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A Mutation in EGF Repeat-8 of Notch Discriminates Between Serrate/Jagged and Delta Family Ligands

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Abstract

Notch signaling affects many developmental and cellular processes and has been implicated in congenital disorders, stroke, and numerous cancers. The Notch receptor binds its ligands Delta and Serrate and is able to discriminate between them in different contexts. However, the specific domains in Notch responsible for this selectivity are poorly defined. Through genetic screens in *Drosophila*, we isolated a mutation, *Notch^{jigsaw}*, that affects Serrate- but not Delta-dependent signaling. *Notch^{jigsaw}* carries a missense mutation in epidermal growth factor repeat-8 (EGFr-8) and is defective in Serrate binding. A homologous point mutation in mammalian Notch2 also exhibits defects in signaling of a mammalian Serrate homolog, Jagged1. Hence, an evolutionarily conserved valine in EGFr-8 is essential for ligand selectivity and provides a molecular handle to study numerous Notch-dependent signaling events.

The evolutionarily conserved Notch (N) signaling pathway affects numerous cell fate and differentiation events as well as proliferation and cell death (1). Signal activation is initiated by the binding of N receptor to ligands, Delta (Dl) or Serrate (Ser) (2). The majority of the extracellular domain of N receptor is composed of epidermal growth factor repeats (EGFr) (Fig. 1A). EGFr-11 and EGFr-12 are necessary for ligand-receptor interactions with both Dl and Ser (3), whereas EGFr-24 to EGFr-29 (Abruptex domain) negatively regulate these interactions (4). Although the in vivo role of most EGFr is unknown, mutations in these repeats are associated with numerous human diseases, including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (5), Alagille syndrome (ALGS) (6), aortic valve diseases (AVDs) (7–9), and squamous cell

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carcinoma (SCC) (10–12). Mammals have four N paralogs (*NOTCH1* to *NOTCH4*) that share similar structural organizations, but only one N gene exists in *Drosophila*, simplifying structure-function analysis in vivo (table S1).

To obtain N mutations, we performed an F3 mosaic genetic screen on the X chromosome (fig. S1) and isolated 42 additional alleles of N. Twenty-one alleles carry different missense mutations and were grouped into eight distinct classes on the basis of molecular and phenotypic features (tables S2 and S3). All mutated residues are conserved in most human N paralogs.

One mutation, Valine361-to-Methionine (V361M) in EGFr-8, *Njigsaw*, exhibits defects in the wing margin without affecting venation or bristle development in mutant clones (Fig. 1, B to F, and table S3). Hemizygous mutants of *Njigsaw* are pupal lethal, and *Njigsaw*^{+/+} flies do not display wing notching. *Njigsaw* fails to complement the lethality of null alleles of N and is rescued by a genomic rescue construct. Homozygous mutant clones in the wing exhibit strong notching and occasional ectopic wing margin formation (Fig. 1, D and E). We did not observe any bristle density or cell fate defects (Fig. 1F and figs. S2 and S3). Hence, *Njigsaw* displays a specific phenotype for a lethal N allele as inductive signaling is impaired, whereas lateral inhibition and lineage decisions remain unaffected.

Because the phenotypes associated with *Njigsaw* are similar to Ser loss of function (13, 14), we determined whether N-Ser signaling is compromised. In the early wing primordium, N is activated at the boundary between the dorsal and ventral compartments (Fig. 1G). The dorsal domain expresses both Ser and Fringe (Fng) (15), whereas Dl is mainly expressed ventrally (16). Fng, a β 3-N-acetylglucosaminyltransferase that adds N-acetylglucosamine (GlcNAc) to O-fucosylated EGFRs, modifies N so that it can be activated by Dl but not Ser (17, 18). As a result, Dl activates N in the dorsal domain, and Ser activates N in the ventral domain in cells flanking the dorsal-ventral boundary (Fig. 1, G and H). In *Njigsaw* hemizygous discs, N activation is severely reduced or lost (Fig. 1I). Furthermore, cells that activate N signaling are present in the dorsal but not ventral compartment (Fig. 1I'). These data indicate that *Njigsaw* is defective in N-Ser but not N-Dl signaling. This was confirmed in mosaic tissues (fig. S4). Last, *Njigsaw* mutant clones can be ectopically activated by overexpression of Dl but not by Ser (fig. S5).

The defects in N-Ser signaling in *Njigsaw* mutants are not confined to the wing. Ser is required during hematopoiesis for crystal cell specification in the lymph glands (19). In *Njigsaw* mutant animals, the crystal cell marker Lozenge is not expressed (fig. S6, A and B), resulting in a loss of circulating crystal cells (fig. S6, C to E). Furthermore, similar to *Njigsaw* (fig. S6, F and G), loss of Ser causes a loss of salivary imaginal ring cells (20). Hence, multiple processes requiring N-Ser signaling are severely impaired in *Njigsaw* mutants.

To determine the cause of ectopic wing margin formation in *Njigsaw* mutant clones (Fig. 1E), we monitored N activity. We observed ectopic N signal activation in wild-type (WT) cells adjacent to *Njigsaw* mutant cells in the ventral compartment near the dorsal-ventral boundary (Fig. 2, A and B). Binding of N to Ser in cis mediates the removal of Ser from the plasma membrane in WT cells (ligand-cis-inhibition) (Fig. 2D) (21), whereas in mutant cells, Ser is up-regulated (Fig. 2C and fig. S7A). The ectopic activation of N in the neighboring WT cells can be suppressed by reducing the levels of Ser specifically in *Njigsaw* mutant cells (fig. S7B), suggesting that *Njigsaw* mutant cells are defective in ligand-cis-inhibition (Fig. 2E). Hence, *Njigsaw* is defective in Ser-mediated signaling, both in trans and in cis, whereas N-Dl signaling appears unaffected.

Changes in the glycosylation status of EGFRs by Fng are known to modulate the ligand selectively of Notch (17, 18). When one copy of *fng* is removed, notching in *Njigsaw* clones

is enhanced, whereas overexpression of Fng in *N^{jigsaw}* mutant clones partially suppresses notching (figs. S8 and S9). These data suggest that *N^{jigsaw}* and Fng affect the same process independently.

To test whether *N^{jigsaw}* affects the glycosylation status of N, we analyzed glycopeptides using mass spectrometry (22, 23). The *N^{jigsaw}* mutation does not cause a significant change in the level of *O*-fucosylation and Fng-mediated elongation of *O*-fucose on EGFr-8 (Fig. 3, A to C, and fig. S10). In addition, other EGFRs do not display alterations in *O*-fucosylation and Fng-mediated elongation (fig. S11), indicating that these EGFRs are properly folded, which is a requirement for glycosylation. Hence, the *N^{jigsaw}* mutation has little or no effect on the sugar modification status of N, again suggesting that the defects in N-Ser signaling are independent of Fng.

Because both trans- and cis-signaling of N-Ser are defective in *N^{jigsaw}* mutant cells, and the expression, exocytosis, and endocytosis of *N^{jigsaw}* are indistinguishable from *N^{WT}* (figs. S12 and 13), we tested whether *N^{jigsaw}* affects Ser binding (17, 24). In contrast to *N^{WT}*, *N^{jigsaw}*-Ser binding is significantly diminished, and this interaction is unaffected by the presence of Fng (Fig. 3D). Moreover, overexpression of O-fut1, a sugar-modifying enzyme and molecular chaperone that can strengthen *N^{WT}*-Ser binding (24), cannot improve *N^{jigsaw}*-Ser binding (Fig. 3D). In contrast, *N^{jigsaw}* interacts with D1 in the presence of Fng (Fig. 3E). These data indicate that *N^{jigsaw}* directly impairs N-Ser binding without disrupting N-D1 binding.

To assess whether *N^{jigsaw}* (V361M) plays a similar role for a mammalian N, we performed a signaling assay (25). We introduced V327M (table S2) into mouse Notch2 and tested its ability to respond to Jagged1 or Delta-like1 (Dll1). Notch2-Jagged1 signaling is severely decreased, whereas Notch2-Dll1 remains unaffected (Fig. 3, F and G). In addition, a V327A mutation causes a further decrease in Notch2-Jagged1 signaling, showing that loss of valine is responsible for the *N^{jigsaw}* phenotype. In contrast, a mutation in the conserved O-fut1/Fng-modification site within Notch2 EGFr-8 (T314A) does not affect Jagged1- or Dll1-mediated signaling.

Among the identified *N* mutations (tables S2 and S3) and other reported *N* alleles (26, 27), *N^{jigsaw}* separates N-Ser from N-D1 binding and signaling in vivo. Previously, two *N* alleles have been shown to exhibit wing margin defects only when mutant cells are in the ventral compartment, suggesting defective Ser-mediated signaling (28). However, these mutations do not affect the EGFRs, and the nature of the signaling defects remains uninvestigated. Our data suggest that in addition to EGFr-11 and EGFr-12, which are required for both D1 and Ser binding (3), EGFr-8 plays a critical role in N-Ser interactions. The *N^{jigsaw}* mutation does not obviously affect the trafficking or glycosylation of N. Therefore, EGFr-8 may directly be involved in ligand-receptor interactions together with EGFr-11 and EGFr-12 or may be crucial for N to adopt a conformation favoring Ser binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Njigsaw*, a V361M mutation in a conserved domain in EGFr-8, is defective in Ser-mediated signaling. **(A)** Schematic representation of N. LNR, Lin12/Notch repeats; TMD, transmembrane domain; RAM, RBP- $\text{j}\kappa$ -associated molecule domain; NLS, nuclear localization signal; ANK, ankyrin repeat domain; TAD, transactivation domain; PEST, Pro-Glu-Ser-Thr rich domain. **(B)** Valine 361 mutated in *Njigsaw* is conserved in Notch paralogs. **(C)** Schematic diagram of EGFr-8. [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.] **(D)** Wings with *Njigsaw* mutant clones exhibit notching. **(E)** Ectopic wing margin in wings with *Njigsaw* mutant clones. **(F)** *Njigsaw* homozygous mutant bristles, marked by *yellow⁻*, are present in their normal stereotypic pattern. Homozygous WT bristles are *singed⁻*, and heterozygous bristles are *yellow⁺ singed⁺*. **(G)** Diagram of wing margin formation. **(H** to **I'**) N activation at the dorsal-ventral boundary of WT and *Njigsaw* mutant discs. [(H) and (I)] Low and [(H') and (I')] high magnification images of third instar wing imaginal discs of [(H) and (H')] WT and [(I) and (I')] *Njigsaw* hemizygous mutant larva. The dorsal domain is labeled with β -galactosidase (LacZ) driven by the *msh* enhancer (*msh-lacZ*: green). N activation is shown by Cut (red). Scale bars, 100 μm (D) to (F); 20 μm (H) to (I').

Fig. 2.

Ligand-cis-inhibition of Ser is impaired in *Njigsaw* mutant cells. (A to B') *Njigsaw* clones in larval wing discs (marked by the absence of GFP, green). Cell-nonautonomous ectopic N activation is indicated by arrows [Wingless (Wg) in (A)] and arrowheads [Cut in (B)]. Clones were induced by (A) *Ubx-FLP* or (B) *hs-FLP*. (C and C') Expression of Ser (blue) is elevated in mutant clones, leading to cell-nonautonomous N activation (monitored by Cut) in surrounding WT neighboring cells. Scale bars, 20 μ m. (D and E) Schematic diagrams of ligand-cis-inhibition and cell-nonautonomous N activation. In *Njigsaw* mutant cells, Ser accumulates at the cell surface and triggers signaling in the neighboring WT cells (E). WT cells are depicted in green, and *Njigsaw* homozygous mutant cells are in gray.

Fig. 3. *N^{jigsaw}* is defective in Ser binding in fly cells and Jagged1 signaling in mammalian cells, independent of Fng. (A and B) Multiple reaction monitoring (MRM) traces of ions show relative levels of unmodified (black line), *O*-fucose monosaccharide (red line), or *O*-fucose disaccharide (blue line) glycoforms of a peptide from EGFr-8 generated in the presence of Fng. Red triangle, fucose; blue square, GlcNAc; black rectangle, peptide. (C) Analysis of relative levels of monosaccharide versus disaccharide forms of the peptide from EGFr-8 based on the average of multiple MRM analyses (fig. S10, C and D). (D and E) Ligand-receptor binding assays in *Drosophila* S2 cells reveal loss of N-Ser binding in *N^{jigsaw}*. Binding of N^{WT} and *N^{jigsaw}* to Ser is shown in (D), whereas binding of N^{WT} and *N^{jigsaw}* to DI is shown in (E). (F and G) Mammalian cell-based assays show that V327M in mouse Notch2 reduces receptor activation by Jagged1 (F) but not Dll1 (G). The V327A reduces Notch2-Jagged1 signaling even more (F), whereas a mutation in the O-fut1/Fng modification site of EGFr-8 (T314A) does not affect Jagged1 or Dll1 signaling [(F) and (G)]. Error bars indicate SEM.