Functional Analysis of the NHR2 Domain Indicates that Oligomerization of Neuralized Regulates Ubiquitination and Endocytosis of Delta during Notch Signaling

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The Notch pathway plays an integral role in development by regulating cell fate in a wide variety of multicellular organisms. A critical step in the activation of Notch signaling is the endocytosis of the Notch ligands Delta and Serrate. Ligand endocytosis is regulated by one of two E3 ubiquitin ligases, Neuralized (Neur) or Mind bomb. Neur is comprised of a C-terminal RING domain, which is required for Delta ubiquitination, and two Neur homology repeat (NHR) domains. We have previously shown that the NHR1 domain is required for Delta trafficking. Here we show that the NHR1 domain also affects the binding and internalization of Serrate. Furthermore, we show that the NHR2 domain is required for Neur function and that a point mutation in the NHR2 domain (Gly430) abolishes Neur ubiquitination activity and affects ligand internalization. Finally, we provide evidence that Neur can form oligomers in both cultured cells and fly tissues, which regulate Neur activity and, by extension, ligand internalization.

The Notch pathway is highly conserved across invertebrates and vertebrates and plays multiple and essential roles in many developmental processes, such as inhibiting differentiation by lateral signaling and regulating cell fate through inductive interactions (7, 23). Notch signaling is induced through direct cell-cell interactions between membrane-bound Notch ligands, Delta, Serrate, and Lag-2 (DSL), and the Notch receptor on adjacent cells. The activation of the Notch receptor results in the cleavage of Notch by the γ -secretase complex, leading to the translocation of the Notch intracellular domain into the nucleus, where it can activate downstream target genes (7). Recessive loss-of-function mutations in key components of the Notch pathway in *Drosophila melanogaster* are embryonic lethal and result in neurogenic phenotypes consisting of an overgrowth of the nervous system at the expense of the epidermis (36).

The ubiquitination and endocytosis of receptors and ligands have been shown to potentiate Notch signaling (32, 33, 54). Currently, there are two models hypothesizing how ligand endocytosis facilitates Notch activation in the signal-receiving cell. The mechanical force or pulling model suggests that Delta endocytosis exerts a force on the Delta-Notch complex that alters the conformation and promotes the cleavage of the Notch extracellular domain (NECD), which is a critical step in Notch activation (17, 41, 42, 51). The recycling model suggests that the modification of an inactive form of Delta in an endosomal compartment makes Delta a more effective ligand, which will be re-presented to the cell surface (perhaps at a microdomain of the plasma membrane) to activate Notch (2, 4, 14, 20, 47, 52). In the signal-sending cell, Neuralized (Neur) (12, 27, 43, 56) and Mindbomb1 (3, 22, 25, 28, 34, 44, 53) are two E3 ubiquitin ligases that regulate the endocytosis of the Notch ligands Delta and Serrate by ubiquitination.

Neur was one of the first five Notch pathway members identified (36). Previous analyses revealed that Neur plays an important role in all three germ layers during embryonic development (10, 18, 46). In addition, Neur is also required for the development of

the adult central and peripheral nervous system, including bristle sense organ patterning and photoreceptor specification (29, 30, 57). Consistent with its role in embryogenesis and adult neurogenesis, Neur is expressed in embryonic neural tissue and in the region of larval imaginal discs that will give rise to adult sensory organs (5). Of note, Neuralized is not required for all Notch signaling events, and evidence suggests that Mind bomb, its functional homologue, performs the same role in different cellular and developmental contexts (28, 34). The presence of either Neur or Mind bomb in the signal-sending cell appears to be required for ligand endocytosis (34). In addition to its role in Notch signaling, Neur was also recently shown to regulate epithelial cell polarity in the embryo (8).

The *neur* locus produces two major transcripts, *neur-RA* and *neur-RC*, which give rise to two proteins, NeurPA and NeurPC, which differ only at their N termini (9). Specifically, NeurPA, which is the predominant isoform during development (5), contains a phosphoinositide (PIP)-binding motif at the N terminus, which is required for Delta endocytosis downstream of Delta ubiquitination by Neur (49). In addition, both isoforms contain three highly conserved domains, including a carboxyl-terminal RING domain and two Neuralized homology repeat (NHR) do-

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mains (NHR1 and NHR2). The RING domain is both necessary and sufficient for Neur E3 ubiquitin ligase activity and is required for the endocytosis of the Notch ligand Delta (27, 43, 56). The NHR1 domain is a protein-protein interaction module that is required for Neur to bind Delta: a point mutation in a highly conserved glycine residue at position 167 disrupts Delta binding (9). Whether the NHR1 domain also mediates the interaction between Neur and Serrate (44) is unknown.

Although several aspects of the role of Neur in Notch signaling have been characterized, the full spectrum of Neur function and regulation still remains to be elucidated, including the function of the NHR2 domain. Previous *in vitro* studies done with mammalian cell cultures demonstrated that the NHR2 domain of *Neur1*, a mouse homologue of *Drosophila* Neur, mediates the interaction between *Neur1* and Jagged1 (26), a mouse homologue of *Drosophila* Serrate. Whether the NHR2 domain is required for Neur function *in vivo* and whether this function depends solely on its interaction with Serrate remain to be determined.

Here we show that Neur binds both Notch ligands via the NHR1 domain. We also show that the NHR2 domain is required for Neur-mediated D1 endocytosis and Notch signaling *in vivo*. Specifically, we show that the deletion of the NHR2 domain and a point mutation in a highly conserved glycine residue within the NHR2 domain reduce D1 internalization. Finally, we demonstrate that Neur can form oligomers, which regulate its ubiquitination activity and, as a result, ligand internalization.

MATERIALS AND METHODS

Plasmid construction and mutagenesis. pAC-NeurWT was previously described (49). By using pAC-NeurWT as a template, Neur^{ΔNHR1}, Neur^{ΔNHR2}, and Neur^{NHR2} were amplified by PCR, cloned into the pENTR vector (Life Technologies, Grand Island, NY), and subsequently flipped into the pAW vector (Murphy Laboratory *Drosophila* Gateway Collection via the *Drosophila* Genome Resource Center [DGRC], Bloomington, IN) or the pMGF vector (gift from A. McQuibban, Toronto, Ontario, Canada) by Gateway cloning (Life Technologies, Grand Island, NY). The Neur^{G430E} and Neur^{ΔNHR1&2} mutants were generated by using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). pMT-D1WT-Ndemyc (24) was obtained from the DGRC (Bloomington, IN). By using pUAST-Ser-myc (gift from C. Delidakis, Heraklion, Crete, Greece) as a template, cDNA encoding Ser was amplified by PCR, cloned into the pENTR vector (Life Technologies, Grand Island, NY), and subsequently flipped into the pMGF vector (gift from A. McQuibban, Toronto, Ontario, Canada) by Gateway cloning (Life Technologies, Grand Island, NY). pRM-HA-Ubiquitin was a gift from P. Rørth (48). Oligonucleotide sequences of PCR primers are available upon request.

Antibodies. Rabbit anti-V5 antibody (Genscript, Piscataway, NJ) was used to detect V5-tagged Neur proteins at a 1:3,000 dilution for Western blots and at 1:500 to 1:1,000 dilutions for immunostaining. Mouse anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) was used to detect FLAG-tagged Neur and Ser proteins by Western blotting (1:3,000 dilution) and immunostaining (1:1,000 dilution). Rabbit anti-myc antibody (Genscript, Piscataway, NJ) was used to detect the myc-tagged D1 protein by Western blotting (1:3,000 dilution). Mouse anti-D1 antibody (C594-9B; DSHB, Iowa City, IA) was used to detect endogenous D1 by immunostaining (1:5,000 dilution). Mouse antitubulin and mouse antiactin antibodies (E7 and JLA20, respectively; DSHB, Iowa City, IA) were used as loading controls on Western blots (1:1,000 dilution). Guinea pig anti-Sens antibody was used to mark sensory organ precursor cells (SOPs) (1:300 dilution; H. Bellen, Houston, TX). Rabbit anti-Neur antibody was used to detect endogenous Neur (1:1,000 dilution; C. Delidakis, Heraklion, Crete, Greece).

Yeast two-hybrid analysis. A yeast two-hybrid (Y2H) analysis was performed by using the Clontech Laboratories (Mountain View, CA) pACT2 GAL4 activating domain (AD) and pGBKT7 GAL4 DNA-binding domain (DB), containing either the NHR1 domain, the NHR2 domain, or Neur Start-NHR2. The positive-control vectors used were the known interactors Jun and cFOS as well as full-length GAL4 (original constructs used for cloning were kindly provided by Charles Boone, Toronto, Ontario, Canada). Negative controls were empty vectors.

Cell culture. *Drosophila* Kc167 (Kc) cells were cultured at 23°C in Schneider's medium (catalog number S9895; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (PAA, Pasching, Austria). Transfections were carried out by using CellfectinII, according to the manufacturer's specifications (Life Technologies, Grand Island, NY). For pMT-D1WT-NdeMYC, pMTF-Ser, and pRM-HA-Ubiquitin, 500 μM CuSO₄ was used to induce protein expression for 24 h prior to the assay.

***Drosophila* stocks and transgenics.** Wild-type controls were *w¹¹¹⁸* flies. Other stocks used in this study include the following: *neur^{A101}GAL4* (catalog number 6393) and *ombGAL4* (catalog number 3605), obtained through the Bloomington Stock Center; *neur^{IF65}*; *neur^{P72}GAL4*; *hs-flp, tub-Gal4, UAS-GFP; FRT82B tub-Gal80/TM6b* (Flybase); *UAS-neur^{PA-V5}* (abbreviated *UAS-Neur^{WT}*); *UAS-neur^{PA5Q-V5}* (49); and *UAS-neur^{PC-V5}*. *neur^{AAS-N67}*, obtained by the excision of the Neur-RC-specific P element from *neur^{GE29607}* (GenExel, Inc., Kaist, Daejeon, Republic of Korea), with a deletion of 1,600 bp that removes most of the first exon encoding Neur^{PC}. Breakpoints were determined by PCR and DNA sequencing (ACTG; SickKids). *UAS-Neur^{PAΔNHR2-V5}* (abbreviated *UAS-Neur^{ΔNHR2}*) and *UAS-Neur^{PAG430E-V5}* (abbreviated *UAS-Neur^{G430E}*) were injected into *w¹¹¹⁸* embryos, and transgenic lines were generated (BestGene, Chino Hills, CA).

The rescue studies were performed as double-blind experiments. Embryos from *w¹¹¹⁸ UAS-Neur^{transgene}/+ neur^{A101}GAL4/TM3Ser* self-crosses were collected. Numbers of wild-type (WT) and neurogenic embryos were scored, and the neurogenic percentage was calculated. Student's *t* test was performed for statistical comparisons. We disregarded those embryos that carried two copies of the balancer chromosome, which are easily identifiable because they are highly disorganized and positive for green fluorescent protein (GFP). Given this protocol, Mendelian inheritance predicts that one-third of embryos will be homozygous for *neur^{A101}GAL4* and therefore neurogenic in the absence of any transgenes.

neur mutant clones overexpressing versions of V5-tagged Neur were generated by using *hs-flp, tub-Gal4, UAS-GFP; UAS-NeurV5/+; FRT82B neurIF65/FRT82B tub-Gal80* flies. Mitotic clones were induced by a 45-min heat shock at 36.5°C during the first-instar larval stage. All crosses were performed at 25°C.

Immunohistochemistry. For immunostaining, Kc cells were grown on 25-mm circular coverslips. Cells were fixed at 48 h after transfection with fresh 4% paraformaldehyde. After fixation, cells were permeabilized, incubated with primary and secondary antibodies, and then mounted in Dako mounting medium (Dako, Burlington, Ontario, Canada). Images were acquired on a Quorum Spinning Disk confocal microscope (Quorum, Guelph, Ontario, Canada). For the quantification of localization, cells were scored for cytoplasmic or membrane localization; a total of ~100 cells were examined per genotype from 3 individual transfections. To examine localization in embryos, fly crosses were set up in cages on grape plates, and 0- to 18-h embryo collections were obtained, followed by standard methanol fixation. Immunostaining protocols were the same as those described above for cell culture. For imaginal disc staining, wandering third-instar larvae were dissected in cold phosphate-buffered saline (PBS). Wing imaginal discs were obtained and treated as described above for cell culture staining.

Endocytosis assay. The endocytosis protocol was modified from a method described previously by Le Borgne et al. (35). Wing discs were dissected in Schneider's *Drosophila* medium (catalog number S9895; Sigma-Aldrich, St. Louis, MO) containing 1% fetal bovine serum (PAA, Pasching, Austria). After dissection, the medium was replaced and sup-

FIG 1 The NHR2 domain and the conserved Gly430 residue are required for Neuralized function *in vivo*. (A) Various V5-tagged Neur constructs used in this study. The NHR1 domain is shown in dark blue, the NHR2 domain is shown in purple, and the RING domain is shown in red. (B) Western blot of embryo lysates showing the expression of V5-tagged Neur driven by *neur*^{A101} GAL4. (C to F) Lateral views of the embryonic nervous system (shown in green) identified with a fluorescein isothiocyanate-conjugated anti-HRP antibody (50). (C and C') A neurogenic embryo (C) and a WT embryo (C') are shown. (D to F) Rescued (with UAS-Neur^{WT} overexpressed) (D) and neurogenic (with UAS-Neur^{ΔNHR2} [E] and UAS-Neur^{G430E} [F] overexpressed) *neur*^{A101} GAL4 embryos showing the expression of exogenous Neur, as detected by anti-V5 staining (red) (panels D', E', and F' [red] and zoomed-in in panels D'', E'', and F''). Only stage 14 embryos with a definable central nervous system architecture were counted. (G) Results from rescue experiments. The proportion of neurogenic embryos from each cross was calculated. (H) Results from overexpression experiments in a heterozygous background. The proportion of wild-type embryos from each cross was calculated.

plemented with 1 μ g/ml 20-hydroxycyclohexanone (Sigma-Aldrich, St. Louis, MO). Wing discs were cultured for 10 min in the presence of the mouse monoclonal anti-Dl antibody (1:100 dilution) (C594-9B; DSHB, Iowa City, IA). Following washes, wing discs were fixed and subjected to secondary antibody treatment.

Western blot analysis and immunoprecipitation. Embryo lysates were prepared from 0- to 18-h collections. Embryos were decapitated and then lysed in 2 $\times$ SDS loading buffer. For immunoprecipitation (IP) experiments, transfected cells ($\sim 1 \times 10^7$ cells) were lysed in 300 μ l radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors (Roche, Basel, Switzerland). The input was precleared with 25 μ l 50% RIPA buffer plus 50% protein G-Sepharose (Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C and incubated with 30 μ l RIPA buffer plus 50% protein G and 3 μ l anti-V5 antibody (Life Technologies, Grand Island,

NY) overnight. Protein G beads were washed and resuspended in 2 $\times$ SDS loading buffer. For the ubiquitination IP, cells were treated with 50 μ M calpain inhibitor IV (MG-132; American Peptide Company, Sunnyvale, CA) for 3 h prior to lysis.

RESULTS

The NHR2 domain is required for Neur function *in vivo*. Neur is comprised of three functional domains: a C-terminal RING finger that is required for E3-ligase activity and two highly conserved Neur homology repeats, NHR1 and NHR2 (Fig. 1A). We have previously shown that the NHR1 domain mediates Neur binding to its ubiquitination target Dl (9). Specifically, we showed that the deletion of the entire NHR1 domain or the mutation of a highly

conserved glycine residue (Gly167) within the NHR1 domain disrupts binding to D1 (9). To date, however, the function of the NHR2 domain remains unknown. To determine whether the NHR2 domain is required for Neur function, we generated transgenic flies carrying a deletion of the entire NHR2 domain (UAS-Neur^{ΔNHR2}) or a mutation at Gly430 (UAS-Neur^{G430E}), which corresponds to the Gly167 residue found in the NHR1 domain (9), under the transcriptional control of the GAL4 upstream activation sequence (UAS) system (Fig. 1A) (6). To ensure that all of our transgenes were expressed at comparable levels, we performed Western blots with embryonic extracts derived from transgenic flies expressing Neur^{WT} (49), Neur^{ΔNHR2}, or Neur^{G430E} using *neur^{A101}GAL4*. We found that all three transgenes were expressed at comparable levels, with slightly higher levels of expression observed for the Neur^{G430E} line (Fig. 1B).

neur^{A101}GAL4 is a hypomorphic allele of *neur*, which shows an embryonic neurogenic phenotype due to the insertion of a P element containing the transcriptional activator GAL4 within the *neur* promoter. To determine if the NHR2 domain was required for Neur function, we examined whether the expression of the NHR2 mutant transgenes could rescue the neurogenic phenotype of *neur^{A101}GAL4* mutant homozygous embryos (Fig. 1C), which we scored by using an anti-horseradish peroxidase (HRP) antibody (50). We observed that 32.91% of embryo progeny of *neur^{A101}GAL4* heterozygotes were neurogenic in the absence of any transgene (Fig. 1C and G). In the presence of the Neur^{WT} transgene, only 7.32% of embryos appeared neurogenic (referred to as rescue) (Fig. 1D and G), as previously shown (49). In contrast, both Neur^{ΔNHR2} and Neur^{G430E} failed to rescue the *neur* mutant phenotype, resulting in significantly higher percentages of embryos appearing neurogenic: 24.46% and 45.10%, respectively (Fig. 1E to G). Although the number of neurogenic embryos in the presence of Neur^{ΔNHR2} (24.46%) was less than that observed for *neur^{A101}GAL4* alone (32.91%), it remained significantly higher than what we observed with Neur^{WT} (7.32%). This finding suggests that this construct may retain some residual activity albeit not enough to fully rescue the *neur* mutant phenotype. The observation that Neur^{G430} resulted in a significant increase in the percentage of neurogenic embryos (Fig. 1G) suggests that this construct may have a dominant negative effect, by inactivating zygotic Neur in the heterozygous progeny. Consistent with this interpretation, the ectopic expression of Neur^{G430E} in a heterozygous background (UAS-Neur^{G430E}/+; *neur^{A101}GAL4*/+) also resulted in a similar increase in the percentage of neurogenic embryos, while the overexpression of Neur^{WT} or Neur^{ΔNHR2} had no effect compared to the effect of heterozygous *neur^{A101}GAL4* by itself (Fig. 1H). Altogether, these data indicate that the deletion of the NHR2 domain significantly reduces Neur activity, and the Gly430 residue is essential for Neur function during embryonic neurogenesis. In addition, our data suggest that the deletion and point mutation in the NHR2 domain have distinct effects on Neur function.

The NHR2 domain is not required for Neur localization at the plasma membrane or its interaction with Notch ligands. Given that the NHR2 domain is important for Neur function, we then asked whether the inability of the NHR2 mutant transgenes to rescue *neur* mutant embryos was due to protein mislocalization *in vivo*. We first analyzed the subcellular localization of the transgenic protein in embryos using *neur^{A101}GAL4* (Fig. 1D", E", and F") and in wing imaginal discs using *ombGAL4* (see Fig. 5J', K',

and L') and found no significant difference between the localizations of the mutant proteins and WT Neur. Neur^{WT}, Neur^{ΔNHR2}, and Neur^{G430E} seemed to be enriched at the plasma membrane.

In previous studies, we showed that in singly transfected S2 cells, Neur and Delta were found primarily at the plasma membrane (49). We also showed that a mutant form of Neur lacking a PIP-binding motif was localized primarily to the cytoplasm in singly transfected cells but could be recruited to the membrane in the presence of D1 (49). To determine if mutations in the NHR2 domain altered the subcellular localization of Neur or its ability to be recruited to the membrane in the presence of Delta, we transfected V5-tagged Neur and/or D1-myc into Kc cells and examined their localization. We found that in singly transfected cells, D1 was localized to the plasma membrane, as previously described (49) (Fig. 2E and O). However, in contrast to what we previously observed for S2 cells, we found that Neur proteins were predominantly cytoplasmic in the absence of D1 (Fig. 2A to C and O) (9, 49). This discrepancy may reflect differences between Kc and S2 cell lines. Nonetheless, when cells were cotransfected with D1 and either WT or NHR2 mutant Neur constructs, Neur relocated to the plasma membrane (Fig. 2F to H and O), demonstrating that the NHR2 domain is not required for Neur localization to the plasma membrane when D1 is present. In contrast, and consistent with our previous findings (9), a mutation of a highly conserved glycine residue in the NHR1 domain (Neur^{G167E}) disrupts the interaction between Neur and D1, leading to the accumulation of Neur within the cytoplasm (Fig. 2I and O) and a concomitant retention of D1 at the membrane (Fig. 2I' and O) (9). The introduction of either Neur^{WT} or Neur^{ΔNHR2} resulted in an increase in the cytoplasmic D1 level (Fig. 2F' and G'), although Neur^{ΔNHR2} was not as efficient as the WT (Fig. 2O). These results are consistent with our rescue data demonstrating that while Neur^{ΔNHR2} does not have wild-type function, it may still have residual activity. In contrast, D1 remained mostly membrane bound in the presence of Neur^{G430E} (Fig. 2H' and O). These data suggest that the NHR2 domain is not required for Neur to bind to Delta; however, the Gly430 residue may be required for D1 internalization. To confirm that the NHR2 domain is not required for Neur to bind D1, we cotransfected D1 and Neur into Kc cells and performed coimmunoprecipitation (co-IP) experiments. As was shown previously, Neur^{G167E} disrupts D1 binding in this assay. We found that D1 coimmunoprecipitated with both NHR2 mutants (Fig. 3A, IP lanes 5 and 6 on the anti-myc blot), further demonstrating that mutations in NHR2 do not affect the interaction between Neur and D1.

Neur binds to both Notch ligands D1 and Ser (16, 49). We have previously shown that the NHR1 domain is both necessary and sufficient to bind to D1 in *Drosophila* (9). Others have shown that the NHR2 domain in mouse Neurl1 mediates the interaction with Jagged1, the mouse orthologue of Ser (26). To determine whether the NHR2 domain is also required for Neur to interact with Ser in *Drosophila*, we first transfected tagged Neur and/or Ser into Kc cells and examined its subcellular localization. Similarly to D1, Ser was localized to the plasma membrane in singly transfected cells (Fig. 2J and O). When Ser was cotransfected with either WT Neur or the NHR2 mutant, Neur was also concentrated at the plasma membrane (Fig. 3K to M and O). Similarly to what we observed with Delta, the expression of Neur^{WT} and Neur^{ΔNHR2} resulted in a dramatic increase in the cytoplasmic Ser level (Fig. 2K', L', and O), while Neur^{G430E} did not (Fig. 2M' and O). Interestingly,

FIG 2 The NHR2 domain is not required for ligand-dependent membrane recruitment of Neur. (A to I) Single confocal slices of Kc cells expressing myc-Dl (E) or Neur^{WT}, Neur^{ΔNHR2}, Neur^{G430E}, or Neur^{G167E} alone (A to D) or in combination with myc-Dl (F to I for Neur and F' to I' for Dl) and merged images of double transfections (F'' to I''). (J to N) Single confocal slices of Kc cells expressing FLAG-Ser (J) or in combination with Neur^{WT}, Neur^{ΔNHR2}, Neur^{G430E}, or Neur^{G167E} (K to N for Neur and K' to N' for Ser) and merged images of double transfections (K'' to N''). Neur is shown in green. Dl and Ser are shown in red. (O) Quantification of Kc cells expressing Dl-myc or FLAG-Ser in combination with Neur^{WT}, Neur^{ΔNHR2}, Neur^{G430E}, or Neur^{G167E}. Percentages of cells showing either a membrane or cytoplasmic localization were calculated for ligands and Neur variants.

Neur^{G167E} remained cytoplasmic in the presence of Ser (Fig. 2N and O), while Ser was predominantly retained at the plasma membrane (Fig. 2N' and O), suggesting that the G167E mutant is not recruited to the plasma membrane, where it is required to induce the internalization of Ser. Furthermore, in coimmunoprecipitation experiments with Neur and Ser, we found that both NHR2 mutants of Neur could be coimmunoprecipitated with Ser (Fig. 3B, IP lanes 9 and 10 on the anti-V5 blot) but not with Neur^{G167E} (Fig. 3B, IP lane 8 on the anti-V5 blot), suggesting that the G167E mutation in the NHR1 domain disrupts Neur-Ser binding. Of note, the interaction between WT Neur and Ser in our co-IP experiment appeared to be very weak; however, given that the expression of Neur^{WT} caused an increase in the cytoplasmic Ser level and other researchers previously reported an interaction between Neur^{WT} and Ser using co-IP experiments (16), we speculated that the weak binding in our experiments might reflect technical differences in co-IP protocols. Taken together, our data show that the NHR2 domain is not required for the plasma membrane localization of Neur in the presence of either Dl or Ser, nor is it required to bind to either ligand. In contrast, the conserved

Gly167 residue in the NHR1 domain is required for Neur to bind to both Dl and Ser in *Drosophila*.

The G430E mutation in the NHR2 domain disrupts Neur E3 ubiquitination activity. Given that the NHR2 domain is not required for Neur to interact with its targets Dl and Ser, we asked whether the NHR2 domain or the Gly430 residue is required for Neur E3-ubiquitin ligase activity. To this end, we performed immunoprecipitation experiments with cells cotransfected with Neur-V5, Dl-myc, and hemagglutinin (HA)-ubiquitin. High levels of HA-tagged ubiquitin were detected in the anti-V5 immunoprecipitate in the presence of Neur^{WT} (Fig. 4A, IP lane 5 on the anti-HA blot). In contrast, in the presence of Neur^{C701S}, a Neur mutant that has no E3 ligase activity (56), the levels of ubiquitinated proteins were significantly reduced (Fig. 4A, compare IP lane 6 to lane 5 on the anti-HA blot). Both the Neur^{G167E} and Neur^{ΔNHR2} mutants appeared to be as efficient at ubiquitination as Neur^{WT} (Fig. 4A, compare IP lanes 7 and 8 to lane 5 on the anti-HA blot). Of note, this assay does not distinguish whether the ubiquitinated proteins correspond to Delta, Neur, or other unidentified partners. Given that Neur^{G167E} does not bind to Dl but

FIG 3 The G167E mutation in the NHR1 domain disrupts the Ser-Neur interaction. (A) Neur^{WT}, Neur^{ΔNHR2}, and Neur^{G430E} can coimmunoprecipitate full-length Df from Kc cell lysates. Neur^{G167E} failed to coimmunoprecipitate full-length Df. (B) Ser can coimmunoprecipitate Neur^{WT}, Neur^{ΔNHR2}, and Neur^{G430E} from Kc cell lysates but fails to pull down Neur^{G167E}. The input contains 1/20 of the lysate used for immunoprecipitation.

we still observed high levels of ubiquitin in the IP, it is possible that Neur is autoubiquitinated or has other targets besides Df. Consistent with this idea, in a similar co-IP experiment, where we expressed Neur and HA-ubiquitin on their own, we observed high levels of HA-tagged ubiquitin coimmunoprecipitating with Neur (Fig. 4B, lanes 3 and 9 on the anti-HA blot), further suggesting that Df may not be the only target of Neur. Nonetheless, the ubiquitination activity was completely abolished in Neur^{G430E} (Fig. 4A, compare IP lane 9 to lane 5 on the anti-HA blot, and B, compare IP lane 10 to lane 9 on the anti-HA blot), which still binds to Df (Fig. 4A, IP lane 9 on the anti-myc blot), while the deletion of either the NHR1 or the NHR2 domain had no effect (Fig. 4A, lane 8 on the anti-HA blot, and B, lanes 4 and 6 on the anti-HA blot), demonstrating that the two mutations in NHR2 have distinct effects on Neur function. Interestingly, when the NHR1 domain was removed from Neur^{G430E}, ubiquitination activity was restored (Fig. 4B, lane 5).

The NHR2 domain is required for Neur-mediated Df endocytosis *in vivo*. We have shown that the deletion of the NHR2 domain does not affect the ability of Neur to bind to Notch ligands or its ubiquitination activity. Furthermore, in Kc cells, Neur^{ΔNHR2} was still able to induce Df and Ser internalization albeit with reduced activity. Switching from cultured cells, we wanted to assess the role of the NHR2 domain in Df endocytosis in flies, a more physiologically relevant context. To address this, we first assessed the effect of the expression of Neur transgenes on Df localization throughout the presumptive wing pouch using *ombGAL4*. Of note, Neur is not endogenously expressed in these cells at this stage of development. In WT larvae, Df is expressed at elevated levels in

the wing vein primordia (Fig. 5E, arrow). The ectopic expression of Neur^{WT} resulted in an increase in the amount of internalized Df, as observed in z sections of wing discs (compare Fig. 5F' to E'). In contrast, we observed relatively normal levels of internalized Df in the presence of either Neur^{ΔNHR2} or Neur^{G430E} (compare Fig. 5G and H to E), suggesting that NHR2 mutant proteins failed to facilitate Df endocytosis.

To further confirm that the differences that we saw in the localization of Df were due to a defect in endocytosis, we used an antibody uptake assay (35) to monitor the endocytosis of Df in the presence of exogenous Neur. Specifically, we used *ombGAL4* to drive the expression of Neur transgenes and examined Df endocytosis using an anti-Df antibody generated against the extracellular domain of Df (Df^{ECD}). Consistent with our previously reported observations (49), we found that the ectopic expression of Neur^{WT} resulted in an increase in Df-antibody internalization (Fig. 5J). Neur^{ΔNHR2} showed significantly decreased levels of Df-antibody internalization (Fig. 5K), indicating that the deletion of the NHR2 domain does affect Df endocytosis *in vivo*. As expected, minimal levels of internalized Df were observed for the Neur^{G430E} mutant (Fig. 5L), since the G430E mutant has no ubiquitination activity.

Neur forms oligomers. RING domain E3 ligases often function as oligomers (13). Evidence for the oligomerization of Neur *in vivo* was obtained by examining the localization of the two isoforms of Neur (Neur^{PA} and Neur^{PC}) during the asymmetric division of sensory organ precursor cells (SOPs). Neur is localized asymmetrically at the anterior cortex when SOPs divide along the anterior-posterior axis to produce a posterior pIIa cell and an anterior pIIb cell (Fig. 6A) (15, 35). We found that the anterior cortical localization of Neur^{PA} in dividing SOPs is not affected in a Neur^{PC}-specific mutant, *neur^{AA8-N67}*, that deletes the first exon of Neur^{PC} specifically (Fig. 6B), suggesting that the asymmetrical localization of Neur^{PA} is independent of Neur^{PC}. Of note, Neur^{PC}, the minor isoform, is essentially an N-terminal truncation of Neur^{PA}. Importantly, Neur^{PC} lacks a PIP-binding motif that was previously shown to be required for the plasma membrane association and function of Neur^{PA} (49) and is cytoplasmic *in vivo* (data not shown).

We then examined the distributions of V5-tagged Neur^{PA} and Neur^{PC} expressed in SOPs using *neur^{A101}GAL4* and found that both Neur^{PA} and Neur^{PC} localized asymmetrically (Fig. 6C and D). However, in the absence of endogenous Neur, i.e., in clones of *neur^{IF65}* mutant cells, we observed that Neur^{PA} but not Neur^{PC} localized asymmetrically (Fig. 6F and G). We therefore conclude that the localization of Neur^{PC} depends on the presence of Neur^{PA}. This observation suggested that Neur^{PA} recruits Neur^{PC} at the anterior cortex.

Neur^{PA} differs from Neur^{PC} by the presence of a PIP-binding motif. We therefore tested the role of this motif in the asymmetric localization of Neur^{PA}. To do so, we examined the distribution of Neur^{PA5Q}, a Neur^{PA} version with a mutated PIP-binding motif (49). In the presence of endogenous Neur, Neur^{PA5Q} localized to an anterior crescent in the metaphase and segregated into the anterior cell (Fig. 6E). However, similarly to Neur^{PC}, this asymmetric distribution of Neur^{PA5Q} was found to require endogenous Neur (Fig. 6H). In contrast, the asymmetric localization of Numb was Neur independent (35). Thus, our analysis of both Neur^{PC} and Neur^{PA5Q} revealed that the asymmetric localization of Neur proteins lacking the N-terminal PIP-binding motif required en-

FIG 4 The G430 residue in the NHR2 domain is required for Neuralized ubiquitination activity. Neur variants and HA-ubiquitin were cotransfected into Kc cells either with DI (A) or without DI (B), followed by anti-V5 immunoprecipitation. The immunoprecipitation of Neur was performed with cells treated with calpain IV (proteasome inhibitor) for 3 h prior to lysis. (A) In the immunoprecipitation blot, myc-DI was pulled down with Neur variants except Neur^{G167E}. HA-tagged ubiquitinated products were present when Neur^{WT}, Neur^{ΔNHR2}, or Neur^{G167E} was expressed. However, the expression of Neur^{G430E} significantly abolished the ubiquitination activity, with a minimum amount of ubiquitinated product being coimmunoprecipitated. (*, full-length DI; ***, C-terminal cleavage product of DI.) (B) In the immunoprecipitation blot, HA-tagged ubiquitinated products were present when Neur^{WT}, Neur^{ΔNHR1}, Neur^{ΔNHR1G430E}, or Neur^{ΔNHR2} was expressed and were absent when Neur^{G430E} was expressed. The input contains 1/20 of the lysate used for immunoprecipitation. *, nonspecific bands and staining.

ogenous Neur. These results suggest that Neur^{PA} can oligomerize *in vivo* with Neur^{PC} and Neur^{PA5Q} to promote their asymmetric localization.

We next verified the ability of Neur to form oligomers *in vitro*. We generated V5- and FLAG-tagged Neur^{WT} constructs (Fig. 7A), transfected these constructs into Kc cells, and performed anti-V5 immunoprecipitations. We found that FLAG-tagged Neur could be immunoprecipitated with Neur^{WT-V5} (Fig. 7B, IP lane 3 on the anti-FLAG blot), further demonstrating that Neur can form homo-oligomers.

To further define the role of the NHR domains in oligomerization, we performed immunoprecipitation experiments with lysates obtained from Kc cells cotransfected with various wild-type and truncated Neur constructs (Fig. 7A). We observed a strong interaction between Neur^{ΔNHR1-V5} and Neur^{ΔNHR1-FLAG} (Fig. 7B, IP lane 7 on the anti-FLAG blot), suggesting that the NHR1 domain cannot be the sole mediator of oligomerization. Similarly, we found that the deletion of the NHR2 domain alone does not affect Neur oligomerization (Fig. 7B, IP lane 9 on the

anti-FLAG blot). We also found that Neur^{ΔNHR1-FLAG} and Neur^{ΔNHR2-V5} could be coimmunoprecipitated from Kc cells (Fig. 7B, IP lane 5 on the anti-FLAG blot). Moreover, when both NHR1 and NHR2 were deleted, the remaining RING domain and the interstitial region could interact with WT Neur (Fig. 7C, IP lanes 5 and 6 on the anti-FLAG blot) and self-associate as well (Fig. 7C, IP lane 7 on the anti-FLAG blot). To determine whether the NHR domains alone are capable of mediating Neur dimerization, we also performed a direct yeast two-hybrid analysis (Fig. 7D). Results from two independent trials indicated that Neur can dimerize via the NHR1 domain (Fig. 7E). Moreover, a construct containing both the NHR1 and NHR2 domains is capable of binding to the NHR2 domain alone (Fig. 7E). Although we did not observe an interaction between the NHR1 and NHR2 domains in Y2H experiments, it is possible that factors required for the NHR1-NHR2 interaction were missing in the *in vitro* system. When we performed immunoprecipitation experiments with lysates obtained from Kc cells cotransfected with NHR1-V5 (9) and NHR2-FLAG, we found that NHR1 can

FIG 5 The NHR2 domain is required for Neuralized-mediated Delta endocytosis. (A to D) Third-instar larval wing discs expressing UAS-enhanced GFP (EGFP) (A), UAS-Neur^{WT} (B), UAS-Neur^{ΔNHR2} (C), and Neur^{G430E} (D) in the developing wing pouch, using *ombGAL4* (green), merged with Df staining (red; shown separately in panels A' to D'). (E to H) Higher magnification of Df staining in the wing pouch (white square in panel A'). Df is expressed at its highest levels in the wing vein primordia (E). The expression of Neur^{WT} results in an increase in the level of internalized Df (F), while Neur^{ΔNHR2} (G) or Neur^{G430E} (H) does not. (E' to H') z-section projections of Df delocalization in disc epithelium obtained from the same x and y regions. The apical view is up. (I) Base level of internalized anti-Df antibodies in *w¹¹¹⁸* wing imaginal disc. (J) Mouse anti-Df antibodies were internalized in the wing imaginal disc expressing Neur^{WT}. (K) Df internalization is largely reduced in the presence of Neur^{ΔNHR2}. (L) Df is not internalized at a detectable level when Neur^{G430E} was expressed. (J' to L') Presence of Neur^{WT}, Neur^{ΔNHR2}, and Neur^{G430E} (green).

interact with NHR2 (Fig. 7F). These data suggest that the NHR domains are sufficient but not necessary for oligomerization, and there may be multiple ways for oligomers to be assembled and maintained.

As shown previously, the Gly430 residue in the NHR2 domain

is essential for Neur ubiquitination activity. To test whether Gly430 plays a role in protein oligomerization, we cotransfected Neur^{G430E} with Neur^{WT-FLAG} into Kc cells, followed by anti-V5 immunoprecipitation. We found that the G430E mutant could still form oligomers with WT Neur but was less efficient than the

FIG 6 Asymmetric localization of Neur lacking a PIP-binding motif depends on endogenous Neur. (A) Neur (red) localizes asymmetrically in an anterior crescent in wild-type dividing SOPs (Senseless [Sens], a marker for SOPs, is shown in green). (B) Neur-PA localizes asymmetrically in *neur*^{AA8-N67} mutant pupae that lack the Neur-PC isoform. (C to E) V5-tagged versions of Neur-PA (C), Neur-PC (D), and Neur-5Q (E) localized asymmetrically in wild-type dividing SOPs (Sens [green]), i.e., in the presence of endogenous Neur. (F to H) In the absence of endogenous Neur, i.e., in *neur*^{IF65} mutant cells (marked by nuclear GFP), only V5-tagged Neur-PA (F) localized asymmetrically. In contrast, Neur-PC (G) and Neur-5Q (H) were not asymmetric.

WT control (Fig. 7G, compare IP lanes 3 and 4 on the anti-FLAG blot). Of note, the difference in binding is unlikely to be due to differences in protein expression levels or immunoprecipitation efficiencies, since we observed comparable levels of wild-type and mutant proteins in both the input (Fig. 7G, input lanes 3 and 4) and immunoprecipitated baits (Fig. 7G, IP lanes 3 and 4 on the anti-V5 blot). Furthermore, when we repeated the co-IP experiments with Kc cells cotransfected with Neur^{G430E-V5} and Neur^{G430E-FLAG} followed by anti-V5 immunoprecipitation, we found that the oligomerization of Neur^{G430E} was compromised (Fig. 7G, lane 9).

We also examined whether the presence of the G430E mutation in the NHR1 deletion protein (Neur^{ΔNHR1G430E-FLAG})

affected the binding to Neur lacking the NHR2 domain (Neur^{ΔNHR2-V5}). As before, we observed similar levels of input (Fig. 7B, input lanes 5 and 11) and immunoprecipitated V5-tagged baits (Fig. 7B, IP lanes 5 and 11), but the level of coimmunoprecipitated FLAG-tagged Neur^{ΔNHR1G430E} was increased compared to that of Neur^{ΔNHR1} (Fig. 7B, compare IP lane 11 to lane 5 on the anti-FLAG blot), suggesting that the G430E mutation allows Neur^{ΔNHR1G430E} to bind more efficiently with Neur^{ΔNHR2} than with Neur^{ΔNHR1}. Taken together, our data suggest that Neur forms oligomers via the two NHR domains; however, without the two NHR domains, the remaining RING domain with the interstitial regions could still direct Neur oligomerization. Nevertheless, the Gly430 residue

FIG 7 Neuralized forms oligomers in Kc cells. (A) Schematic of tagged wild-type and deletion mutants of Neur for coimmunoprecipitation. Kc cells were cotransfected with differently tagged Neur protein variants, followed by an anti-V5 immunoprecipitation. (B and C) In each co-IP, pairs of Neur variants were tested for oligomerization. The deletion of either NHR domain or both NHR domains did not affect Neur oligomerization. FLAG-tagged deletion mutant proteins of both NHR domains are indicated by the two-headed arrow (C) (*, nonspecific bands). Yeast two-hybrid analysis revealed that Neur interacts with itself via the NHR domains, particularly NHR1. (D) Schematic of constructs used for yeast two-hybrid analysis. (E) Yeast two-hybrid results demonstrating that Neuralized is capable of interacting with itself via its NHR domains. “+++” indicates an observed “strong” interaction, “++” indicates a moderate interaction, “+” indicates a weak interaction, and “–” indicates no observed interaction. (F) The FLAG-tagged NHR2 domain was pulled down by the V5-tagged NHR1 domain in co-IP experiments. The input contains 1/20 of the lysate used for immunoprecipitation. (G) In the immunoprecipitation blot, FLAG-Neur^{WT} was coimmunoprecipitated with V5-Neur^{WT}, while FLAG-Neur^{WT} was coimmunoprecipitated with Neur^{G430E} with reduced efficiency. Moreover, Neur^{G430E} failed to self-associate.

in NHR2 plays an important role in this oligomerization process.

DISCUSSION

Neur is an E3-ubiquitin ligase that plays an essential role in Notch signaling by regulating the endocytosis of Notch ligands. It contains NHR domains, which are rare and conserved between vertebrates and invertebrates but not present in viruses, bacteria, fungi, or plants. In the *Drosophila* proteome, besides Neuralized, there are two other NHR-containing proteins, CG3894 and Bluestreak.

In mammals, proteins containing NHR domains (also known as NEUZ) include the β -catenin regulator OzzE3 (40) and lung-inducible Neuralized-related C3HC4 RING protein (LINCR) (21). Recent studies reported that the human homologue of Bluestreak serves to localize to the centrosome (1, 37). Although the general role of the NHR domains is unclear, these domains tend to cluster, and most proteins contain two to six NHR domains. The significance of having more than one NHR domain in one protein is to yet be determined.

Here we have investigated the role of the highly conserved

NHR domains in Neur function. We show that the NHR1 domain alone mediates the interaction between Neur and both Delta and Serrate. We also show that the NHR2 domain is required for Neur function, and while it is not required for the interaction with Notch ligands, it is involved in DI internalization, a critical step in Notch activation. Moreover, the NHR domains play a role in Neur oligomerization, which in turn could contribute to Neur ubiquitination activity and ligand endocytosis.

The NHR1 domain mediates the interaction between Neur and the Notch ligands Delta and Serrate. We have previously shown that the NHR1 domain of Neur is both necessary and sufficient for the interaction with the Notch ligand DI (9). Specifically, we found that a point mutation in a highly conserved glycine residue within the NHR1 domain (G167E) abolishes the ability of Neur to bind Delta. Whether the NHR1 domain was also required to bind to Serrate, however, was unknown. In fact, *in vitro* studies in vertebrates suggested that the NHR2 domain in mouse Neuralized-like 1 (Neur11) was sufficient to bind to Jagged1, the mouse orthologue of Serrate (26). In contrast, we find that the NHR2 domain is not required for Neur to bind Serrate in *Drosophila* and that the interaction is mediated entirely by the NHR1 domain. Other studies reported previously that the motifs on DI and Ser that mediate the interaction with Neur are conserved (11, 16). In comparisons of protein sequences, Jagged1 and Serrate share 40.7% similarity overall, while the overall similarity between Jagged1 and DI is 33.8% (39). The NHR1 and NHR2 domains from *Drosophila* Neuralized have the same degree of amino acid similarity with the mouse Neur11 NHR2 domain (33%). Since there is no clear correlation between protein sequence similarity and the ability of either the NHR1 or the NHR2 domain to interact with Notch ligands, whether NHR1 or NHR2 is important for interacting with ligands is likely to be species dependent. We found that the Neur-Ser interaction is abrogated by the G167E mutation in the NHR1 domain. Given that the Neur^{G167E} mutant still retains ubiquitination activity, it is unlikely to affect overall protein folding. A previously reported structural analysis of the *Drosophila* NHR1 domain suggested that Gly167 resides in a hydrophobic core and that the Gly167 mutation presumably destabilizes the surrounding microenvironment (19). Therefore, the Gly167 mutation may result in spatial changes in the neighboring residues of the core, thus abolishing binding to ligands.

The G430E mutation reveals a distinct role for the NHR2 domain in the regulation of Neur activity and Delta trafficking. Our data demonstrate that the NHR2 domain is required for Neur function *in vivo*. Neur^{G430E} fails to rescue *neur* mutant embryos, while Neur^{ΔNHR2} has some residual activity, which suggests that they affect different aspects of Neur function. The expression of Neur^{G430} in a heterozygous background, which does not have a neurogenic phenotype on its own, resulted in a significant increase in the percentage of neurogenic embryos, suggesting that Neur^{G430E} has a negative effect on Neur function. In contrast, Neur^{ΔNHR2} overexpression did not have any effect on heterozygous embryos, suggesting that it behaves as a loss-of-function allele.

Despite the fact that the two NHR2 mutant proteins Neur^{G430E} and Neur^{ΔNHR2} behave differently, they both localize to the plasma membrane in the presence of Delta both *in vitro* and *in vivo*, and they are both capable of binding to the Notch ligands Delta and Serrate. However, both mutant proteins affect the extent of DI internalization to various degrees. Neur^{G430E} exhibits

severely compromised ubiquitination activity and is no longer capable of inducing Delta internalization. Neur^{ΔNHR2}, on the other hand, retains ubiquitination activity but is much less efficient than WT Neur at directing DI internalization *in vivo* or in Kc cells. The precise mechanism by which Neur^{ΔNHR2} affects Delta endocytosis is unclear.

One possibility is that the NHR2 domain is required for Neur oligomerization. We have shown that Neur forms NHR domain-mediated oligomers by coimmunoprecipitation experiments. Therefore, the deletion of the NHR2 domain (Neur^{ΔNHR2}) may simply reduce the oligomerization potential of Neur, leading to a decrease in ligand endocytosis. In contrast, the point mutation (Neur^{G430}) might disrupt the overall structure of the NHR2 domain, preventing oligomerization and resulting in a protein that has no ubiquitination activity and therefore can no longer internalize ligands. However, this model cannot fully explain our data, which show that ΔNHR2 does not prevent Neur oligomerization and that Neur oligomerization still occurs in the absence of any NHR domains, suggesting that while the NHR domains may play a role in oligomerization, they are not necessary for this process. Our data also show that although the G430E mutant loses ubiquitination activity, the double mutant containing the NHR1 deletion and the G430E mutation retains ubiquitination activity, which argues that G430E does not affect the overall folding of the NHR2 domain.

Another possibility is that the NHR1 and NHR2 domains initially form an intramolecular structure that is inactive and must be resolved for ubiquitination to promote ligand internalization (Fig. 8B). The G430E mutation may lock Neur into an intramolecular conformation through an NHR1-NHR2 interaction, such that this inactive form can still bind to DI and Ser but cannot form oligomers and has no ubiquitination activity as a consequence of dysfunctional oligomerization (Fig. 8C). This model, in contrast to the former one, is supported by our data, which demonstrate that the G430E mutant no longer forms oligomers and has no ubiquitination activity. Furthermore, when the NHR1 domain was removed from the G430E mutant (Neur^{ΔNHR1G430E}), the ubiquitination activity was restored, suggesting that the intramolecular loop can no longer form, whereas intermolecular interactions between NHR1 and NHR2 domains can occur. It may also explain the negative effect of Neur^{G430E} on Neur^{WT}: although Neur^{G430E} has a reduced ability to bind WT Neur, it is still able to sequester some portion of Neur^{WT} into a nonfunctional intermolecular oligomer that can no longer ubiquitinate targets. In contrast, the deletion of the NHR2 domain would prevent intramolecular interactions but would be expected to have a reduced ability to form productive oligomers, leading to a defect in ligand internalization. Whether the NHR2 domain has additional roles in recruiting a protein(s) (marked as “X?” in Fig. 8) that promotes Notch ligand ubiquitination and endocytosis remains to be determined.

Like Neuralized, other RING domain E3 ligases often function as oligomers, and they multimerize in different ways: some form heterodimers, such as Mdm2-MdmX (38), and some can form homo-oligomers, such as TRAF (55). The functional significance of RING E3 oligomerization is poorly defined. One previously proposed model is that the oligomerization of E3 ligases may functionally resemble the dimerization of receptor tyrosine kinases in such a way that autoubiquitination yields a mark that serves as a platform to assemble a signaling complex (31). Consis-

FIG 8 Possible mechanisms for Neur oligomerization. (A) Schematic of wild-type Neur. (B) Neur^{WT} activity is inactivated by the intramolecular interactions between NHR1 and NHR2 in a looped-back conformation. The resolution of the intramolecular interactions allows Neur to form intermolecular dimers between NHR domains, which in turn promote the binding and ubiquitination of the ligand DI or Ser to activate Notch signaling. (C) Neur^{G430E} is locked in a looped-back conformation due to the G430E mutation. Although Neur^{G430E} still binds to DI or Ser and is able to bind other proteins, Neur^{G430E} loses the ubiquitination activity and is no longer able to ubiquitinate DI or Ser to activate Notch signaling. (D) Neur^{ΔNHR2} stays active in an open conformation due to the deletion of the NHR2 domain. Although Neur^{ΔNHR2} still binds to DI or Ser and has ubiquitination activity, the deletion of the NHR2 domain results in a failure to recruit other unidentified proteins (marked as “X?”) required for ligand endocytosis and Notch signaling.

tent with this idea, we did see ubiquitination in anti-Neur IPs when DI was not present, consistent with the idea that Neur may be autoubiquitinated (12). Whether this autoubiquitination can initiate a cascade of further downstream ubiquitination events remains to be determined. It is possible that oligomerization is mediated via autoubiquitination and the interaction of ubiquitinated Neur with itself through a ubiquitin-binding motif (UIM). If so, then the NHR1-NHR2 interaction could also function to keep Neur in an inactive state by occluding the putative UIM. Such a model of Ubi-UIM complex formation was previously proposed for other endocytic proteins (45).

In summary, we have shown that NHR domains are protein-protein interaction modules that are required for many aspects of Neur function. The NHR1 domain mediates the interaction between Neur and its targets DI and Ser. Both NHR domains appear to regulate Neur activity by affecting its ability to form oligomers and/or interact with proteins required for the endocytosis of Notch ligands. Interestingly, NHR domains have been identified in several other proteins that are conserved between flies and humans. Whether Neur can form heterodimers with these other NHR-containing proteins and whether these heterodimers play a role in Notch signaling and other developmental processes remain to be determined.

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