single band

around 110 kDa in all the reactions except the one carried out in the presence of GST-XNeur where a significant fraction had a lower mobility (Figure 5D, lane 2). Similar results were obtained when one-half of the same in vitro ubiquitination reactions was immunoprecipitated with anti-Myc antibodies, analyzed by SDS-PAGE, and subjected to autoradiography (Figure 5E). Again, [^{35}S]XDelta1-Myc migrated as a single band in all samples expect for GST-XNeur, where it migrated as two bands (Figure 5E, lane 2), roughly one ubiquitin moiety (8 kDa) apart in size. By contrast, when the same assay was performed using [^{35}S]mNotch1-Myc or [^{35}S]NF-protocadherin as control proteins, no shift in their migration was observed following in vitro ubiquitination (data not shown). These results indicate that XNeur promotes the ubiquitination of XDelta1 and that monoubiquitinated-XDelta1 constitutes the species efficiently generated in vitro. Significantly, even though DNeur functions as efficiently as XNeur to activate substrate-independent ubiquitination, it seems unable to transfer ubiquitin to XDelta1 (Figures 5C–5E, lane 3).

Interaction between XNeur, XDelta1, and *Drosophila* Delta In Vivo

Although ubiquitination is primarily thought to target proteins for degradation by the proteasome, the ubiquitin modification has recently been found to promote a variety of alternative mechanisms for protein turnover (Bonifacino and Weissman, 1998; Hicke, 2001). For example, ubiquitination of membrane proteins may serve as a tag for endocytosis or for lysosomal targeting. Thus, one possibility, consistent with XNeurΔRF localization at the cell surface in embryos and in cell culture (Figure 4B, and data not shown) is that XNeur ubiquitinates XDelta1 at the cell surface, tagging it for downregulation. To test this possibility, we expressed a Myc-tagged form of XDelta1 along with XNeur in *Xenopus* embryos by RNA injection and assayed the injected embryos in whole-mount by HRP immunohistochemistry with an anti-Myc antibody (Figure 6A). In this assay, XNeur, at higher levels of expression, consistently reduced the levels of XDelta1-Myc staining at the cell surface, leaving residual Myc staining in the cytoplasm (Figure 6A, right panels). By contrast, the levels of mNotch1-Myc or NF-Protocadherin-Myc appeared unchanged in response to coinjection of XNeur (data not shown). Furthermore, when forms of XNeur lacking a functional RF domain were tested in this assay, they consistently increased the levels of XDelta1-Myc at the cell surface (Figure 6B), indicating that XNeur and the RF mutants have opposite effects on XDelta1 expression levels, even though they both inhibit Notch signaling when overexpressed. We also tested another Notch ligand, XDelta2-Myc, in the same assay and observed similar reduction or increase in XDelta2-Myc levels when coexpressed with full-length XNeur or XNeurΔRF, respectively (data not shown). We next determined the amounts of XDelta1-Myc on the cell surface by biotinylating animal cap cells that were removed from RNA-injected embryos at blastula stages. Western blot analysis of whole embryo extracts shows that the total levels of XDelta1 were slightly reduced in the presence of XNeur relative to control or those expressing XNeurΔRF at this stage (Figure 6C, lanes 6–8). Western analysis of the biotinylated membrane fraction isolated on streptavidin beads indicates that cell surface levels of XDelta1-Myc were reduced in the presence of Flag-XNeur and increased by the presence of Flag-XNeurΔRF (Figure 6C, lanes 1–3). We note that the reduction in biotinylated XDelta1 may not be as dramatic as the reduction of XDelta1-Myc levels observed in whole-mount staining because the cells were assayed earlier by the former than by the latter experiment for technical reasons. Together, these results support the idea that XNeur promotes a loss of XDelta1 from the cell surface and that XNeurΔRF does not function as a stable version of XNeur but most likely as an antagonist of XNeur, i.e., as a dominant-negative.

DNeur also promotes a loss of both endogenous and ectopic *Drosophila* Delta when assayed in vivo by misexpression in the developing imaginal wing disc (Lai et al., 2001 [this issue of *Developmental Cell*]). Since XNeur misexpression blocks Notch signaling in *Drosophila* (Figure 3), we next used the *Drosophila* assay to determine whether XNeur similarly promotes the internalization and degradation of *Drosophila* Delta. XNeur was expressed in a stripe along the anterior-posterior boundary of the wing imaginal disc using *dpp-Gal4* and the levels of endogenous Dl determined by immunohistochemisty. Double-labeling to mark cells in which *dpp-Gal4* is active showed that the expression of Dl (Figure 6Da, arrow) contained within the *dpp-Gal4* domain (Figure 6Db, yellow) is markedly reduced by XNeur expression (Figure 6Dc, arrow), indicating that XNeur promotes the loss of Dl. Since this effect could conceivably be mediated by a decrease in transcription of Dl, we performed a second assay in which Dl was ectopically and ubiquitously expressed using a *hs-Dl* transgene. As is the case for endogenous Dl in the wing disc, ectopic Dl was primarily localized to the apical plasma membrane following heat shock induction (Figure 6Dd) but in a background containing *dppGal4>UAS-Xneur*, rapidly internalized in the presence of XNeur, with complete vesicularization observed by 40 min following heat shock (Figure 6De, bar). Internalized Dl was highly unstable and was degraded by 90 min following heat shock, resulting in a cleared stripe in the ectopic Dl pattern (Figure 6Df, bar). Misexpression of XNeur did not affect accumulation of Notch, indicating that it does not generally affect the localization or stability of transmembrane proteins (data not shown). These data clearly indicate that XNeur downregulates Dl and specifically promotes internalization and degradation of newly synthesized Dl, precisely paralleling the activity of DNeur in these assays (Lai et al., 2001).

Discussion

Although *neuralized* has been known for two decades as a neurogenic mutant and its genetic interaction with genes involved in Notch signaling have been clearly demonstrated in *Drosophila*, no satisfactory biochemical description of its function exists to date. In addition, vertebrate genes related to *Drosophila neuralized* have been identified but whether or not these are involved in Notch signaling has not been tested (Nakamura et al., 1998; Ruan et al., 2001). Here we provide evidence that

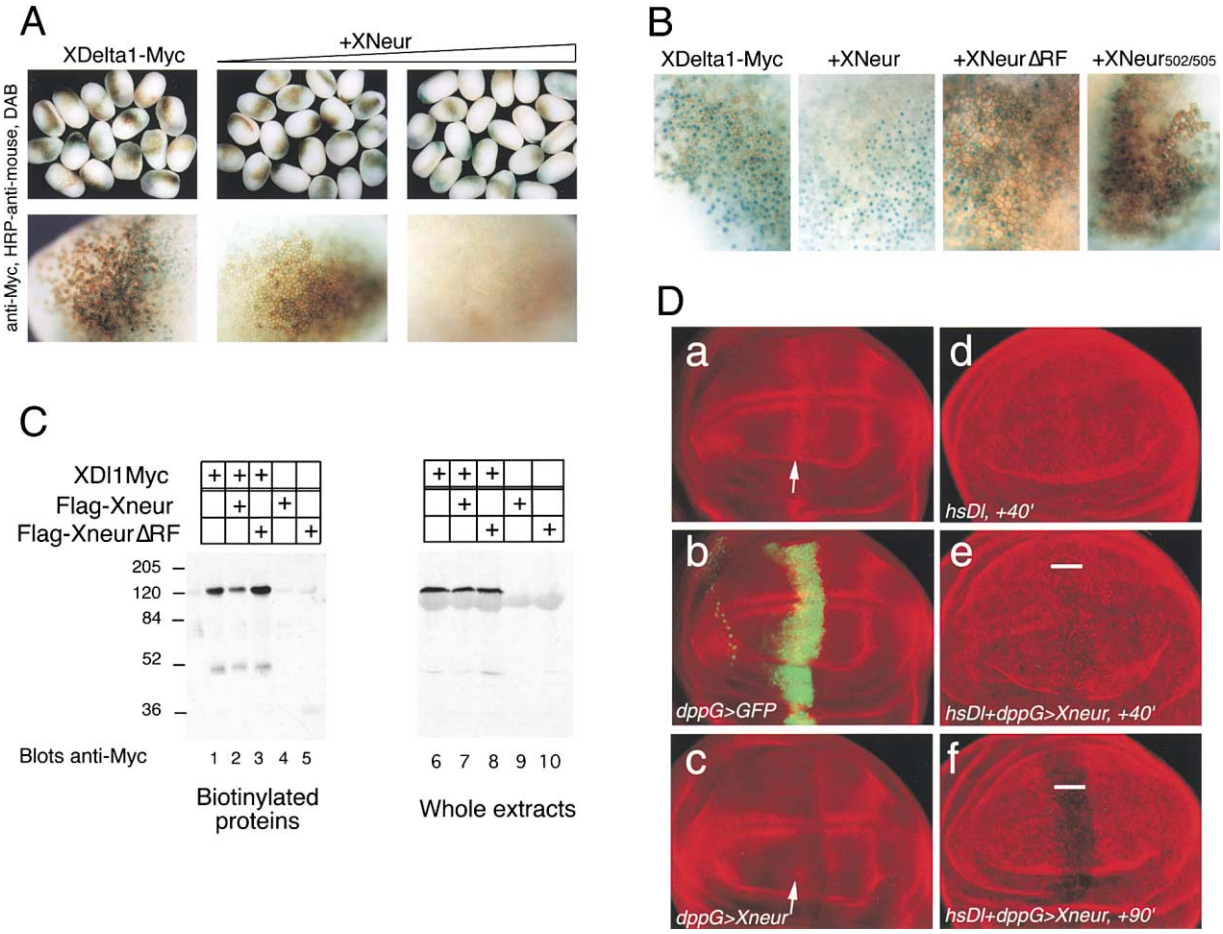

Figure 6. In Vivo Interactions between XNeur, XDelta1, and D-Dl

(A) XNeur decreases XDelta1 staining in *Xenopus* embryos. Embryos injected with RNA (0.2 ng) encoding XDelta1-Myc alone (left column) or with 0.2 ng or 0.4 ng of XNeur RNA (middle and right column, respectively) and then processed at neural plate stages using HRP immunohistochemistry with the 9E10 anti-Myc antibody (brown staining).

(B) RF mutants of XNeur increase XDelta1-Myc steady-state levels. Embryos injected with 0.1 ng of XDelta1-Myc RNA along with 0.4 ng of the different versions of *Xneur* RNAs and processed as in (A). In both (A) and (B), injected areas are marked with a *nlacZ* tracer and X-gal staining (light blue).

(C) Animal caps were dissected from stage nine embryos injected with the indicated RNAs and biotinylated. Shown are Western blots probed by anti-Myc antibody of whole extracts on the right or after purification of extracts on streptavidin beads on the left.

(D) XNeur promotes internalization and degradation of D-Dl. Wing pouches from third instar larvae labeled for Dl ([a–f], red) or GFP ([b], green). (a and b) *dppGal4>UAS-GFP*: L3 wing vein expression of Dl ([a], arrow) is contained within the dpp-Gal4 domain ([b], yellow). (c) In *dpp-Gal4>UAS-Xneur:* steady-state levels of Dl are decreased (arrow). (d–f) Third instar larvae were heat shocked at 38°C for 40 min to induce the *hs-Dl* transgene and then returned to 22°C for the indicated periods of time prior to dissection. (d) *hs-Dl* alone; Dl is ubiquitously expressed following heat shock and a 40 min rest. (e) *dpp-Gal4>Xneur*: heat shock-induced Dl is completely internalized by 40 min after heat shock (white bar) and (f) completely degraded after 90 min, resulting in a cleared stripe (white bar).

XNeur, acting as an E3 ubiquitin ligase, can regulate Notch signaling during embryogenesis by changing the levels of XDelta1 at the cell surface.

Conserved Role of the Neuralized Proteins in Notch Signaling as Ubiquitin Ligases

Three lines of evidence indicate that XNeur plays a conserved role in Notch signaling by acting as a ubiquitin ligase. First, the expression pattern of *Xneur* in embryos is highly reminiscent of that reported for *Drosophila neur* (Boulianne et al., 1991; Price et al., 1993). *Xneur* is initially expressed at low levels in a diffuse pattern within the developing neural plate and skin but is then upregulated in primary neurons and ciliated-cell precursors, both of which are selected out to undergo differentiation by Notch signaling during the process of lateral inhibition. Second, overexpressing *Xneur* or the putative inhibitory mutant *XneurΔRF* in embryos inhibits Notch signaling, as evidenced by a dramatic increase in the number of ciliated-cell precursors. Third, *Xneur* also perturbs Notch signaling when misexpressed in *Drosophila*, resulting in loss-of-Notch signaling phenotypes, although occasional gain-of-Notch signaling phenotypes do occur. Finally, XNeur fulfills a number of requirements expected for an E3 ubiquitin ligase, as recently proposed for DNeur (Yeh et al., 2001). XNeur is relatively unstable in both embryos and cell lines. XNeur is stabilized when cells are treated with proteasome inhibitors or when the

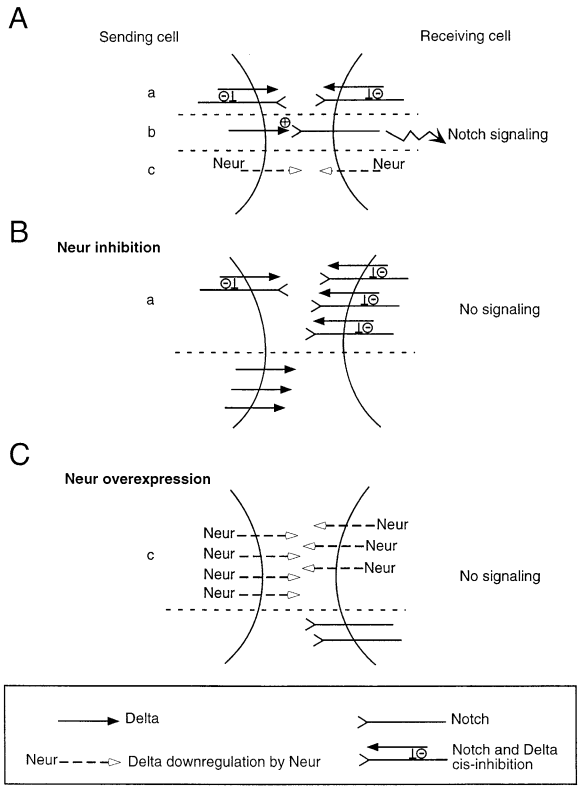

Figure 7. A Model for XNeur Function

(A) The Delta ligand is proposed to undergo at least three molecular interactions: (a) a *cis*-inhibitory interaction that blocks the Notch receptor, (b) a *trans* interaction with Notch on neighboring cells that activate signaling, and (c) an interaction with Neur that removes it from the cell surface.

(B) Blocking Neur function increases the levels of cell surface Delta, driving most of it into an inhibitory interaction with Notch and blocking signaling cell autonomously.

(C) Increasing Neur function decreases the levels of cell surface Delta to the point where too little is available for the *trans* interaction with Notch.

RF domain is deleted or mutated at critical residues, indicating that XNeur is degraded by the proteasome in a mechanism requiring the RF domain. XNeur, like many E3 proteins, is polyubiquitinated in cells, and this modification is inhibited by mutations in the RF motif. Finally, the XNeur RF domain fused to GST activates an E2 conjugase in vitro, resulting in substrate-independent ubiquitination, while mutated forms of XNeur RF domain in the same assay are inactive. These data suggest strongly that XNeur plays a conserved role in regulating Notch signaling during lateral inhibition as an E3 ubiquitin ligase.

XNeur Regulates XDelta1 Levels by Ubiquitination

How might the neuralized proteins modulate the levels of Notch signaling as ubiquitin ligases? For reasons discussed further below, the Notch ligands used during lateral inhibition are likely targets for XNeur activity, a possibility supported here by several lines of evidence. First, as also reported for DNeur (Lai and Rubin, 2001a; Yeh et al., 2000), the stabilized form of XNeur, XNeurΔRF, accumulates at the plasma membrane when overexpressed in *Xenopus* embryos and in cultured cells, where it colocalizes with XDelta1 at the plasma membrane. Second, overexpression of *Xneur* in *Xenopus* embryos markedly reduces the levels of Myc-tagged XDelta1 detected on the cell surface by antibody staining. Conversely, XNeurΔRF or XNeur with RF mutations markedly increases the levels of XDelta1-Myc, consistent with these forms acting as dominant-negative mutants. Third, the amount of XDelta1 that can be biotinylated in intact cells is decreased by XNeur but increased by XNeurΔRF. Finally, misexpression of *Xneur* in *Drosophila* wing imaginal discs promotes a loss of both endogenous and ectopic *Drosophila* Delta, as predicted by the observation that *Xneur* and *Dneur* produce similar overexpression phenotypes in *Drosophila*. Thus, XNeur can target the Delta ligands and promote their loss from the plasma membrane.

We note that XNeur requires an intact RF domain to promote the loss of cell surface Delta, indicating that its function relies on its ubiquitin ligase activity. Significantly, ubiquitination has recently been shown to be a mechanism for targeting membrane proteins to endocytosis (Strous and Govers, 1999; Hicke, 2001) or for targeting endocytosed membrane proteins to degradation by the lysosome rather than recycling to the plasma membrane (Katzmann et al., 2001; Levkowitz et al., 1998). Thus, one likely possibility is that XNeur facilitates the loss of XDelta1 from the cell surface by changing its pattern of trafficking to and from the cell surface. These changes could occur because XNeur directly ubiquitinates XDelta1, as observed in vitro where XNeur promotes the monoubiquitination of XDelta1. However, further work is needed to determine whether the in vitro ubiquitination of XDelta1 by XNeur is physiologically relevant or whether XNeur may target XDelta1 indirectly by ubiquitinating other substrates involved in ligand trafficking. Indeed, a simple, direct interaction seems unlikely since XNeur can decrease the levels of XDelta1 and XDelta2 in the *Xenopus* embryo assay, and *Drosophila* Dl in the *Drosophila* imaginal disc assay, even though the cytoplasmic domains of the different Delta ligands are not conserved in primary sequence.

Ubiquitination and the Requirement for Delta Internalization

How might XNeur be both necessary and inhibitory for Notch signaling by promoting the loss of the XDelta1 from the cell surface? One possibility is based on the observation that *Drosophila* Delta is concentrated in endocytic vesicles, often in a pattern associated with areas where Notch signaling is active, such as in cells surrounding the SOP or in the prospective wing vein cells at the time of their specification (Huppert et al., 1997; Kooh et al., 1993; Parks et al., 1997). Muskavitch and colleagues have suggested that this endocytosis of Delta is required for signaling to take place (Parks et al., 2000). Given that the *neur* genes are highly expressed in differentiating cells, i.e., the cells producing the most signal during lateral inhibition, one attractive hypothesis is to make *Dneur* responsible for driving Delta endocytosis in these cells, hence for generating a productive signal. However, the results of clonal analysis

show that *Dneur* function is cell autonomous: mutant cells signal normally while they are unable to respond to Notch signal (Lai and Rubin, 2001a; Yeh et al., 2000). Moreover, *Dneur* is genetically dispensable for a number of events requiring Notch signaling, including the formation of wing veins, indicating that Delta can signal in its absence. Thus, the Neur proteins are unlikely to be part of the machinery by which the Notch ligands signal.

An alternative model is that the Neur proteins regulate Delta levels in ways that can affect the reception of the signal (Figure 7). This model is based on findings showing that the Delta ligands are also very effective at inhibiting Notch signaling when overexpressed, presumably because they are strong inhibitors of Notch in *cis* (cell autonomously) (de Celis and Bray, 1997, 2000; Heitzler and Simpson, 1993; Jacobsen et al., 1998). Indeed, the Delta ligands share with the Neur proteins the ability to inhibit Notch signaling when present in either too low or high amounts. Thus, reduction in XNeur activity may block Notch signaling cell autonomously by allowing ligands to accumulate on the cell surface where they inhibit Notch in *cis* (Figure 7B). Conversely, increasing XNeur activity may decrease ligand levels, potentially improving Notch reception in receiving cells but ultimately blocking the ligand signaling from sending cells (Figure 7C). Another feature of the Delta-like ligands consistent with this model is that forms lacking the intracellular domain are extremely potent inhibitors of Notch signaling, possibly because they are unable to signal as proposed by Muskavitch and colleagues, but also because they cannot be regulated by endocytosis and thus accumulate to levels that block Notch signaling in *cis* (Chitnis et al., 1995; Parks et al., 2000; Sun and Artavanis-Tsakonas, 1996). Indeed, clonal analysis of a truncated Delta in chick indicates that it blocks Notch signaling in a cell-autonomous manner (Henrique et al., 1997).

In conclusion, we propose that Neuralized proteins play a conserved role as ubiquitin ligases in the Notch pathway and that they function by maintaining Delta levels into a range compatible with signaling. The exact mechanism used by Neuralized proteins to regulate Delta accumulation at the cell surface remains to be investigated. Finally, our study uncovers a novel level of control of Notch signaling relying on ligand-receptor interactions and on protein degradation pathways and stresses thus the need to understand the molecular basis of the cell-autonomous inhibition of Notch by Delta.

Experimental Procedures

Embryos, RNA Injections, and Analysis

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg laying and in vitro fertilization using standard methods. Embryos were staged according to Nieuwkoop and Faber (1967). For RNA injection, synthetic-capped RNAs encoding XNeur, XNeurΔRF, HT-XNeur, and HT-XNeurΔRF were generated in vitro from coding sequences cloned into the pCS2+ vectors (Turner and Weintraub, 1994). RNA templates encoding Notch-ICD and X-Su(H)DBM were described previously (Chitnis et al., 1995; Coffman et al., 1993; Wettstein et al., 1997). Unless stated otherwise, 1–2 ng of test RNA was injected into single blastomeres of albino embryos at the two- or four-cell stage along with 20 pg of *n-lacZ* RNA as a tracer (Coffman et al., 1993). Injected embryos were first stained for β-galactosidase activity with X-gal to localize the tracer. Analysis by WISH of *Xenopus* embryos is as described previously using RNA probes for *Xenopus* α-*tubulin*, *N-tubulin* (Deblandre et al., 1999). Immunostaining of injected embryos for His-tagged or Myc-tagged proteins used an anti-RGSHis antibody (Qiagen) and the 9E10 hybridoma supernatant, respectively.

Isolation of cDNA Encoding XNeuralized and Generation of XNeur Mutants

Degenerate PCR was carried out using a λgt10 *Xenopus* stage 17 embryo cDNA library as a template and oligonucleotide primers corresponding to two regions conserved in *Dneur* and human and mouse ESTs homolog to *Dneu*: AITFS (CGGGATCCGHATHACHT TYWS) and WAKAL (CGGAATTCDGCYTTDGCCCARAA). This reaction yielded a 215 nucleotide product subsequently used to isolate full-length *Xneur* cDNAs. Nucleotide sequence of the *Xneur* cDNAs has been submitted to GenBank under the accession number AF419159. The region coding for full-length XNeur or for XNeurΔRF (deletion of the 78 C-terminal residues corresponding to the RF domain) were amplified using PCR and cloned into pCS2 or into forms of pCS2 with Flag and His tags. Forms of XNeur with mutations in the RF domain as described in Figure 1 were generated using the QuikChange method (Stratagene) by PCR amplification of CS2-XNeur template with pairs of sense/antisense primers. PCR products were confirmed by DNA sequencing.

Cell Culture and Transfections

HEK-293T were grown in DMEM high glucose containing 10% FBS, glutamine, pyruvate, penicillin, and streptomycine. Cells were plated 1 day before transfection at 5×10^5 cells per well in 6-well plates, transfected for 6 hr using the calcium phosphate precipitate method as described with 2–3 μg DNA per well (Chen and Okayama, 1988). MG132, lactacystin (Calbiochem), or *clasto*-lactacystin β-lactone (Boston Biochem) were added after 24 hr at a concentration of 2 μM. For analysis of Flag-tagged proteins, cells were harvested 48 hr after transfection in 400 μl of lysis buffer (1% Triton X-100, 25 mM Tris, pH 7.5, 300 mM NaCl, 1 mM PMSF, 1 μM pepstatin, 1 μM leupeptin). Insoluble debris was removed by centrifugation at 13,000 rpms at 4°C for 15 min. Supernatants were collected, and 10 or 20 μl of whole-cell extracts were separated on 10% polyacrylamide gels followed by Western blotting with anti-Flag M2 antibody. For analysis of His_6-tagged proteins, cells were harvested 48 hr after transfection in 1 ml of cold PBS, pelleted by brief centrifugation, and lysed in 1 ml of 6 M guanidium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) plus 5 mM imidazole. The lysate was sonicated for 30 s then mixed with 25 μl of a 50% slurry of Ni^{2+}-NTA-agarose (Qiagen) for 5 hr at room temperature. The beads were washed and eluted in 50 μl of protein buffer containing 200 mM imidazole as described (Treier et al., 1994). One-fourth of the eluted material was loaded on 10% polyacrilamide gels and analyzed by Western blotting with anti-RGSHis antibodies and with rabbit polyclonal anti-HA antibodies HA.11 (Covance).

Analysis of Proteins Expressed in *Xenopus* Embryos

Proteins were overexpressed in embryos by injection of the corresponding RNA in the two blastomeres of two-cell stage embryos. Extraction of total protein and analysis by Western blotting was performed as above for tissue culture cells. Analysis of cell surface proteins by biotinylation was as described previously (Bang and Kintner, 2000).

In Vitro Ubiquitination Assays

GST fusion proteins containing different forms of XNeur or DNeur were generated by PCR amplification of coding sequences corresponding to the appropriate region and subcloning into pGEX-4T3. GST fusion protein purification and in vitro ubiquitination assays were carried out as described previously (Joazeiro et al., 1999). Reaction products were analyzed by immunoblotting, using mouse anti-ubiquitin antibodies (Zymed) or mouse anti-GST antibodies (Santa Cruz), followed by HRP-anti-mouse antibodies and ECL detection. For in vitro substrate ubiquitination assays, XDelta1-Myc

was translated in vitro from 0.5 μg circular pCS2-XDelta1-Myc vector using the TnT SP6 Coupled Reticulocyte Lysate System (Promega) with 2 μl of Tran^{35}S-label 10 mCi/ml (ICN Biomedicals).

Analysis of *Xneur* in *Drosophila*

XNeur was misexpressed in *Drosophila* using the Gal4-UAS system (Brand and Perrimon, 1993). Coding sequences for *Xneur* were inserted into *pUAST*, and transgenic flies carrying *UAS-Xneur* were established by standard methods using w^{1118} as a recipient (Rubin and Spradling, 1982). Eleven independent lines were obtained, all of which conferred similar misexpression phenotypes. References to Gal4 lines used in this study can be found in (Lai and Rubin, 2001a) and (Lai et al., 2001). Indirect immunofluorescence was performed according to Lai and Rubin (2001a) using the C594.9B (DHSB) mouse anti-Delta antibody (1:100 dilution).

Acknowledgments

We thank Drs. A. Bang, E. Lamar, and N. Koyano-Nakagawa for comments on the manuscript. HA-ubiquitin was kindly provided by Dr. D. Bohmann and constructs for in vitro ubiquitination were kindly provided by Drs. Joazeiro and Hunter. The *Drosophila* studies were carried out in the laboratory of Dr. James Posakony, to whom we are especially grateful. We also would like to thank Gary Struhl for *hs-Dl* flies and the Developmental Hybridoma Studies Bank for maintaining the Dl antibody. This work was supported by an NIH grant (to C.K.). E.C.L. was supported by a Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG 1632.

Received September 21, 2001; revised November 1, 2001.

References

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770–776.

Bang, A.G., and Kintner, C. (2000). Rhomboid and Star facilitate presentation and processing of the Drosophila TGF-alpha homolog Spitz. Genes Dev., *14*, 177–186.

Bonifacino, J.S., and Weissman, A.M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell Dev. Biol. *14*, 19–57.

Boulianne, G.L., de la Concha, A., Campos-Ortega, J.A., Jan, L.Y., and Jan, Y.N. (1991). The Drosophila neurogenic gene neuralized encodes a novel protein and is expressed in precursors of larval and adult neurons. EMBO J. *10*, 2975–2983.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Chen, C.A., and Okayama, H. (1988). Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques *6*, 632–638.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature *375*, 761–766.

Coffman, C.R., Skoglund, P., Harris, W.A., and Kintner, C.R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos. Cell *73*, 659–671.

de Celis, J.F., and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. Development *124*, 3241–3251.

de Celis, J.F., and Bray, S.J. (2000). The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. Development *127*, 1291–1302.

Deblandre, G.A., Wettstein, D.A., Koyano-Nakagawa, N., and Kintner, C. (1999). A two-step mechanism generates the spacing pattern of the ciliated cells in the skin of Xenopus embryos. Development *126*, 4715–4728.

Dietrich, U., and Campos-Ortega, J.A. (1984). The expression of neurogenic loci in imaginal epidermal cells of Drosophila melanogaster. J. Neurogenet. *1*, 315–332.

Hartenstein, A.Y., Rugendorff, A., Tepass, U., and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the Drosophila embryo. Development *116*, 1203–1220.

Heitzler, P., and Simpson, P. (1993). Altered epidermal growth factor-like sequences provide evidence for a role of Notch as a receptor in cell fate decisions. Development *117*, 1113–1123.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr. Biol. *7*, 661–670.

Hicke, L. (2001). A new ticket for entry into budding vesicles-ubiquitin. Cell *106*, 527–530.

Huang, F., Dambly-Chaudiere, C., and Ghysen, A. (1991). The emergence of sense organs in the wing disc of Drosophila. Development *111*, 1087–1095.

Huppert, S.S., Jacobsen, T.L., and Muskavitch, M.A. (1997). Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. Development *124*, 3283–3291.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. *10*, 429–439.

Jacobsen, T.L., Brennan, K., Arias, A.M., and Muskavitch, M.A. (1998). Cis-interactions between Delta and Notch modulate neurogenic signalling in Drosophila. Development *125*, 4531–4540.

Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T., and Liu, Y.C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science *286*, 309–312.

Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145–155.

Kooh, P.J., Fehon, R.G., and Muskavitch, M.A. (1993). Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. Development *117*, 493–507.

Lai, E.C., and Rubin, G.M. (2001a). neuralized functions cell-autonomously to regulate a subset of notch-dependent processes during adult Drosophila development. Dev. Biol. *231*, 217–233.

Lai, E.C., and Rubin, G.M. (2001b). Neuralized is essential for a subset of Notch pathway-dependent cell fate decisions during Drosophila eye development. Proc. Natl. Acad. Sci. USA *98*, 5637–5642.

Lai, E.C., Deblandre, G.A., Kintner, C., and Rubin, G.M. (2001). *Drosophila* Neuralized is a ubiquitin ligase that promotes the internalization and degradation of Delta. Dev. Cell *1*, this issue, 783–794.

Lehmann, R., Jimenez, F., Dietrich, U., and Campos-Ortega, J.A. (1983). On the phenotype and development of mutants of early neurogenesis in Drosophila melanogaster. Roux's Arch. Dev. Biol. *192*, 62–74.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. *12*, 3663–3674.

Nakamura, H., Yoshida, M., Tsuiki, H., Ito, K., Ueno, M., Nakao, M., Oka, K., Tada, M., Kochi, M., Kuratsu, J., et al. (1998). Identification of a human homolog of the Drosophila neuralized gene within the 10q25.1 malignant astrocytoma deletion region. Oncogene *16*, 1009–1019.

Nieuwkoop, P.D., and Faber, J. (1967). Normal table of *Xenopus laevis* (Amsterdam, North Holland).

Parks, A.L., Huppert, S.S., and Muskavitch, M.A. (1997). The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster. Mech. Dev. *63*, 61–74.

Parks, A.L., Klueg, K.M., Stout, J.R., and Muskavitch, M.A. (2000).

Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development *127*, 1373–1385.

Petcherski, A.G., and Kimble, J. (2000). Mastermind is a putative activator for Notch. Curr. Biol. *10*, R471–R473.

Price, B.D., Chang, Z., Smith, R., Bockheim, S., and Laughon, A. (1993). The Drosophila neuralized gene encodes a C3HC4 zinc finger. EMBO J. *12*, 2411–2418.

Ruan, Y., Tecott, L., Jiang, M.M., Jan, L.Y., and Jan, Y.N. (2001). Ethanol hypersensitivity and olfactory discrimination defect in mice lacking a homolog of Drosophila neuralized. Proc. Natl. Acad. Sci. USA *98*, 9907–9912.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348–353.

Strous, G.J., and Govers, R. (1999). The ubiquitin-proteasome system and endocytosis. J. Cell Sci. *112*, 1417–1423.

Sun, X., and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. Development *122*, 2465–2474.

Treier, M., Staszewski, L.M., and Bohmann, D. (1994). Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell *78*, 787–798.

Turner, D.L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. *8*, 1434–1447.

Wettstein, D.A., Turner, D.L., and Kintner, C. (1997). The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. Development *124*, 693–702.

Yeh, E., Zhou, L., Rudzik, N., and Boulianne, G.L. (2000). Neuralized functions cell autonomously to regulate Drosophila sense organ development. EMBO J. *19*, 4827–4837.

Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C.J., and Boulianne, G.L. (2001). Neuralized functions as an E3 ubiquitin ligase during *Drosophila* development. Curr. Biol. *11*, 1675–1679.