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Neuralized-2 Regulates a Notch Ligand in Cooperation with Mind Bomb-1*[S]

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Mutations in *Drosophila neuralized* (*Dneur*) result in a variety of developmental defects that closely resemble those of Notch mutants and other Notch pathway mutants. However, mice with disrupted *neur1* do not show any aberrant cell fate specifications in neurogenesis and somitogenesis. Thus, we speculated that other vertebrate *neur* homolog(s) might compensate for loss of the *neur* gene. Here, we report the paralog of mouse Neur1, named Neuralized-2 (Neur2), which is a ubiquitin-protein isopeptide ligase (E3) that interacts with and ubiquitinates Delta. Both murine Neur1 and Neur2 have similar degrees of homology to DNeur, and *neur2* is expressed in patterns similar to those of *neur1* in embryos, suggesting potential functional redundancy. Interestingly, two distinct classes of E3 ligases, Mind bomb-1 (Mib1) and Neur2, have cooperative but distinct roles in Delta endocytosis to Hrs-positive vesicles, *i.e.* Mib1 functions in the initial step of Delta endocytosis, and Neur2 is required for targeting endocytosed Delta to Hrs-positive vesicles. Thus, our study provides a new insight into how distinct E3 ligases work together in the endocytic pathways for Notch signaling.

The Notch pathway is a signaling module that is conserved in all metazoans and has been implicated in a variety of developmental processes (1). It is best known for its role in selecting cells to become neuroblasts or sensory organ precursors in *Drosophila* by the process of lateral inhibition, in which cells adopting a neural fate inhibit their neighbors from adopting a similar fate (1, 2). The core components in Notch signaling include the ligands, the receptor (Notch), and the transcription factor (Suppressor of Hairless (Su(H))) in *Drosophila*.

Notch signaling is initiated by the interaction of the Notch receptor with its ligands (3, 4). These interactions induce proteolytic cleavage (S2) of the Notch receptors, which results in membrane-bound Notch fragments (5). After the S2 cleavage, the remaining receptor fragments are cleaved at a third site (S3) within the membrane by γ-secretase complexes (6, 7). The released intracellular fragments of Notch (Notch intracellular domain) translocate to the nucleus to form transcriptional activator complexes with Su(H)/CBF1/RBP-J. These complexes activate Notch target genes such as Hairy/E(spl)-related basic helix-loop-helix repressors (8).

Although much is known about Notch signal transduction after the receptor undergoes the ligand-dependent S2 cleavage, the mechanism by which the Notch ligands engage Notch receptors and trigger their cleavage is less understood. Recent studies demonstrated that the endocytosis of Notch ligands in the signal-sending cells is required for the effective activation of Notch signaling (9). Key components for the endocytosis of Notch ligands have been identified in *Drosophila*, zebrafish, and mice; these are Neuralized (Neur), Mind bomb (Mib)-1/2, Liquid facets/epsin, and auxilin (10–19).

In *Drosophila*, Neuralized (DNeur)[3] is required for a subset of the developmental events that are known to be regulated by Notch signaling (20–23). Recent studies have suggested that DNeur is a ubiquitin-protein isopeptide ligase (E3) that promotes the internalization and degradation of Delta to activate the Notch signaling pathway (10, 11, 24). However, mice with a disrupted *neur1* (*neur1* was heretofore known as *neuralized* in mice) gene are viable; they do not show any Notch-related phenotype during embryogenesis but do exhibit defects as adults, including impaired olfactory discrimination, hypersensitivity to ethanol, and abnormalities both in axoneme organization in the spermatozoa and in mammary gland maturation during pregnancy (25, 26). Thus, we speculate that other vertebrate *neur* homolog(s) might compensate for loss of the *neur* gene in mammals based on the conservation between *Drosophila* and mammalian Notch signaling.

Here, we report two Neuralized ortholog/paralogs, Neur1 and Neuralized-2 (Neur2), both of which have similar degrees of homology to DNeur. We show that Neur2 is another E3 ligase that ubiquitinates the Notch ligand Delta. The expression patterns of *neur2* in embryos were similar to those of *neur1*, suggesting a possible compensatory role of Neur2 to Neur1. In

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[3] The abbreviations used are: DNeur, *Drosophila* Neuralized; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; *zath1*, zebrafish atonal homolog-1a; HEK, human embryonic kidney; Ub, ubiquitin; E1, ubiquitin-activating enzyme; GST, glutathione *S*-transferase; XD, *Xenopus* Delta; NHR, Neuralized homology repeat; E2, ubiquitin carrier protein; GFP, green fluorescent protein.

contrast to DNeur, which promotes the internalization and degradation of Delta, Neur2 alone does not induce the endocytosis of Delta. It is intriguing that Neur2 readily leads to the endocytosis of Delta to the Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate)-positive vesicles in the presence of Mib1, suggesting that Mib1 and Neur2 work cooperatively in the endocytic pathway of Delta. Our results indicate a novel functional relationship between the E3 ligases of Delta, Mib1, and Neur2.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse neur2 Gene and Generation of Mutations in the Neur2 RING Finger Domain—PCR was carried out using mouse brain cDNA as the template and oligonucleotide primers designed to obtain the open reading frame of the mouse *neur2* gene. The PCR for *neur2* yielded a product of ~1641 bp, which was cloned into the hemagglutinin (HA)-tagged pcDNA vector (Invitrogen) or the pEGFPN3 vector (Clontech). The *neur2* mutants with mutations in the RING finger domain were generated using DpnI-mediated site-directed mutagenesis. All cloned cDNA vectors were confirmed by restriction enzyme digestion and DNA sequencing.

In Situ Hybridization—For whole-mount *in situ* hybridization, mouse embryos were isolated in ice-cold phosphate-buffered saline, fixed overnight in 4% paraformaldehyde, and processed following previously described procedures (16). *In situ* probes for mouse *neur1* and *neur2* were cloned into pBluescript II KS vectors and synthesized with EcoRI-cleaved vectors by T7 RNA polymerase (Fermentas). The PCR primers for the mouse *neur1* probe were GCCGAGACCATCTTCATCA and CAGGAGTAGCACAGGCACAT. The PCR primers for the mouse *neur2* probe were CTTCGCTGACACGCTGAC and ATTCTGATGCTGAGGACGAC. For *in situ* hybridizations with zebrafish embryos, zebrafish *ath1* (*zath1*) was linearized with BamHI and transcribed with T7 RNA polymerase for the digoxigenin-labeled antisense RNA probe (27). Whole-mount *in situ* hybridization was performed as described (28).

Northern Blot Analysis—Two probes were generated to detect the N- and C-terminal regions of Neur2. The PCR primers for the N-terminal probe (5′-probe) were Neur2NL (TCTGCAACGGAGTCACCT) and Neur2NR (GTCGATGAGTGCCCAGAGT). The PCR primers for the C-terminal probe (3′-probe) were Neur2CL (AGGATGACAGCGATTCAGAC) and Neur2CR (CTACGGCCTGTAGATCTTG). For expression analysis in adult mouse tissues, the MTN™ mouse multiple tissue Northern blot (Clontech) was hybridized with both probes.

Transfections and Western Blotting—Human embryonic kidney (HEK) 293A cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Cells were transfected with appropriate amounts of plasmid DNA using Lipofectamine Plus (Invitrogen). At 24–48 h post-transfection, cells were harvested in 1 ml of lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 5 mM EDTA) containing protease inhibitors (Roche Applied Science). Western blotting was performed as described (16).

Immunoprecipitation—HEK 293A cells were transfected with 4 μg of plasmid DNA/10-cm plate and then resuspended in immunoprecipitation buffer (50 mM HEPES/NaOH (pH 7.5), 3 mM EDTA, 3 mM $CaCl_2$, 80 mM NaCl, 1% Triton X-100, and 5 mM dithiothreitol). Immunoprecipitation was performed as described (16). Immunoreactive proteins were analyzed by immunoblotting with anti-Myc antibody (Santa Cruz Biotechnology, Inc.) or anti-ubiquitin (Ub) antibody (clone P4D1; Santa Cruz Biotechnology, Inc.), followed by either horseradish peroxidase-conjugated goat anti-mouse antibody (Promega Corp.) or horseradish peroxidase-conjugated anti-HA antibody (Santa Cruz Biotechnology, Inc.).

In Vitro and in Vivo Ubiquitination Assays—*In vitro* ubiquitination assays were carried out using ubiquitin-activating enzyme (E1; 250 nM; Calbiochem), glutathione *S*-transferase (GST)-UbcH5a (2 μM; Calbiochem), and N-terminally His-tagged ubiquitin (100 μM; Sigma) in ubiquitination assay buffer (50 mM Tris (pH 7.4), 2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 mM creatine phosphate, and 15 units of creatine phosphokinase). The purified GST-fused Neur1 and Neur2 RING finger domains and their mutants were added to the reactions. After incubation at room temperature for up to 90 min, the reactions were analyzed by Western blotting to detect ubiquitinated proteins.

To detect ubiquitination activity *in vivo*, HEK 293A cells were transfected with 4 μg of plasmid DNA/10-cm plate. MG132 (2 μM) was added 24 h after transfection, and the cells were harvested 24 h later. Immunoprecipitation was performed as described (16).

Subcellular Localization Analysis—Three COS-7 cell lines were analyzed: one natural and two engineered. Of the engineered cell lines, one line overproduced *Xenopus* Delta (XD), and the other line did not. These were generated by infecting cells with murine stem cell virus carrying XD and murine stem cell virus that did not, respectively, and then using puromycin for 6 days to select for stable integrants. The three cell lines were transfected with various plasmids. At 24 h post-transfection, the cells were washed with phosphate-buffered saline and fixed in 4% paraformaldehyde with 3% sucrose for 30 min at 4 °C. The fixed cells were incubated overnight at 4 °C in blocking solution (3% skim milk and 0.1% Triton X-100 in phosphate-buffered saline) and stained with mouse anti-Myc and anti-HA antibodies for 1 h at room temperature in 3% skim milk in phosphate-buffered saline. The cells were then incubated with either Alexa 546- or Alexa 350-conjugated anti-mouse antibody (Molecular Probes) for 30 min at room temperature, washed, and stained with Hoechst dye (10 μg/ml) for 2 min. After three washes, the cells were mounted on glass slides and analyzed with a Zeiss fluorescence microscope. All images were collected using an Olympus DP70 camera. Retrovirus-mediated gene transfer was accomplished using a retroviral expression system (Clontech) following the manufacturer's suggested protocol.

Zebrafish Maintenance and mRNA Microinjections—Zebrafish were raised and maintained under standard conditions. The allele of mib^{ta52b} was used as the *mib1* mutant (13). The mouse *neur2* cDNA was subcloned into the $pCS2^{+}$ vector, and the sense RNA encoding full-length mouse *neur2* was transcribed *in vitro* using an SP6 mMESSAGE mMACHINE (Ambion, Inc., Austin, TX). The synthesized *neur2* mRNA was

microinjected into one- or two-cell stage embryos. The amount of mRNA (~100 pg/embryo) injected into the embryos was estimated visually from the injection volume using a hemocytometer.

RESULTS

Neur2 Is a Neur1 Paralog—The *Drosophila* and *Xenopus* Neur proteins are E3 ligases that are essential components of Notch signaling (10, 11, 24, 29). By searching the expressed sequence tag and genomic data bases, we identified a *neur* paralog, *neuralized-2* (*neur2*; GenBank™ accession number DQ839448), in mice. To investigate the function of Neur2, its cDNA was cloned and amplified by reverse transcription-PCR from mouse brain RNA. Mouse Neur1 and Neur2 have 40% base pair identity and 53% similarity in their amino acid sequences, and they also have remarkable resemblance in their domain organization (Fig. 1, *A* and *B*). Mouse Neur1 and Neur2 both have two Neuralized homology repeat (NHR) domains and a RING finger domain (25, 26, 30). Furthermore, comparison between mouse Neur1 and Neur2 subdomains showed a higher degree of homology, with 49, 42, and 70% identities in the NHR1, NHR2, and RING finger domains, respectively (data not shown).

Drosophila has a *neur* gene without known paralogs. *Xenopus* Neur has 27% identity to DNeur (Fig. 1*D*) and shares functional similarity (29), suggesting that *Xenopus* Neur might be a DNeur ortholog, albeit we cannot exclude the possibility of unidentified *Xenopus* Neur ortholog(s). To test whether Neur1 and Neur2 are the ortholog and paralog of DNeur, respectively, we compared sequence identity and similarity. The protein sequence identities of Neur1 and Neur2 to DNeur are almost equal: 26% over the full length and 33–48% within domains (Fig. 1*B*), which raises the question of whether Neur2 is a paralog or another ortholog of DNeur. In examination of other species, we found a putative *neur2* gene in zebrafish and in human by a genome BLAST search (supplemental Fig. 1), but not in *Xenopus* because of incomplete genome sequence (Fig. 1*C*).

In the comparison of sequence similarity between species, murine Neur2 has higher homology to the putative zebrafish and human Neur2 proteins than to zebrafish Neur1, *Xenopus* Neur, and human Neur1. Furthermore, *Xenopus* Neur has very high homology to Neur1 from zebrafish and humans, suggesting that *Xenopus* Neur is the ortholog of Neur1 (Fig. 1, *C* and *D*). Intriguingly, DNeur has similar identities to both Neur1 and Neur2 in all species examined, suggesting functional similarity of Neur1 and Neur2. Further functional analysis will determine whether Neur2 is a paralog or an ortholog of DNeur.

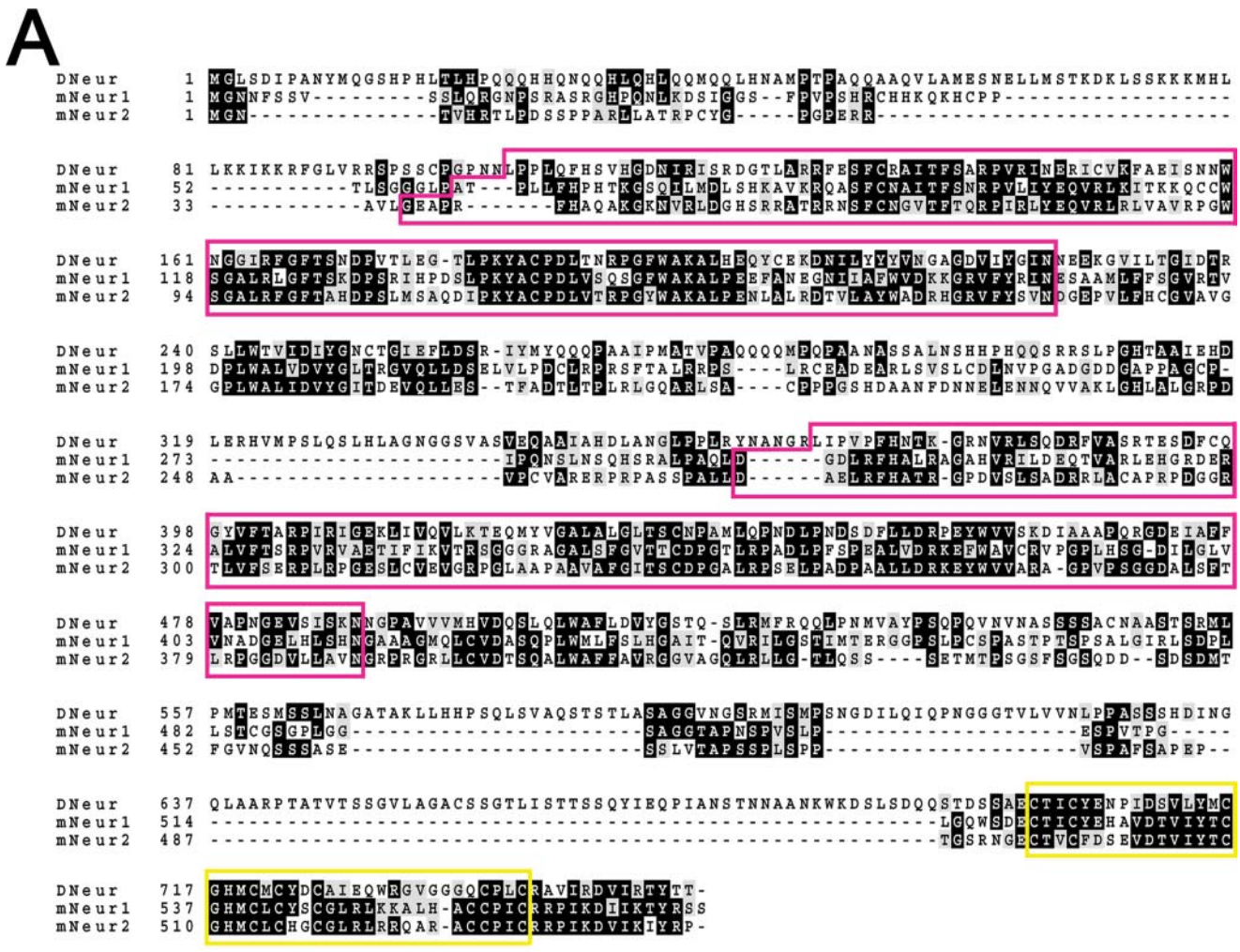
To test the expression patterns in embryos, we analyzed the spatial distribution of *neur1* and *neur2* transcripts by whole-mount *in situ* hybridization. At embryonic day 10.5, *neur1* transcripts were detected in the limb buds and dorsal root ganglia, as reported previously (30). Interestingly, *neur2* was also expressed in the same regions where *neur1* transcripts were detected (Fig. 1*E*). These similar expression patterns of *neur1* and *neur2* suggest that both Neur1 and Neur2 might be functionally redundant during embryogenesis. Northern blot analyses using 5′- and 3′-probes revealed a 7.5-kb *neur2* transcript, which could include its open reading frame (~1.6 kb). *neur2* was expressed at high levels in the brain and kidney and at low levels in the heart (Fig. 1*F*), whereas *neur1* was expressed at high levels in the brain and skeletal muscle (25, 26). The different expression patterns of these two genes suggest distinct roles of Neur1 and Neur2 in adults.

Neur2 Functions as an E3 Ligase—Because the *Drosophila* and *Xenopus* Neur proteins function as E3 ligases (10, 11, 24, 29), we tested whether murine Neur1 and Neur2 also possess similar biochemical activity. Many RING finger domain-containing E3 ligases can mediate auto-ubiquitination, which facilitates degradation by the proteasome (31). Thus, we tested the protein levels of Neur1 and Neur2 in HEK 293A cells using transfection assays. As expected, both HA-Neur1 and HA-Neur2 were hardly detectable (Fig. 2*A*, *lanes 2* and *4*). However, when transfected cells were treated with the proteasome inhibitor MG132 (2 μM) for 24 h, the expression levels of both HA-Neur1 and HA-Neur2 were dramatically increased (Fig. 2*A*, *lanes 3* and *5*), suggesting proteasome-dependent degradation of both Neur1 and Neur2.

To test whether this degradation is caused by self-ubiquitination through the RING finger domain, HA-Neur1 or HA-Neur2 was expressed in HEK 293A cells in the presence or absence of MG132 and then immunoprecipitated from the lysates with anti-HA antibody. The immunoprecipitates were subjected to immunoblotting with anti-HA antibody to detect Neur1 and Neur2 as well as with anti-Ub antibody (clone P4D1) to detect ubiquitin chains. Although polyubiquitinated Neur1 and Neur2 proteins were observed compared with the mock-transfected control (Fig. 2*B*, *lanes 3* and *5*), the ubiquitination was dramatically increased in the presence of MG132 (Fig. 2*B*, *lanes 4* and *6*), suggesting proteasomal degradation of these ubiquitinated proteins.

Neur2 contains a RING finger domain that may confer E3 ligase activity. To test this possibility, we generated a RING finger domain deletion mutant (Neur2ΔRING) and two mutant proteins (Neur2 mut1 and mut2) by inducing point mutations of critical residues in the RING finger domain (29). The mutations were C494S/C497S in Neur2 mut1 and C509S/H511N in Neur2 mut2 (Fig. 2*C*). When HEK 293A cells were transfected with expression vectors encoding two HA-tagged Neur2 mutants and Neur2ΔRING, these mutant proteins were expressed at higher levels compared with full-length HA-Neur2 (Fig. 2*D*, *lanes 3–5*). These results suggest that the degradation of Neur2 depends on the RING finger domain, which may be important for E3 ligase activity.

Next, to investigate the intrinsic ubiquitin ligase activity of the Neur2 RING finger domain *in vitro*, bacterially expressed and purified GST-Neur2 RING finger domain fusion proteins were incubated with E1, ubiquitin carrier protein (E2; UbcH5a), ubiquitin, and ATP. The presence of ubiquitinated substrates was detected by Western blotting. As expected, a typical reaction mixture containing GST-Neur1 RING resulted in a smear of high molecular mass polyubiquitinated substrates (Fig. 2*E*, *lane 2*), whereas a similar reaction containing GST was devoid of such activity (*lane 1*). When GST-Neur2 RING was tested in this assay, we observed robust ubiquitination in an E1- and E2 (UbcH5a)-dependent manner (Fig. 2*E*, *lanes 3–5*), indicating that the Neur2 RING finger domain possesses E3 ligase activ-



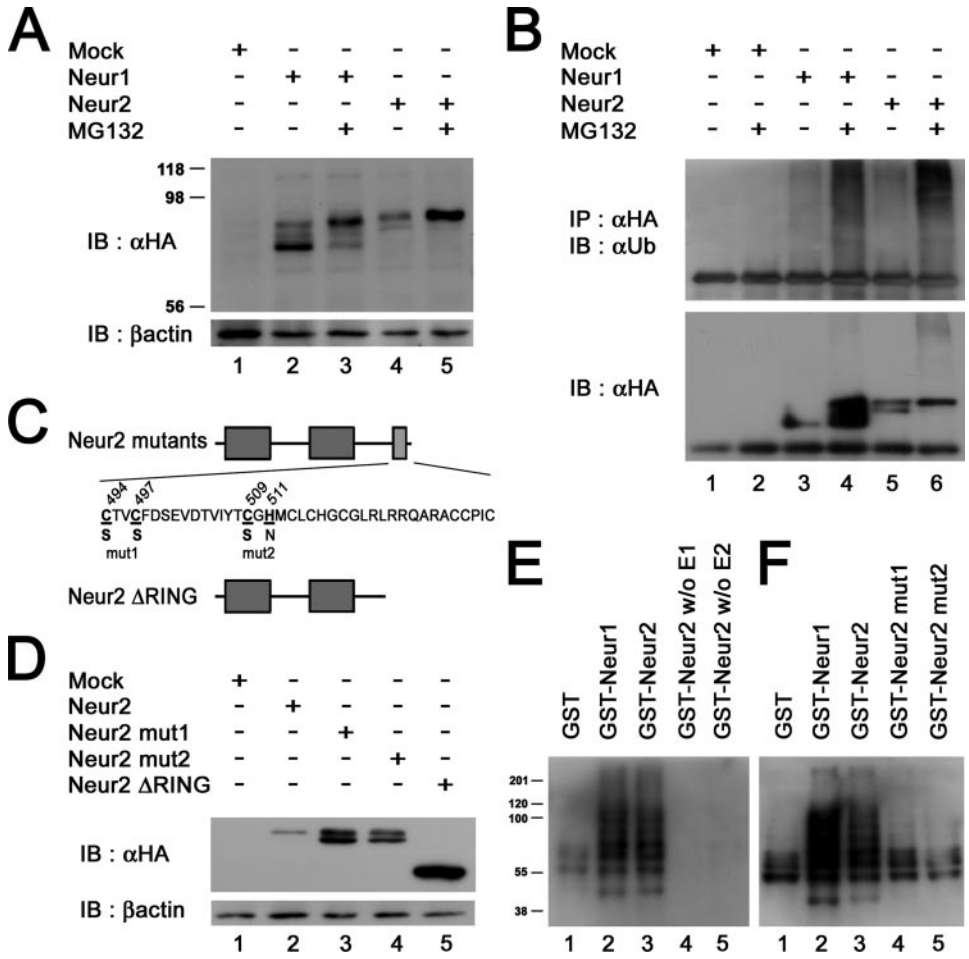


FIGURE 2. **Neur2 as an E3 ligase.** *A*, proteasome-dependent degradation of Neur1 and Neur2. HEK 293A cells were transfected with plasmids expressing the indicated proteins, followed by incubation in the presence or absence of 2 μM MG132 for 24 h. Cell lysates were immunoblotted (*IB*) with anti-HA antibody. *B*, self-ubiquitination of Neur1 and Neur2 *in vivo*. HEK 293A cells were transfected with plasmids encoding HA-Neur1 and HA-Neur2 in the presence or absence of 2 μM MG132 for 24 h. At 48 h post-transfection, whole cell lysates were immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted with anti-Ub antibody (clone P4D1). *C*, schematic drawing of Neur2 mutant constructs. Neur2 mut1 contains C494S/C497S mutations, and Neur2 mut2 contains C509S/H511N mutations. *D*, RING finger domain-dependent degradation of Neur2 proteins. HEK 293A cells were transfected with plasmids expressing the indicated proteins, and cell lysates were immunoblotted with anti-HA antibody. *E*, E1- and E2-dependent self-ubiquitination by the Neur2 RING finger domain. GST fusion proteins of the Neur2 RING finger domain were incubated in a reaction mixture in the presence or absence of E1 and E2 (UbcH5a). GST and GST-Neur1 RING were used as negative and positive controls, respectively. Ubiquitination was detected with anti-Ub antibody. *F*, RING finger domain-dependent self-ubiquitination. The Neur2 RING finger domain mutants were assayed as described for *E*.

ity. To confirm the E3 ligase activity of the RING finger domain of Neur2, we generated a GST fusion with Neur2 bearing either the C494S/C497S or C509S/H511N mutations and then performed an *in vitro* ubiquitination assay as described above. These mutant proteins showed no ubiquitination activity (Fig. 2*F*).

Neur2 Interacts with the XD Ligand—DNeur interacts with Delta and ubiquitinates it to target it to the cellular endocytic machinery (10, 11). To test whether murine Neur1 and Neur2 also interact with Delta, HA-Neur1 and HA-Neur2 were cotransfected with either Myc-tagged XD or XD without its intracellular domain in HEK 293A cells. HA-Neur2 was immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody to detect the co-immunoprecipitation of Myc-tagged ligands. The results showed that both Neur1 and Neur2 interacted with XD, but not with XD lacking its intracellular domain (Fig. 3*A*). These results indicate that both Neur1 and Neur2 bind to XD and that the intracellular domain of the Delta ligand is essential for the interaction with murine Neur proteins.

To identify the domain of murine Neur2 that interacts with the Delta ligand, XD-Myc was cotransfected with HA-Neur2 or a truncated form of Neur2: the NHR1 domain (amino acids 1–202), the NHR2 domain (amino acids 201–487), the combined NHR1 and NHR2 domains (amino acids 1–487), or the Neur2 RING finger domain (Fig. 3*B*). All of the Neur2 forms were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody to detect the co-immunoprecipitation of XD-Myc. All of the Neur2 forms except the Neur2 RING finger domain were co-immunoprecipitated with XD-Myc (Fig. 3*C*), whereas DNeur lacking the first NHR domain failed to interact with Delta (11). Taken together, these results indicate that Neur2 interacts with the intracellular domain of Delta through each NHR domain.

Neur2 Interacts with and Ubiquitinates Deltalike-1—Because murine Neur2 binds to XD through its NHR domains and has ubiquitin ligase activity, we tested whether Neur2 also binds to murine Deltalike-1 (Dll1) and ubiquitinates it using the RING finger domain. As expected, both HA-Neur1 and HA-Neur2 interacted with Dll1-Myc (Fig. 4*A*, *lanes 2*

FIGURE 1. **Cloning of mouse Neur1 and Neur2.** *A*, alignment of the amino acid sequences of mouse Neur1 and Neur2 with DNeur. The *colored boxes* indicate domains depicted in *B*. *m*, mouse. *B*, structures of the Neur1 and Neur2 proteins with predicted domains, with percent identity to DNeur. *C*, identity and similarity of Neur proteins in different species. For each species comparison, the percentages of identity and similarity (in *parentheses*) were predicted from sequence alignments using BLAST 2 Sequences. Zebrafish *neur2* (*zneur2*) and human *neur2* (*hneur2*) are putative genes identified by a genome BLAST search (see supplemental Fig. 1). *X*, *Xenopus*. *D*, schematic drawing of a comparative analysis of Neur proteins from *Drosophila* to human. Both Neur1 and Neur2 exist in parallel in different species except *Drosophila*. *E*, whole-mount *in situ* hybridization using *neur1* (*left*) and *neur2* (*right*) antisense probes in embryonic day 10.5 mice. Note that *neur1* was expressed in the limb buds (*LB*) and dorsal root ganglia (*DRG*) as reported previously (30), and *neur2* was also expressed in the same regions where *neur1* transcripts were detected. *F*, Northern blot analysis of *neur2* expression in adult mouse tissues.

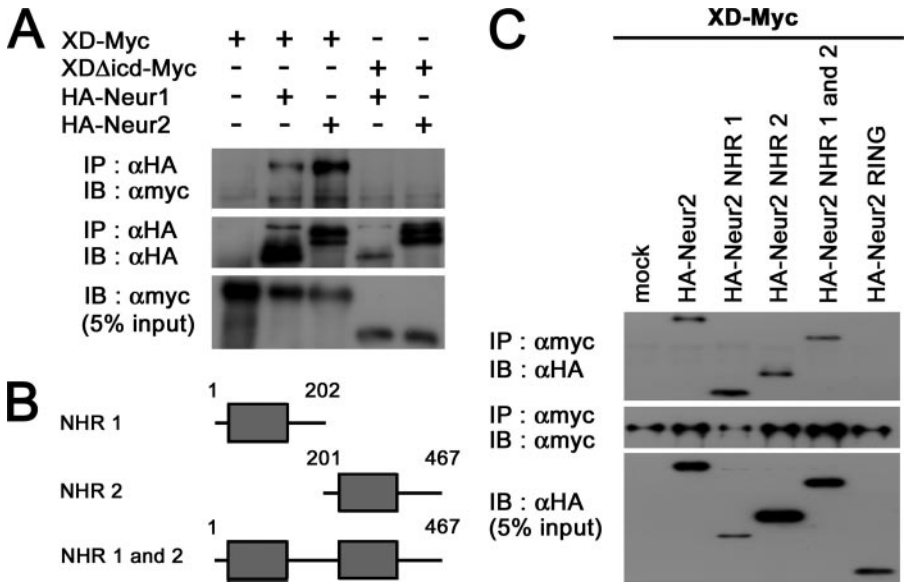


FIGURE 3. **Interaction between Neur2 and the XD ligand.** *A*, interaction between Neur2 and XD through the C-terminal cytoplasmic domain of XD. HEK 293A cells were cotransfected with various plasmid constructs. Cell lysates were immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted (*IB*) with anti-Myc antibody. The *upper panel* shows immunoprecipitations of XD by the Neur1 and Neur2 constructs, and the *middle* and *lower panels* show the expression of the Neur1/Neur2 and Delta constructs, respectively, in cell lysates. *XDΔicd-Myc*, Myc-tagged XD without its intracellular domain. *B*, schematic drawing of HA-tagged NHR domain constructs. *C*, interaction between Neur2 and XD through the NHR domain. The details of the experiment were as described for *A*.

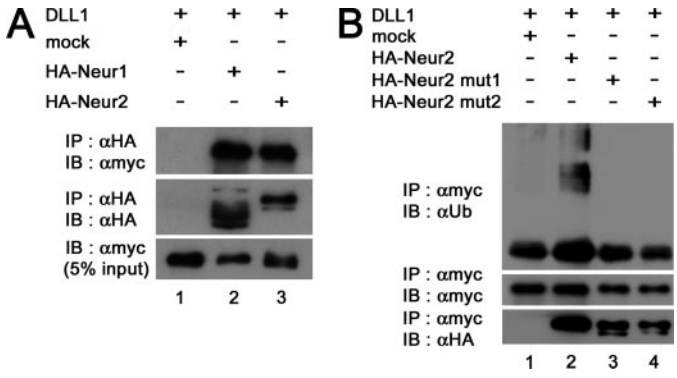


FIGURE 4. **Interaction with and ubiquitination of the Dll1 ligand by Neur2.** *A*, interaction between Neur2 and Dll1. The details of the experiment were as described in the legend to Fig. 3*A*. *B*, ubiquitination of Dll1 by Neur2. HEK 293A cells were cotransfected with various plasmid constructs. Cell lysates were immunoprecipitated (*IP*) with anti-Myc antibody and immunoblotted (*IB*) with anti-Ub antibody. The *upper panel* shows the ubiquitination of Dll1 by Neur2, and the *middle* and *lower panels* show the expression of the Dll1 and Neur2 constructs, respectively.

and *3*), suggesting that Dll1 could be a natural ligand of Neur1 and Neur2.

We next examined whether Neur2 promotes ubiquitination of Dll1. Dll1-Myc and the candidate E3 ligases were cotransfected into HEK 293A cells. Dll1-Myc was immunoprecipitated with anti-Myc antibody, and the presence of ubiquitinated Dll1-Myc was detected with anti-Ub antibody. The ubiquitination of Dll1-Myc was readily observed in the presence of HA-Neur2 (Fig. 4*B*, *lane 2*). As expected, Neur2 mut1 and mut2, which have mutations of critical residues in the RING finger domain, did not show any ubiquitination of Dll1-Myc (Fig. 4*B*, *lanes 3* and *4*). From these data, we concluded that Neur2 interacts with and ubiquitinates Dll1 through its NHR and RING finger domains, respectively.

Subcellular Localization of Neur2—To characterize the subcellular localization of Neur2, we used a subcellular marker that distinguishes the intracellular subcompartments (32). In *Drosophila*, Delta is endocytosed by DNeur and then passed into the endocytic pathway targeted to Hrs-positive vesicles (33, 34). Hrs binds directly to ubiquitin by way of a ubiquitin-interacting motif and is involved in the endosomal sorting of ubiquitinated membrane proteins (35). COS-7 cells transfected with Neur2-green fluorescent protein (GFP) were stained with an antibody for HA-Hrs. Neur2-GFP co-localized with Hrs (Fig. 5*A*″). Interestingly, Neur2 co-localized with Neur1 in the cytoplasm as a punctate structure (Fig. 5*B*″), but not with Mib1 (Fig. 5*C*″), another E3 ligase that promotes the endocytosis of Delta (12, 13, 36, 37). In contrast to Neur2, Mib1 did not co-localize with Hrs (Fig. 5*D*″), suggesting that Neur2 and Mib1 might have distinct roles in the endocytic pathway. Taken together, these results indicate that Neur2 is an endosomal protein localized in early endosomal compartments.

Cooperation of Neur2 and Mib1 in the Endocytic Pathway of Delta—To test whether Neur2 regulates the endocytosis of Delta to Hrs-positive vesicles, XD-Myc, Neur2-GFP, and HA-Hrs were coexpressed in COS-7 cells. When COS-7 cells were cotransfected with XD-Myc and HA-Hrs, XD-Myc was expressed on the plasma membrane, but HA-Hrs localized in the cytoplasm as a vesicular structure (Fig. 6*A*). Unexpectedly, in contrast to DNeur, when XD-Myc, Neur2-GFP, and HA-Hrs were coexpressed in COS-7 cells, XD-Myc still accumulated on the cell surface despite the co-localization of Neur2 and Hrs (Fig. 6, *B* and *B*′), suggesting that Neur2 is not involved in the initial endocytosis of Delta despite its binding to Delta (Fig. 3*A*).

To date, there are two distinct E3 ligases (Neur and Mib) that regulate the endocytosis of Delta. In this study, Neur2 and Mib1 exhibited distinct subcellular localization (Fig. 5*C*″). Thus, we speculate that these two E3 ligases might have different roles in the endocytic pathway of Delta. To test this possibility, XD-Myc was coexpressed along with Neur2-GFP and/or Mib1-GFP in COS-7 cells. When XD-Myc and HA-Hrs were coexpressed with murine Mib1, XD-Myc localized in the cytoplasm as a vesicular structure, where it co-localized with Mib1-GFP, but not with HA-Hrs (Fig. 6, *C* and *C*′). In contrast, when XD-Myc and HA-Hrs were coexpressed with Mib1-GFP in the presence of Neur2-GFP, XD-Myc was endocytosed to the cytoplasm and co-localized with HA-Hrs (Fig. 6*D*). These results suggest a cooperative role of Mib1 and Neur2 in the endocytic pathway of Delta, *i.e.* Mib1 regulates the initial endocytosis of

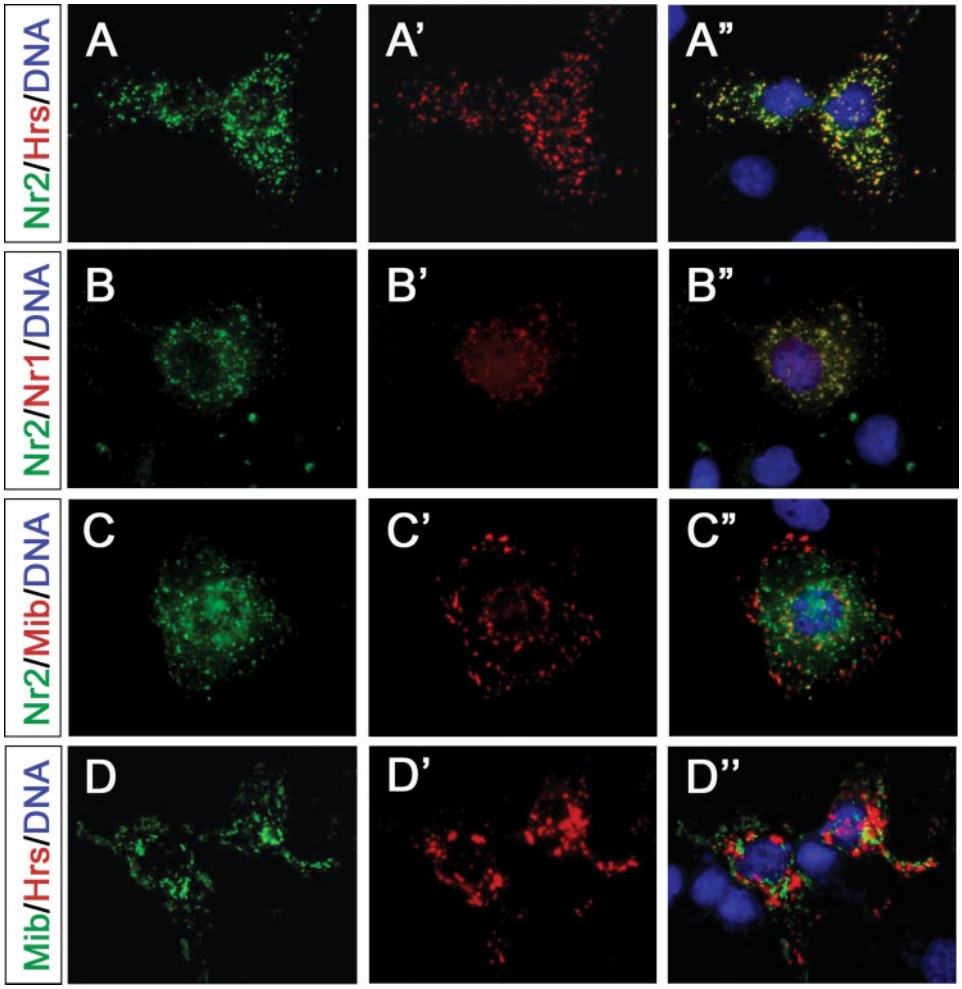


FIGURE 5. **Subcellular localization of Neur2.** *A*, co-localization of Neur2 (*Nr2*) and Hrs; *B*, co-localization of Neur2 and Neur1 (*Nr1*); *C*, distinct subcellular localization of Neur2 and Mib1; *D*, localization of Mib1 and Hrs. COS-7 cells were transfected with Neur2-GFP (*A–C*) and Mib1-GFP (*D*) in the presence of HA-Hrs (*A′* and *D′*), HA-Neur1 (*B′*), or HA-Mib1 (*C′*). The HA epitope was detected with mouse anti-HA antibody, followed by Alexa 546-conjugated anti-mouse antibody (in *red*). Nuclear DNA was stained with Hoechst dye (in *blue*). Overlapping expression is shown in *yellow* (*A″–D″*). Magnification ×400.

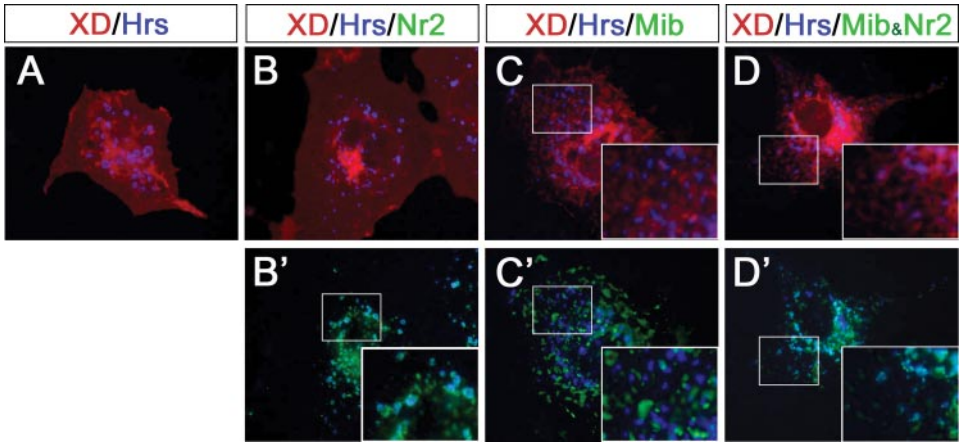


FIGURE 6. **Targeting of XD to Hrs-positive vesicles by Neur2 and Mib1.** *A*, distinct subcellular localization of XD and Hrs. COS-7 cells were transfected with XD-Myc and HA-Hrs. *B*, co-localization of Neur2 (*Nr2*) and Hrs without endocytosis of XD. COS-7 cells were cotransfected with XD-Myc, Neur2-GFP, and HA-Hrs (*B* and *B′*). *C*, endocytosis of XD by Mib1, but distinct localization of XD and Hrs. COS-7 cells were cotransfected with XD-Myc, Mib1-GFP, and HA-Hrs (*C* and *C′*). *D*, targeting of XD to Hrs-positive vesicles and co-localization of XD, Neur2, Mib1, and Hrs. COS-7 cells were cotransfected with XD-Myc, Mib1-GFP, Neur2-GFP, and HA-Hrs (*D* and *D′*). Myc epitopes were detected with rabbit anti-Myc antibody, followed by Alexa 546-conjugated anti-rabbit antibody (in *red*). HA epitopes were detected with mouse anti-HA antibody, followed by Alexa 350-conjugated anti-mouse antibody (in *blue*). Magnification ×400.

Delta to early endosomal compartments, and Neur2 is needed for targeting endocytosed Delta to Hrs-positive vesicles.

Co-localization of Neur2 and Mib1 in the Presence of Delta—To further investigate the cooperative role of Neur2 and Mib1 in the endocytic pathway, we generated COS-7 cells expressing XD and a control vector. When control vector-expressing COS-7 cells were transfected with Neur2-GFP and HA-Mib1, these two E3 ligases did not co-localize (Fig. 7*A*, *panel a″*). In XD-expressing COS-7 cells, however, Neur2-GFP and HA-Mib co-localized in the cytoplasm (Fig. 7*A*, *panel b″*). To visualize the endocytosis of Delta by Mib1 and Neur2, COS-7 cells were transfected with Mib1-GFP, HA-Neur2, and XD-Myc. As expected, HA-Mib1 co-localized with Neur2-GFP where XD-Myc was expressed (Fig. 7*B*). These results indicate that the cooperative role of Mib1 and Neur2 is dependent on the expression of Delta and that both Mib1 and Neur2 are required for targeting Delta to Hrs-positive vesicles.

Non-redundant Role of Mib1 and Neur2 in Zebrafish Notch Signaling—Recent studies in *Drosophila* have shown that *Drosophila* Mib1 is necessary for signal sending by both Delta and Serrate in establishment of the wing dorsoventral boundary, a well characterized instance of Notch signaling (12, 36–38). Because phenotypes caused by the absence of DMib1 can be rescued by ectopic provision of DNeur (36–38), we tested whether Neur2 can rescue the defects of the zebrafish *mib1* mutant mib^{ta52b}. The zebrafish mib^{ta52b} mutants have an increased number of *zath1*-positive hair cells because of a failure of lateral inhibition in the mechanosensory organs, neuromasts (39). Zebrafish embryos from mib^{ta52b} heterozygous intercrosses were injected at the one- or two-cell stage with mRNA encoding mouse *neur2*, and its effects on the development of hair cells were assayed by examining *zath1* expression 36 h post-

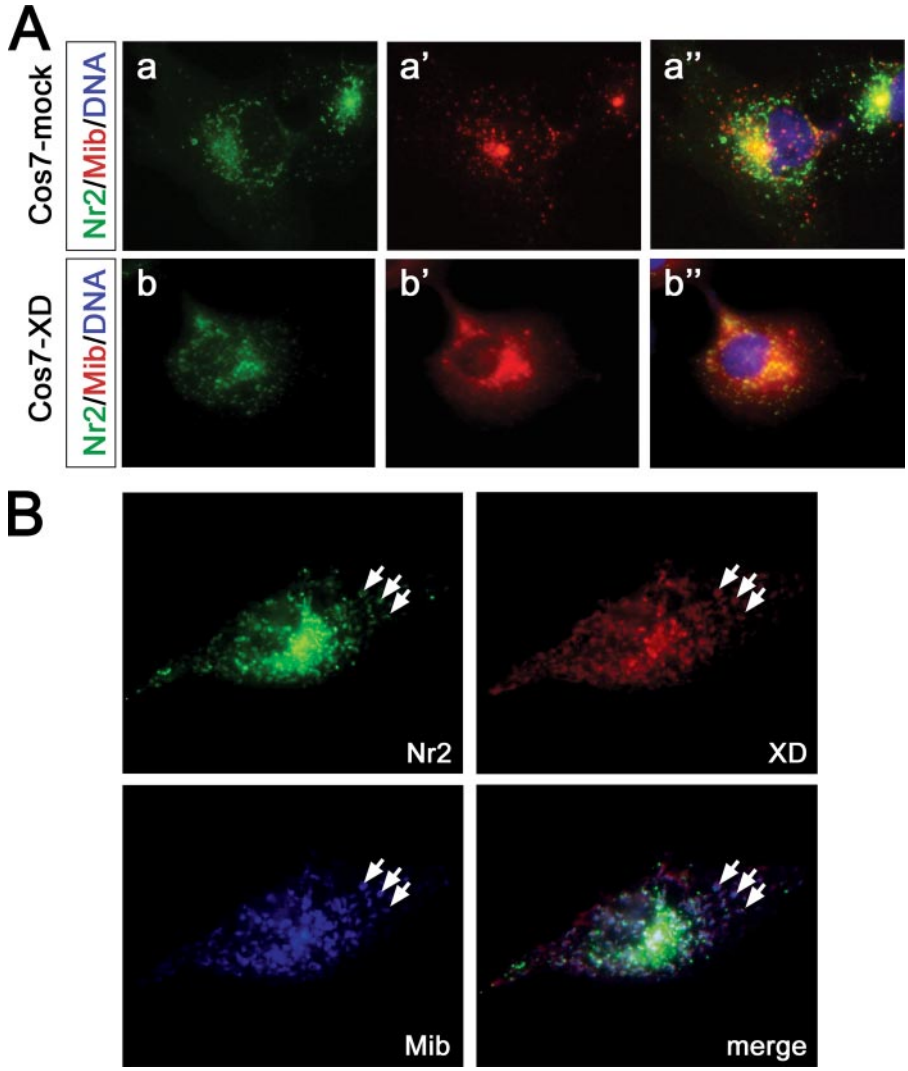


FIGURE 7. **Co-localization of Neur2 and Mib1 in the presence of XD.** *A*, co-localization of Neur2 (*Nr2*) and Mib1 in COS-7 cells overexpressing XD. COS-7 cell lines overexpressing XD (*Cos7-XD*) and control constructs (*Cos7-mock*) were generated using murine stem cell virus retroviral vectors and transfected with Neur2-GFP (*panels a* and *b*) and HA-Mib1 (*panels a'* and *b'*). HA epitopes were detected with mouse anti-HA antibody, followed by Alexa 546 anti-mouse antibody (in *red*). Nuclear DNA was stained with Hoechst dye and is show in *blue* (*panels a''* and *b''*). *B*, co-localization of Neur2, Mib1, and XD. COS-7 cells were cotransfected with XD-Myc, Neur2-GFP, and HA-Mib1. Myc epitopes were detected with rabbit anti-Myc antibody, followed by Alexa 546-conjugated anti-rabbit antibody (in *red*). HA epitopes were detected with mouse anti-HA antibody, followed by Alexa 350-conjugated anti-mouse antibody (in *blue*). Magnification ×400.

fertilization. In contrast to *Dneur*, the ectopic overexpression of mouse *neur2* could not reduce the massive *zath1* overexpression in mib^{ta52b} mutants (supplemental Fig. 2*C*), indicating that zebrafish Mib1 cannot be functionally replaced by ectopically expressed Neur2. These results suggest that Neur2 and Mib1 work independently and non-redundantly in Notch signaling in zebrafish.

DISCUSSION

Many E3 ligases are known to regulate Notch signaling (40). They are divided into two groups: one that ubiquitinates Notch receptors and another that regulates Notch ligands. Sel-10 ubiquitinates the intracellular domain of Notch, and Itch cooperates with Numb to stimulate the endocytosis of Notch receptors (41, 42). Both Neur and Mib ubiquitinate Delta and thus promote its endocytosis (10, 11, 13, 14, 24, 29). In this study, we have cloned mouse Neur2, another protein homologous to *Drosophila* Neur. Neur2 is another E3 ligase that targets the Notch ligand in the Notch signaling pathway and works cooperatively in the endocytic pathway with Mib1.

In *Drosophila* neurogenesis, Neur acts in a subset of Notch-dependent cell fate decisions, including lateral inhibition, by acting as a ubiquitin ligase and triggering the endocytosis of the ligand Delta (21, 23). Mutations in *Drosophila neur* result in a variety of developmental defects that closely resemble those of Notch mutants and other Notch pathway mutants (20, 22, 23). Targeted disruption of *notch1* expression or disruption of its downstream targets in mice leads to embryonic lethality by developmental defects (43–46). However, mice with disrupted *neur1* exhibit no abnormal cell fate specifications during neurogenesis and somitogenesis, two processes in which Notch signaling has been shown to be involved (25, 26). This discrepancy might be due to functional redundancy provided by Neur2. In this study, we have shown that Neur2 also interacts with and ubiquitinates Delta. Furthermore, it works in the endocytic pathway of Delta in cooperation with Mib1. Thus, Neur2 is another E3 ligase that might regulate Notch signaling.

One of the essential steps in Notch signaling is the endocytosis of the Notch ligand (9, 47, 48). Two proteins, Neur and Mib, which are known to be E3 ligases, are potent components involved in the endocytosis of Notch ligands (10–14, 24, 29, 36–38). However, their amino acid sequences show little similarity, and their protein structures are distinct, suggesting potential differential roles in the endocytic pathway of Delta. In this study, the subcellular localization analysis established that both Mib1 and Neur2 are required for the endocytosis of Delta to Hrs-positive vesicles. XD localized on the cell surface and Hrs-negative vesicles in the presence of Neur2 and Mib1, respectively. XD was endocytosed and co-localized with Hrs-positive vesicles in COS-7 cells only when coexpressed with both Mib1 and Neur2. These results are consistent with the previous reports that Dll1 and DeltaD accumulate on the plasma membrane in $mib1^{-/-}$ mice (15) and zebrafish $mib1^{tfi91}$ mutants (13). These results suggest that Mib1 functions in the initial step of Delta endocytosis and that Neur2 is required in targeting endocytosed Delta to Hrs-positive vesicles, *i.e.* that

ubiquitination of Delta by Mib may be needed to recruit adaptor proteins for proper endocytosis and that additional ubiquitination of Neur may be required to sort Delta endosome to Hrs vesicles.

In *Drosophila*, both Mib and Neur are involved in regulating Notch ligand endocytosis, but Mib is expressed in different patterns than is Neur (36–38). Ectopic expression of *Drosophila* Neur bypasses the requirement for *Drosophila* Mib, so they appear to be interchangeable in mediating the ubiquitination and internalization of the DSL ligand in *Drosophila* (36–38). Furthermore, in *Drosophila*, Delta is endocytosed by DNeur and then passed into the endocytic pathway targeted to Hrs-positive vesicles (33, 34). In this study, however, murine Neur2 did not reduce the increased *zath1*-positive hair cells in the zebrafish *mib*^{ta52b} mutants because of a failure of lateral inhibition in the mechanosensory organs, neuromasts (39). In addition, Mib1 and Neur2 have distinct and non-redundant but cooperative roles in the endocytic pathway of Delta. It is possible that the molecular functions of Mib1 and Neur2 are not same in different animal species because Neur mediates lateral inhibition of neural precursors in *Drosophila*, whereas Mib1 does so in zebrafish and mice.

There are multiple Notch ligands such as three Delta-like ligands (Dll-1, -3, and -4) and two Serrate-like ligands (Jagged-1 and Jagged-2) in mammals. For the regulation of these Notch ligands, three E3 regulators (Mib1, Mib2 and Neur) have been identified so far (15, 16, 25, 26, 30). In this study, we have identified a new E3 ligase (Neur2) that regulates Notch signaling. Our data suggest that two distinct classes of E3 ligases (Mib and Neur) have cooperative but distinct roles in Delta endocytosis to Hrs-positive vesicle. Why do these two distinct E3 ligases exist to regulate the same Notch ligands in the cell fate decisions? Delta endocytosis facilitates S2 cleavage and removal of the Notch extracellular domain, a critical step in Notch activation. *In vivo*, Delta is endocytosed and then passed to a recycling pathway targeted to Rab11-positive vesicles or to an endocytic pathway targeted to Hrs-positive vesicles (33, 34). Rab11 regulates trafficking of vesicular cargo through the recycling endosomal compartment, and Hrs sorts the ubiquitinated membrane proteins into the clathrin-coated microdomains of early endosomes, thereby preventing their recycling to the cell surface (35, 49). Therefore, our study on Mib1 and Neur2 provides new and exciting insights into how distinct E3 ligases work together in the endocytic pathways (initial internalization, recycling, and degradation) for Notch signaling. Because Notch signaling has multiple and essential roles in many cell fate decisions and in patterning events, further work is needed to clarify the cooperation of these four E3 ligases in the Notch signaling pathways.

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