Development 140, 2039-2049 (2013) doi:10.1242/dev.087916

An extracellular region of Serrate is essential for ligand-induced cis-inhibition of Notch signaling

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SUMMARY

Cell-to-cell communication via the Notch pathway is mediated between the membrane-bound Notch receptor and either of its canonical membrane-bound ligands Delta or Serrate. Notch ligands mediate receptor transactivation between cells and also mediate receptor cis-inhibition when Notch and ligand are co-expressed on the same cell. We demonstrate in *Drosophila* that removal of any of the EGF-like repeats (ELRs) 4, 5 or 6 results in a Serrate molecule capable of transactivating Notch but exhibiting little or no Notch cis-inhibition capacity. These forms of Serrate require Epsin (Liquid facets) to transduce a signal, suggesting that ELR 4-6-deficient ligands still require endocytosis for Notch activation. We also demonstrate that ELRs 4-6 are responsible for the dominant-negative effects of Serrate ligand forms that lack the intracellular domain and are therefore incapable of endocytosis in the ligand-expressing cell. We find that ELRs 4-6 of Serrate are conserved across species but do not appear to be conserved in Delta homologs.

KEY WORDS: Notch, Cis-inhibition, Serrate ligand, Extracellular domain

INTRODUCTION

Cell-to-cell communication mediated via the Notch signaling system controls cellular differentiation processes in a wide variety of multicellular animals. Notch signals have been documented to control cell fate, survival, growth, proliferation, differentiation and morphogenesis (reviewed by Lai, 2004). Both the Notch receptor and its ligands, Delta and Serrate (Ser; also known as Jagged in vertebrates), are single-pass type I transmembrane molecules that mediate communication between adjacent cells. Active Notch signals are usually generated when a ligand-expressing (sending) cell contacts Notch on an adjacent (receiving) cell. During this event, there are many cellular processes that regulate signal transmission. The prevailing notion is that, in the sending cell, ligand endocytosis is required to trigger Notch activation in the receiving cell (Overstreet et al., 2004; Parks et al., 2000; Seugnet et al., 1997) (reviewed by Le Borgne, 2006), an event associated with ligand monoubiquitylation by Neuralized or Mind bomb (Le Borgne et al., 2005; Wang and Struhl, 2005). Cells deficient in specific endocytic components such as Dynamin and Epsin are incapable of signaling to an adjacent Notch-expressing cell (Wang and Struhl, 2005). In receiving cells, Notch molecules are apparently cleaved by a furin-like protease prior to being placed in the cell membrane as a heteromeric receptor (Kidd and Lieber, 2002; Logeat et al., 1998). Interaction with ligand triggers a cascade of proteolytic processing of the Notch receptor that ultimately releases the Notch intracellular domain (NICD) from the membrane (reviewed by Schweisguth, 2004). The NICD is then translocated to the nucleus where it interacts with a CSL [CBF1/RBPjκ/Su(H)/Lag-1] protein and recruits co-activators, including Mastermind, to drive Notch-dependent gene expression (Bray and Furriols, 2001; Petcherski and Kimble, 2000). The steady-state level of Notch receptor on cell surfaces is regulated by several ubiquitin ligases, an intracellular PEST domain and interactions with regulatory proteins such as Numb and α-adaptin (reviewed by Le Borgne, 2006).

All of the aforementioned regulatory events control Notch activity during transactivation by its ligands. However, an important aspect of the intricate control of the Notch signaling pathway relies on the ability of the ligands to repress Notch activity when the receptor and ligand are co-expressed on the same cell (de Celis and Bray, 1997; Micchelli et al., 1997) (reviewed by del Álamo et al., 2011). This interaction, termed cis-inhibition, is dependent on the relative concentrations of ligand and receptor and remains enigmatic. Notch interactions with ligand generate a graded activation response to ligand levels in trans, but a sharper, threshold type of inhibitory response to ligand interactions in cis (Sprinzak et al., 2010). These differences are likely to be essential to signal directionality, particularly in regions where cells initially express both ligand and receptor as they establish signal-sending and signal-receiving cell types. An example is the neurogenic region of the *Drosophila* ectoderm, where Notch sending versus receiving cells are segregated from a field of developmentally equivalent cells expressing both the receptor and the ligand.

Ligands lacking only the intracellular domain or lacking both the intracellular and transmembrane domains (i.e. secreted forms) lose the ability to transactivate Notch but retain strong inhibitory interactions with the receptor (Hukriede et al., 1997; Hukriede and Fleming, 1997; Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997). These findings indicate that inhibition of Notch by its ligands requires sequences found in their extracellular domains.

Since it has been reported in *Drosophila* that Ser demonstrates stronger cis-inhibitory properties than Delta (Klein et al., 1997; Li and Baker, 2004), we have undertaken a systematic analysis of the extracellular domain of Ser to localize sequences involved in Notch inhibition. We have identified EGF-like repeats (ELRs) 4 through 6 as dispensable for Notch transactivation but required for Notch cis-inhibition. Consistent with these observations, the region defined by ELRs 4, 5 and 6 is conserved among Ser (Jagged) family ligands

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Accepted 28 February 2013

in other species. Our studies indicate that this same ELR region is responsible for both cis-inhibition and Notch inhibitory properties associated with secreted forms of Ser. Moreover, we find that forms lacking the ability to inhibit Notch still require endocytosis in order to activate Notch, thereby indicating that deleted forms do not mimic activated ligand.

MATERIALS AND METHODS

Drosophila cultures and strains

UAS-Ser expression constructs were generated and transgenic lines were produced by Genetic Services. Other stocks were obtained from the Bloomington Stock Center. Cultures were maintained on standard cornmeal/dextrose/agar medium supplemented with active dry yeast at 25°C except when expression was by the *Gal4ptc* promoter (Hinz et al., 1994) or when generating single-cell clones, where they were maintained at 18°C. Two copies of a *Ser* wing promoter [Gal4^{Ser2} (Hukriede et al., 1997)] were used to express constructs along the dorsal/ventral wing boundary.

Construction of ELR deletions

All constructs were generated from the wild-type *Ser* cDNA sequence (Fleming et al., 1990) and all nucleotide sequences and amino acid numbering in the following constructs originate from that reference. Each construct was placed in the pUAST transformation vector (Brand and Perrimon, 1993). Effects were examined for a minimum of two independent inserts for each construct.

SerDel3

Amino acids 351-390 were deleted by cutting the *Ser* cDNA with *Dra*I (position 1484) and using the 5′-CGAGATCGTTTAAATTTCTGTGCC-ACCAGGCCATGCCGCAACGGC-3′ primer to place an artificial in-frame *Dra*I site at position 1598 at the beginning of ELR 4.

SerDel4

Amino acids 391-490 were deleted by placing an artificial *Ppu*MI site at position 1619 of the *Ser* cDNA using the 5′-GCCGCCGTTGCGGCAG-GGACCCGTGGCACATGGGTGCTC-3′ primer, fusing that with the *Ppu*MI site at position 1921.

SerDel5

Amino acids 491-528 were deleted by placing an artificial *Kas*I site at position 1916 of the *Ser* cDNA using 5′-GCCACCATGCTCGCAG-GGCGGCGCCTCGCACTCGTCGATATTTAT-3′ and fusing that with the *Kas*I site at position 2031.

SerDel6

Amino acids 529-610 were deleted by placing an artificial *Kas*I site at position 2277 of the *Ser* cDNA using 5′-GACTGTGTGGCGCCGT-GCCGGAATGGAGCC-3′ and fusing that with the *Kas*I site at position 2031.

SerhydroΔ6

Amino acids 532-574 were deleted and Ile575 changed to Leu575 using primer 5′-CCGGATCGATGCGGGAGCTCGCACTCGTTCACATCCA-3′ to introduce a unique *Sac*I site at position 2029 and using primer 5′-CACATCCTTGGAGCTCGGACCCTGCATCAATGC-3′ to introduce a unique *Sac*I site at position 2161 and fusing the cDNA at the *Sac*I site.

SerDel4-6

Amino acids 388-607 were deleted by placing an artificial *Xba*I site at position 1603 using primer 5′-GGCACATGGGTCTAGAACGATCTC-GCACTGCTCGCC-3′and fusing that with the *Xba*I site at position 2262.

SerDel7

Amino acids 611-647 were deleted by placing an artificial *Xba*I site at position 2373 using primer 5′-GCGAGACGGATCTAGACGAGTGCG-CCACTTCCC-3′ and fusing that with the *Xba*I site at position 2262.

Ser^{sec} and Ser^{sec}Del6

The N-terminus through the first *Bam*HI site (position 3496) of the wild-type *Ser* cDNA was inserted into pUAST to produce a secreted, extracellular form of Ser encoding all 14 ELRs plus an additional 72 amino acids. Ser^{sec}Del6 was produced by swapping the 5′ coding region of SerDel6 with the wild-type 5′-end of Ser^{sec} at the *Xba*I site at position 2262.

Nterm6 and NTerm6Del4

The N-terminal encoding region of Ser was taken from the wild-type *Ser* cDNA and cut with *Eco*RI and *Xba*I at position 2262 near the end of ELR 6 and fused in-frame with a tomato tag generated with the N-terminal primer 5′-TGCCGAGAATCTAGATGACATGGTGAGCAAGGGCGAGGAGG-TC-3′ that contains a compatible 5′ *Xba*I site and a C-terminal primer 5′-TGATCGGAATTCTTACTTGTACAGCTCGTCCATGCC-3′ that has an in-frame stop codon followed by an *Eco*RI site. The NTerm6Del4 construct was similarly constructed using the N-terminal-encoding *Eco*RI to *Xba*I portion of the SerDel4 cDNA construct described above.

NIRtom

A *Bgl*II site was introduced into the N-terminal region of Ser after the signal peptide using the primer 5′-GGTATTTGAGATTTCTAAGATCTCC-AGCTCGAAGTTACC-3′. The N-terminal segment was fused with the beginning of ELR 4 at amino acid 391 by generating a second *Bgl*II site with primer 5′-TGCGAGATCGTGGAGCAGATCTGTGCCACCAG-GCCATGC-3′. The construct was terminated following ELR 6 with the same tomato tag and fusion point as in the Nterm6 construct.

Tomato-tagged Ser

A tomato tag (Shaner et al., 2004) was generated using the N-terminal primer 5′-GCTCCGGAAATGGTGAGCAAGGGCGAGGAG-3′ and the C-terminal 5′-TAATTCCGGAGCCTTGTACAGCTCGTCCATGCC-3′. The PCR fragment generated was cut with *Bsp*EI and inserted into the *Bsp*EI site of the *Ser* cDNA at position 4357. This tag was inserted into Ser wild type, SerDel3 and SerDel4-6.

Generation of wing disc clones

Lqf (Epsin)-deficient cell clones

Females of genotype *y w HsFLP1.22 TubP.Gal4 UAS.CD8-GFP/y w HsFLP1.22 TubP.Gal4 UAS.CD8-GFP; Tub.Gal80 FRT 2A/TM6B* (provided by Dr Gary Struhl, Columbia University, NY, USA) were crossed with either *UAS-Ser*/UAS-Ser*; FRT 2A/FRT 2A* males [the asterisk indicates either UAS-Ser (wild type) or UAS-SerDel6] to generate Ser-expressing clones in a wild-type background or to *UAS-Ser*/UAS-Ser*; FRT 2A lqf^{SO11027}/TM6B* males to generate similar clones in an *lqf*$^{-}$ background (Wang and Struhl, 2005). Progeny of these crosses were heat shocked 24-36 hours after egg laying at 37°C for 1 hour and then returned to 25°C until crawling third instar stage for dissection and immunohistochemistry.

Single-cell clones

By crossing *w^{1118}; UASmCD8GFP/UASmCD8GFP; Act5C >y+ >Gal4/Act5C >y+ >Gal4* females (Lee and Luo, 1999) to *HsFLP1.22/Y; UAS-Ser*/UAS-Ser** males [the asterisk indicates either UAS-Ser (wild type) or UAS-SerDel6], animals capable of expressing a UAS-Ser form were produced. To induce single-cell clones, animals were raised at 18°C and were heat shocked at 37°C for 1 hour during early third instar stage. Wing discs were dissected from these animals 12-20 hours after heat shock and processed.

Immunostaining

Wing discs were dissected in PBS, fixed in 4% paraformaldehyde and blocked in 3% normal goat serum, 0.2% saponin in PBS. Primary antibodies were mouse anti-Cut [Developmental Studies Hybridoma Bank (DSHB)] at 1:250 or mouse anti-Notch 9C6 (Fehon et al., 1990) at 1:500. Secondary antibody was Alexa-Fluor 546 goat anti-mouse IgG1 or Alexa-Fluor 488 goat anti-mouse IgG1 (Invitrogen) at 1:1000. GFP, EGFP and tomato expression were observed by intrinsic immunofluorescence. For the Ser cell surface staining assay, cells expressing Ser+Tomato or SerDel6+Tomato were fixed with 4% paraformaldehyde and incubated with anti-Ser antibody [gift of Kenneth Irvine (Rutgers, NY, USA) (Papayannopoulos et al., 1998)] in PBS. Cells were washed three times with PBS followed by staining with Alexa-Fluor 488 goat anti-rat IgG (Invitrogen) at 1:1000. Quantification of immunostaining was performed using ImageJ (NIH) software.

S2 aggregation assays

Cells stably expressing Notch+EGFP or Ser+Tomato were cultured in standard M3 medium (Gibco) supplemented with 10% fetal bovine serum and 100 µg/ml hygromycin (Invitrogen) (Fehon et al., 1990). Different concentrations (2.0, 0.2, 0.02 µg and no-plasmid control) of pMK33-Ser *Bsp* tom, pMK33-Del6 Ser *Bsp* tom or pUAST-Del3 Ser *Bsp* tom were transfected into Notch+EGFP cells using Effectene (Qiagen). To induce the expression of Del3 Ser *Bsp* tom, pMT-Gal4 was co-transfected. Equal total amounts of DNA were transfected by adding empty vector. One day after transfection, plasmid expression was induced with 0.35 mM $CuSO_4$. Four hours after induction began, the cells were mixed with Ser+Tomato cells in equal numbers and allowed to aggregate overnight by rotating on a cell rocker. Aggregates were defined as clusters of four or more cells. For all values, at least 100 cell units (single cells or cell clusters) were scored. Transient transfection efficiencies were determined by isolation of total cells and detection of the intrinsic tomato tag present within the Ser molecules with rabbit anti-dsRED (1:1000 dilution; Clontech No. 632496). As a loading control for cell numbers, levels of β-tubulin were detected using mouse anti-β-tubulin E7 (1:20,000 dilution; DSHB).

Surface biotinylation assay

Cells stably expressing Ser+Tomato or SerDel6+Tomato were used. The surface biotinylation assay (Cell Surface Protein Isolation Kit, Pierce) was carried out as previously reported (Hsouna et al., 2010).

Sequence alignments

DNA sequences for *Ser* and *Delta* homologs were obtained through NCBI and were manually aligned using the CLC Sequence Viewer (CLC Bio). The sequences used were those encoding *Homo sapiens* jagged 1, NM_000214.2; *Xenopus laevis* Jagged 1, NM_001090307; *Gallus gallus*, serrate 1, X95283; *Danio rerio* Jagged 1a, NM_131861; and *Drosophila melanogaster* Delta, Y00222.

RESULTS

Targeted deletion analysis of Ser

To define inhibitory domains in the extracellular region of the Notch ligands we carried out a deletion analysis of the *Drosophila* Notch ligand Ser, which has 14 EGF-like repeats (ELRs) in its extracellular domain (Fig. 1). Transgenic fly lines carrying deletions for ELRs were generated and the constructs were expressed either via the *patched* Gal4 driver [*Gal4ptc* (Hinz et al., 1994)] at the anterior/posterior (AP) border of the wing or by a partial *Ser* gene promoter $Gal4^{Ser2}$ (Hukriede et al., 1997) driving expression along the marginal region in the dorsal domain of the wing disc. Using *Gal4ptc*, the influence of ectopic Ser expression on Notch activity was measured by examining the expression of Cut, a transcriptional target of Notch signals and a classic indicator of Notch activity in the wing disc (Doherty et al., 1996).

The result of expressing the wild-type *Ser* cDNA at the AP boundary (green) on Notch signaling (red) is shown in Fig. 2A-C. Cells expressing Ser do not express Cut, in spite of having endogenous Notch expression. This is an indication that, in these cells, Ser expression inhibits Notch receptor activation [cis-inhibition (de Celis and Bray, 1997; Micchelli et al., 1997)]. By contrast, Ser expression triggers Notch activity (transactivation) on the anterior and posterior sides of the *ptc* stripe in the ventral region of the disc, i.e. in cells expressing the receptor and apposed to cells expressing the wild-type ligand. Expression of the transgene carrying a deletion of ELR 7 (SerDel7; Fig. 2P-R) behaves indistinguishably from wild type, demonstrating that ELR 7 is required neither for transactivation nor for cis-inhibition of Notch in this assay. A transgene lacking ELR 6 (SerDel6; Fig. 2M-O) driven by *Gal4ptc* transactivates Notch comparably to expression of wild-type Ser (Fig. 2O, arrows). However, cis-inhibition of Notch is nearly eliminated (Cut is expressed within the Ser-expressing stripe; Fig. 2N,O). Thus, ELR 6 appears to be necessary for cis-inhibition of Notch but not for transactivation.

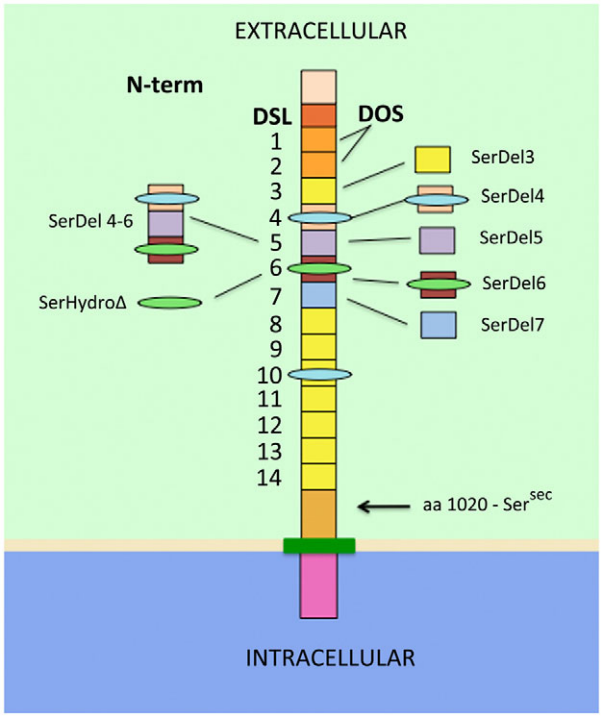

Fig. 1. Ser protein structure and deletion constructs. The predicted structure of the *Drosophila* Ser protein is shown. The extracellular N-terminal region includes N-terminal sequences that are conserved among Notch ligands, (N-term), the DSL (Delta, Ser and Lag-2) domain and the 14 ELRs. ELRs 1 and 2 constitute the DOS (Delta and OSM-11) domain (Komatsu et al., 2008); ELRs 4, 6 and 10 of Ser are interrupted with non-EGF-like sequences (ovals) (Fleming et al., 1990; Thomas et al., 1991). The transmembrane segment is depicted in dark green and the intracellular region is shown in pink. Regions of Ser deleted in the individual constructs are shown alongside. The approximate location of the terminal amino acid for Ser^{sec} is indicated by an arrow at amino acid 1020 [amino acid numbering as in Fleming et al. (Fleming et al., 1990)].

Within ELR 6 of *Drosophila* Ser there is a non-EGF-like stretch of hydrophobic amino acids that is not conserved in Ser-like molecules outside of insects (Fleming et al., 1990; Thomas et al., 1991). To test whether this hydrophobic region is required for cis-inhibition by Ser, we removed only that sequence and generated the SerhydroΔ6 transgene. When SerhydroΔ6 is expressed by *Gal4ptc* (Fig. 2S-U), transactivation is observed adjacent to the *ptc* stripe but Cut activity is not observed within the *ptc* expression stripe. Thus, SerhydroΔ6 still retains both Notch transactivation and cis-inhibition roles. To further define the cis-inhibition region of Ser, we deleted ELR 5 (SerDel5) or ELR 4 (SerDel4) and expressed these transgenes via *Gal4ptc*. In both cases, Notch transactivation occurs as with wild-type Ser expression but, as with the ELR 6 deletion, we see almost no evidence of cis-inhibition (Fig. 2G-L). Evidence for weak Notch inhibition is occasionally observed in regions away from the margin along the posterior *Gal4ptc* expression stripe (see Fig. 2K in the ventral posterior regions of SerDel5 expression). This might indicate the presence of residual cis-inhibition by the constructs, but the effects are only evident where Notch activation is reduced in regions away from the dorsal/ventral margin (Irvine and Vogt, 1997). This suggests that ligand-induced Notch inhibition

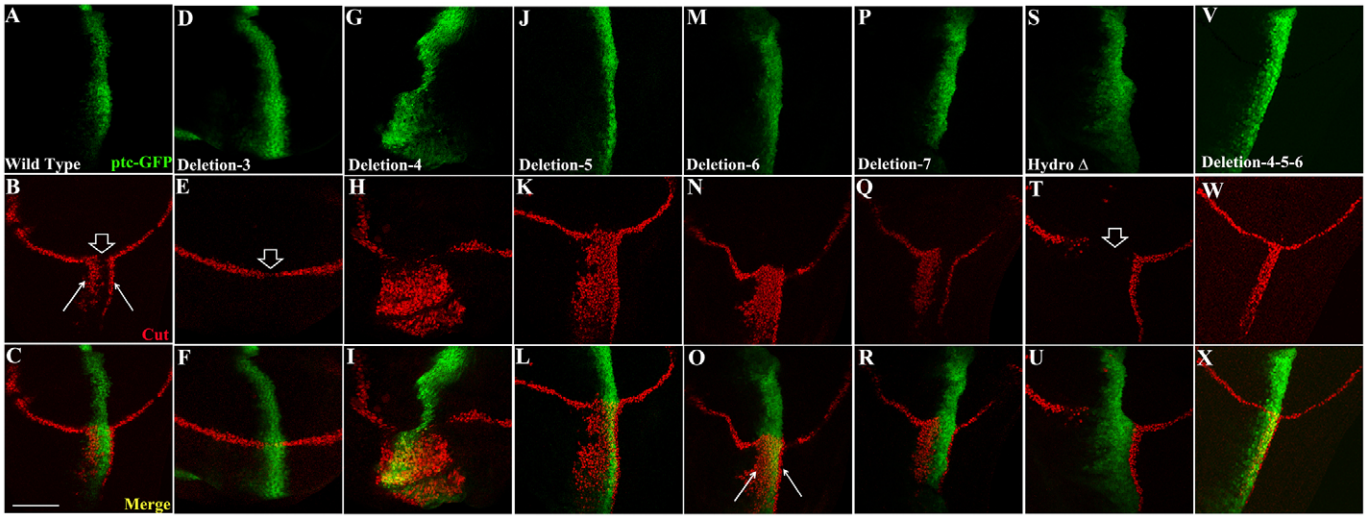

Fig. 2. Ectopic expression of Ser deletion constructs via *Gal4ptc*. Ser constructs were expressed in the third instar imaginal wing disc via *Gal4ptc*. Notch activity was assessed by examination of Cut expression induced in response to Notch activity (Neumann and Cohen, 1996). For all discs, dorsal is up and anterior is left. Each column demonstrates the expression of a single construct: the top panel (green) depicts the cells expressing the construct; the middle panel (red) shows cells expressing Cut; and the lower panel is a merge. (**A-C**) Expression of wild-type Ser induces Notch activity adjacent to the expression stripe (arrows in B) as well as cis-inhibition within the expression stripe (arrowhead in B). (**D-F**) SerDel3 fails to activate Notch adjacent to the expression stripe yet demonstrates weak cis-inhibition (arrowhead in E) when expressed across the dorsal/ventral boundary. An inserted red fluorescent tomato tag (Shaner et al., 2004) in the intracellular domain of SerDel3tom placed at an identical position to a tomato tag inserted into the wild-type Ser construct demonstrates that the construct is expressed (see Materials and methods; not shown). (**G-O**) SerDel4 (G-I) behaves similarly to SerDel5 (J-L) and SerDel6 (M-O). Expression of SerDel6 activates Notch similarly to wild type (arrows in O) but fails to inhibit Notch within the expression stripe. (**P-R**) Expression of SerDel7 provides Ser activity indistinguishable from wild type. (**S-U**) Removal of only the hydrophobic insertion sequences within ELR 6 of Ser (see Fig. 1) produces inhibition (arrowhead in T) and activation along the posterior border of expression. (**V-X**) Expression of a construct that simultaneously removes ELRs 4, 5 and 6 generates similar results to the individual removal of these repeats (see G-O). Images show representative expression levels for each construct. Variations in Notch transactivation levels are comparable for all constructs with the exception of SerhydroΔ6 and SerDel3 as noted in the text. Scale bar: 100 μm.

might occur by more than one mechanism (see Discussion). Similar results were obtained when SerDel4-6, which deletes ELRs 4-6 simultaneously, was expressed via *Gal4ptc* (Fig. 2V-X) suggesting that these ELRs might function as a unit.

Notch transactivation was not observed when we deleted only ELR 3 (SerDel3tom) and drove its expression with *Gal4ptc* (Fig. 2D-F). Expression of SerDel3tom by *Gal4ptc*, however, does show reduced, variable Notch cis-inhibition (Fig. 2E). These results suggest that ELR 3 is necessary for Notch transactivation yet might not be involved in cis-inhibition.

Weakly expressed forms of wild-type Ser can lead to irregular activation of Cut within the *ptc* expression domain that appears superficially similar to that seen in Fig. 2N (Fleming et al., 1997). We therefore examined expression of our deleted Ser forms using the Gal4^{Ser2} promoter. Expression of either wild-type Ser or SerDel7 via the Gal4^{Ser2} promoter (Fig. 3A) produces a notched margin phenotype and wing vein deltas in adult wings (Fig. 3C,D). These phenotypes are characteristic of the intrinsic Notch-inhibiting dominant-negative effect that has been associated with excessive expression of wild-type Ser, as seen in the *Ser^{D}* mutant allele or by wild-type Ser transgene misexpression (Klein et al., 1997; Thomas et al., 1995). Similarly, expression by Gal4^{Ser2} of SerhydroΔ6, which retains the cis-inhibition property (see above), causes wing margin loss similar to that seen by wild-type Ser (Fig. 3E). Thus, the hydrophobic amino acids within ELR 6 are not required for cis-inhibition. By contrast, when SerDel6, SerDel5, SelDel4 or SerDel4-6 is expressed via Gal4^{Ser2}, no notching of the wing margins or wing vein deltas is observed; however, severe wing vein loss is observed within the expression domain of this promoter (Fig. 3F-I). Therefore, Gal4^{Ser2} expression of these constructs generates a novel phenotype distinct from that associated with expression of wild-type Ser forms. Given that only cis-inhibition is affected by deletions of ELRs 4 through 6, we presume that this wing vein loss phenotype is due to abnormal activation of Notch within the vein-forming territories, leading to the specification of intervein cell fates (Huppert et al., 1997). Finally, expression of SerDel3tom by Gal4^{Ser2} demonstrates wing margin loss and delta formation of wing veins comparable to wild-type Ser expression in this pattern (Fig. 3J), supporting our conclusion that SerDel3tom retains the ability to inhibit Notch in cis.

It has been demonstrated that cis-inhibition occurs at the cell surface and that mutations within ligands that affect their cellular distribution and decrease their surface accumulation can reduce cis-inhibition (Glittenberg et al., 2006). We examined the localization and accumulation of the SerDel6 or SerDel4-6 proteins relative to wild-type Ser. Careful examination of confocal images revealed no differences in the cellular localization or expression levels of wild-type Ser and these transgenic deletion constructs (see supplementary material Fig. S1). Additionally, the cellular localization and accumulation of SerDel6 was indistinguishable from that of wild-type Ser in S2 tissue culture cells (supplementary material Fig. S2). We conclude that these extracellular deletion forms of Ser are not significantly altered in cellular trafficking and ligand distribution.

The above analyses limit the cis-inhibitory region of Ser to ELRs 4 through 6, which we have thus termed the Notch inhibitory region

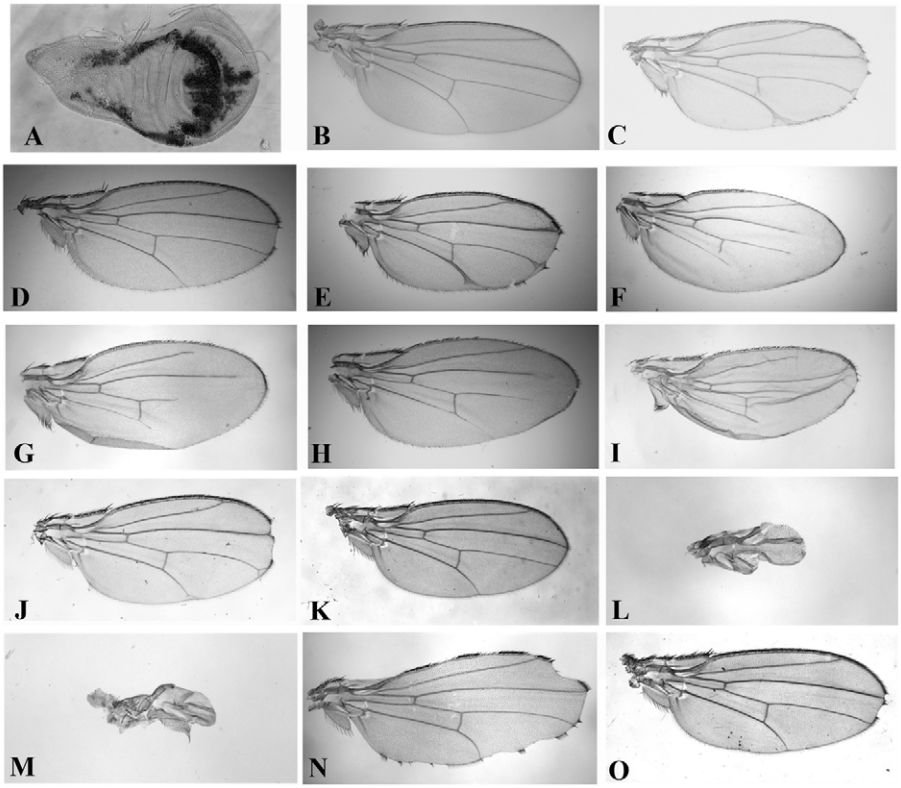

Fig. 3. Ectopic expression of Ser deletion constructs via Gal4^{Ser2}. Ser constructs were expressed in the wing via Gal4^{Ser2} and assayed in the adult. (**A**) Expression of Gal4^{Ser2} in the late third instar imaginal wing disc (dorsal to left and posterior to bottom) is mostly in the dorsal compartment along the dorsal/ventral boundary in the same pattern as endogenous Ser expression. (**B**) Wild-type wing. (**C**) Ectopic expression of wild-type Ser produces wings with serrated margins and delta-like wing veins. (**D**) SerDel7 expression demonstrates wing nicks similar to wild-type Ser expression. (**E**) Effect of SerhydroΔ6 expression appears similar to that of wild-type Ser, with wing margin nicks and delta-like wing veins. (**F**) SerDel6 expression generates normal wing margins but incomplete wing venation. (**G-I**) SerDel5 (G), SerDel4 (H) and SerDel4-6 (I) expression produce wings indistinguishable from those produced by SerDel6 expression (see F). (**J**) Expression of SerDel3 produces wing nicking and vein deltas similar to wild-type Ser expression. (**K**) Expression of NIRtom does not alter the wild-type wing morphology. (**L**) Expression of Ser^{sec} demonstrates its dominant-negative effect in reduced wing size and exaggerated wing venation (image at same magnification as in B). (**M**) Expression of dominant-negative Nterm6tom generates wings indistinguishable from those resulting from expression of Ser^{sec}. (**N**,**O**) Removal of ELR 6 from Ser^{sec} (Ser^{sec}Del6; N) or removal of ELR 4 from Nterm6tom, (Nterm6Del4; O) greatly reduces the dominant-negative effect of these constructs.

(NIR). To address whether the NIR is sufficient for the cis-inhibitory function, we expressed a construct (NIRtom) that expresses only ELRs 4, 5 and 6 followed by an in-frame tomato tag (Shaner et al., 2004) to follow expression efficiency. This construct produces a soluble protein that is abundant and secreted from cells (data not shown). Expression of NIRtom by the Gal4^{Ser2} promoter fails to generate any detectable phenotype in the adult wing (Fig. 3K), even though expression levels are robust (not shown). These results demonstrate that the NIR is necessary but not sufficient for Notch inhibition.

Given that Notch inhibition by its ligands is likely to be a general property and has been documented for both Ser and Delta ligands across species (Dorsky et al., 1997; Ladi et al., 2005; Lowell and Watt, 2001), we were interested in comparing the primary sequences around the NIR in the various ligands. Homology searches (supplementary material Fig. S3) show that the sequences involved in cis-inhibition are well conserved within the Ser ligand family but not between Ser and Delta. We constructed pairwise alignments for each of the sequences versus Ser ELRs 4-6 using bl2seq (NCBI), then merged them manually to create the multiple alignments shown. Pairwise alignment scores against Ser for the individual sequences are shown in supplementary material Table S1.

Notch activation by NIR-deficient Ser depends on endocytosis

Endocytosis plays a crucial role within the signal-sending cell as it may be essential for the recycling of ligands on the surface after they have presumably been modified to generate a ligand form that is competent of interacting with and activating the receptor (Wang and Struhl, 2004). A crucial element of the endocytic processes that allows ligand-induced Notch transactivation relies on the activity of Epsin [Liquid facets (Lqf) in *Drosophila*] (Overstreet et al., 2004; Wang and Struhl, 2004). Given that NIR-deleted Ser forms transactivate but do not cis-inhibit Notch, we probed the potential role that the NIR plays in the *lqf*-dependent events that generate active, receptor-competent ligands.

When wild-type Ser is expressed in a normal *lqf*$^{+}$ background, Notch is activated in the cells surrounding the clone but not within the clone, demonstrating both transactivation and cis-inhibition by the wild-type form (Fig. 4A-C). By contrast, when wild-type Ser is expressed in an *lqf*-deficient background, Notch activation is not observed around the clone (Fig. 4D-F). Lqf is thus, as expected, required in the ligand-expressing cell to activate Notch in the apposing cell (Overstreet et al., 2004; Wang and Struhl, 2004).

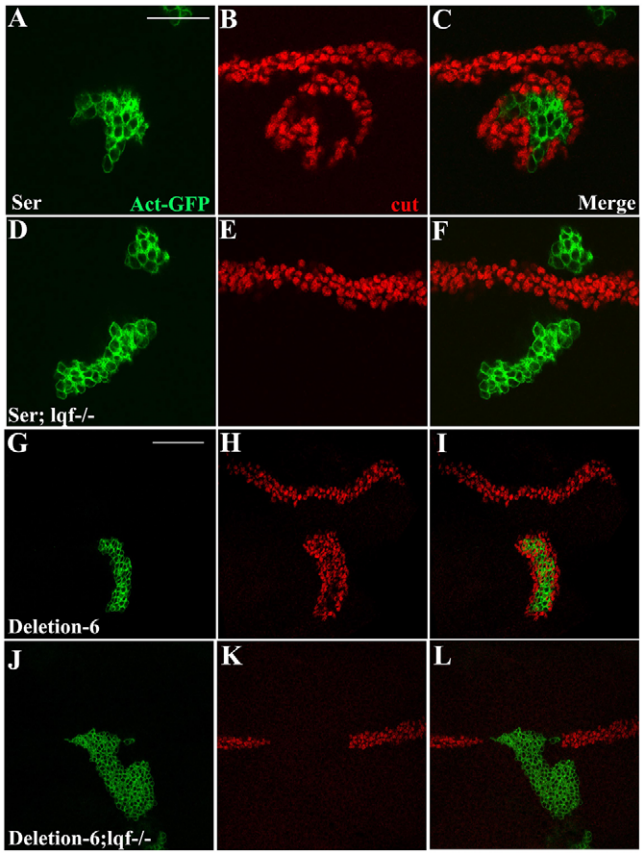

Fig. 4. Requirement for endocytosis in Ser-induced Notch activity. Clones were ectopically expressed with either wild-type Ser (A-F) or SerDel6 (G-L) in third instar imaginal discs and the requirements for *lqf* in mediating the signal transduction capacity of these forms was examined. In each row, the left panel shows cells expressing Ser or SerDel6; the middle panel shows Notch activation (Cut expression); and the right panel shows the merged images. (**A-C**) Wild-type Ser expression in a wild-type genetic background shows activation of Notch in cells adjacent to the clone (transactivation) but not within the clone (cis-inhibition). (**D-F**) In the absence of *lqf*, Notch is not activated within or around clones expressing wild-type Ser. (**G-I**) When SerDel6 is expressed in clones in a wild-type genetic background, Notch is activated around the clone (transactivation) and within the clone (loss of cis-inhibition). (**J-L**) When this same construct is expressed in a *lqf*$^{-}$ background, Notch is not activated around or within the clone. A-F were taken at a slightly higher magnification than G-L. Scale bars: 10 μm.

When analogous clones of NIR-deficient SerDel6 are expressed in an *lqf*$^{+}$ background, Notch is activated both within and around the clone, consistent with retention of transactivation and loss of cis-inhibition for Notch by this form (Fig. 4G-I). Cells expressing SerDel6 in the *lqf*-deficient background can no longer activate Notch within the clone or in cells surrounding the clone (Fig. 4J-L). Thus, the ability of the mutant ligand SerDel6, which lacks the NIR, to transactivate Notch still depends on the functional presence of Lqf.

Dominant-negative Ser forms

Membrane-bound or secreted mutant forms of Notch ligands that lack an intracellular (IC) domain have been shown to inhibit Notch signaling activity, although the mechanism(s) of such negative activity are not understood (Hukriede and Fleming, 1997; Mishra-Gorur et al., 2002; Parks et al., 2000; Qi et al., 1999; Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997). Given that the NIR is retained in all known dominant-negative forms of Ser, we sought to determine whether this region is also responsible for the dominant-negative effects of Ser on Notch. We generated a Ser form, termed Nterm6tom, that expresses the N-terminal sequences of Ser through the end of ELR 6 followed by an in-frame red fluorescent tomato tag to allow us to track expression of the construct (Shaner et al., 2004). As a control, we used a secreted, untagged, dominant-negative molecule called Ser^{sec} that is slightly longer than the known dominant-negative Bd^{G} form [Ser^{sec} includes the N-terminus through amino acid 1020 of Ser and contains all 14 ELRs plus an additional 272 amino acids (Hukriede et al., 1997; Hukriede and Fleming, 1997)]. The results of expression of these Ser forms by the Gal4^{Ser2} promoter are shown in Fig. 3L-O.

Expression of Ser^{sec} via the Gal4^{Ser2} driver (Fig. 3L) elicits a rudimentary wing, characteristic of dominant-negative ligand forms expressed in this pattern. Indistinguishable wing loss phenotypes are seen with similarly expressed NTerm6tom (Fig. 3M). This indicates that the dominant-negative properties of this Ser form reside within the first six ELRs, an area encompassing the NIR. Given that ELRs 4 and 6 are necessary for cis-inhibition, we refined the analysis by deleting either ELR 6 (Ser^{sec}Del6) or ELR 4 (Nterm6Del4) of these secreted Ser molecules. Expression of these forms by Gal4^{Ser2} revealed that the loss of ELR 6 in Ser^{sec}Del6 and of ELR 4 in Nterm6Del4 greatly reduces the dominant-negative attributes of these molecules (Fig. 3N,O). These findings demonstrate that the NIR is crucial for the dominant-negative qualities of secreted Ser forms.

Cis- and trans-interactions between Notch and Ser

Triggering the activation of the Notch receptor on the cell surface might depend on competition between cis- and trans-interactions between Notch and its ligand. We investigated this competition by examining interactions between Notch-expressing and Ser-expressing S2 tissue culture cells. Cells expressing Notch will physically bind and aggregate with cells expressing Ser or Delta (Fehon et al., 1990; Rebay et al., 1991). These interactions can be seen between adjacent cells (in trans) as judged by cell aggregation and by the colocalization of both molecules when expressed on the same cell (in cis). We reasoned that if the mechanism of Notch cis-inhibition results from competition between ligand and receptor in cis versus in trans, then interactions between Notch-expressing cells and Ser-expressing cells should be reduced or eliminated by co-expressing Ser in the cells that simultaneously express Notch.

S2 cells stably expressing tomato-tagged Ser (Sertom) were mixed with S2 cells stably expressing EGFP-labeled Notch and allowed to aggregate with one another to determine baseline levels of Notch-Ser interactions (Fig. 5). Subsequent experiments transiently transfected the stable Notch EGFP line with either Sertom (wild type), SerDel6tom or SerDel3tom transgenes at varying concentrations to promote cis-inhibition within the Notch-expressing cells. Increasing levels of co-transfected wild-type Sertom DNA in the Notch-expressing cells effectively reduces cellular aggregation when these cells are mixed with Ser-expressing cells (Fig. 5A,A′). This suggests that cis-expression of Ser on Notch cells generates competition for Notch binding in trans, effectively reducing aggregation.

When SerDel6, a mutant that has little or no ability to cis-inhibit Notch in our wing expression assays (Figs 2, 3), is expressed in cis with Notch in S2 cells, aggregation is not inhibited (Fig. 5B,B′).

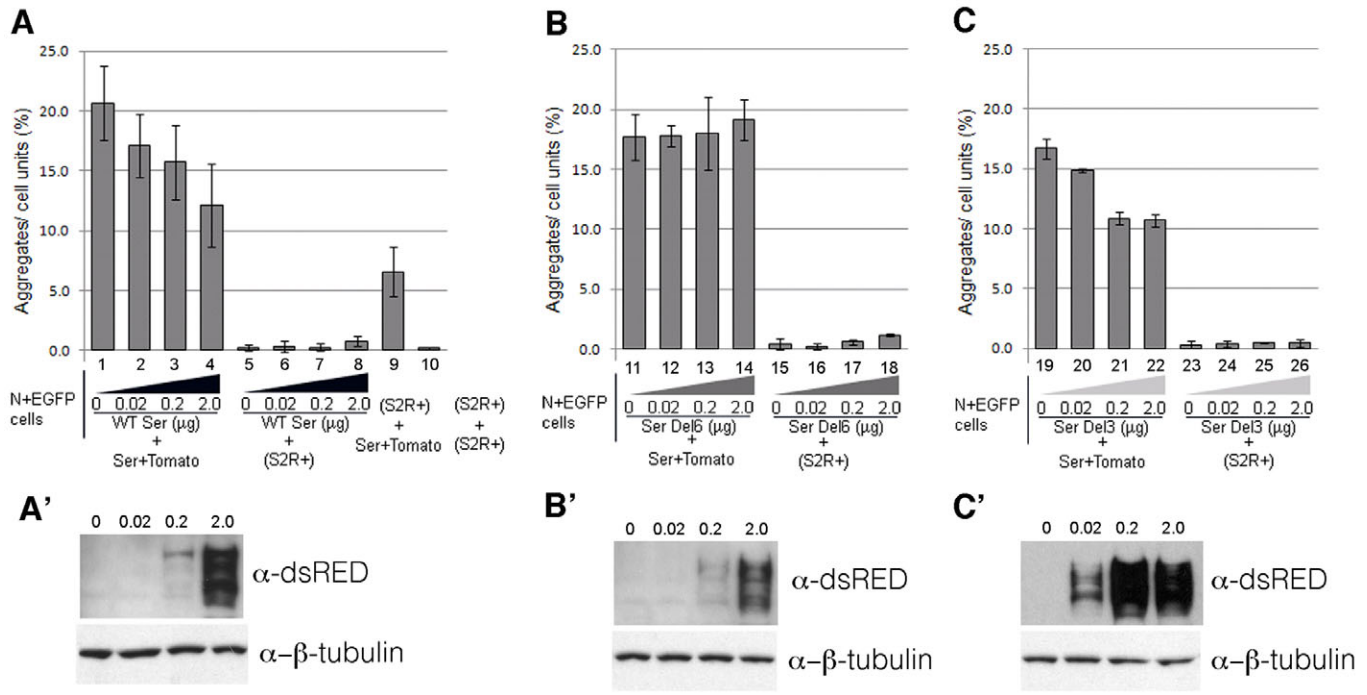

Fig. 5. Binding competition between cis- and trans-expressed forms of Ser in *Drosophila* S2 cell aggregation. (**A-C**) Stable lines of EGFP-labeled Notch cells were added to stable lines of tomato tagged Ser-expressing cells, allowed to mix for 24 hours at room temperature and then assayed for the formation of interacting cellular aggregates (Fehon et al., 1990). The Notch-expressing cells were transiently transfected with 0.0 to 2.0 μg tomato-tagged *Ser* DNA prior to mixing with Ser-expressing cells to induce cis-inhibition by co-expression of Ser with Notch. When performed with wild-type *Ser* DNA (A), effective inhibition of aggregation occurs in a dose-dependent manner when the Ser-expressing cells are mixed with the transfected Notch-expressing cells. When Ser-expressing cells are not mixed with the transfected Notch-expressing cells (S2R+), self-aggregate formation is minimal (the aggregates contain substantially fewer cells than Ser-Notch aggregates; A, compare bars 9 and 1). When SerDel6 is used for this assay (B), aggregation is not significantly inhibited by cis-expression. When the SerDel3 form that does not activate Notch is used in this assay (C), efficient inhibition of aggregation is observed between these cell types. (**A′-C′**) Expression levels of wild-type Ser (A′), SerDel6 (B′) and SerDel3 (C′) in transient transfections demonstrated using anti-dsRED antibody, which detects the tomato tag of the expressed Ser molecules. Staining with anti-β-tubulin antibody (beneath) demonstrates that consistent total cell numbers were loaded in each assay. Error bars indicate s.d. of triplicate experiments.

Thus, this altered Ser form lacks the ability to compete in cis for Notch binding. To further examine this phenomenon, we repeated the experiment but used the SerDel3tom construct that fails to transactivate Notch but demonstrates the ability to cis-inhibit Notch *in vivo* (Fig. 2D-F). When SerDel3tom was transfected into the Notch-expressing cells cellular aggregation was significantly reduced (Fig. 5C,C′), indicating that it can effectively compete in cis for Notch binding and demonstrating that the ability to transactivate Notch can be separated from the ability to bind with Notch in trans. These observations also confirm that SerDel3 retains the functional NIR of Ser.

Transactivation versus auto-activation of Notch by SerDel6

Because both wild-type Ser and SerDel6 appear capable of binding with and activating Notch, yet the SerDel6 form fails to cis-inhibit Notch, we examined the cis interaction of Notch and Ser further. It is reasonable to presume that the balance between cis and trans Notch-Ser interactions in cells expressing both ligand and the receptor determines the directionality of the signal. We used our constructs to probe whether a ligand can activate the receptor on the same cell. Although our studies show that SerDel6 fails to cis-inhibit Notch, thereby allowing activation of Notch within the ligand-expressing stripe (Fig. 2M-O), there are always multiple adjacent cells expressing ligand in these assays. Therefore, we cannot determine whether activation of Notch within the ligand stripe is due to transactivation from one cell onto another in the absence of cis-inhibition or whether it results from activation of Notch by ligand expressed on the same cell surface (auto-activation).

To determine if auto-activation of Notch is possible in this system, we generated single-cell clones in the wing disc capable of expressing wild-type Ser or SerDel6 in an otherwise wild-type background. As expected, a single cell expressing wild-type Ser can only transactivate Notch on adjacent cells, while keeping Notch inactive on its own cell surface (Fig. 6A-C). Thus, wild-type Ser is incapable of auto-activating Notch when cis-inhibition is active in the ligand. When the non-inhibiting SerDel6 form is expressed in single-cell clones, the effects are comparable to wild-type Ser expression (Fig. 6D-F). This demonstrates that Notch is not activated on the surface of a single cell expressing SerDel6. We conclude that a Ser molecule, whether it has an intact NIR or not, does not activate the Notch receptor when expressed in the same cell.

DISCUSSION

In spite of the complexity of the genetic circuitry controlling Notch receptor activity, the developmental logic of the pathway is constant in linking, as a rule, the fate of one cell to that of its neighbor. As two cells communicate utilizing Notch signals, it is often – indeed

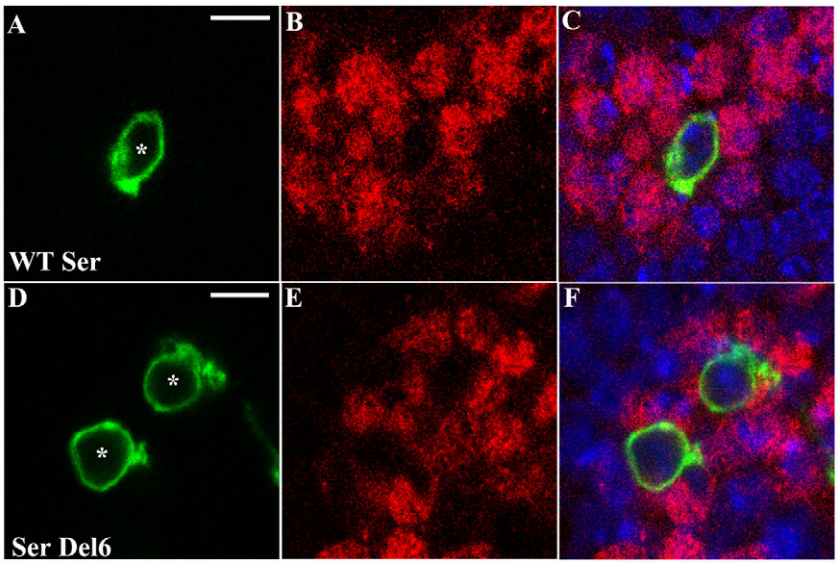

Fig. 6. Single-cell clones expressing Ser and SerDel6 do not auto-activate Notch. Cell clones were generated in third instar wing discs that were dissected and processed 12-16 hours after induction to induce single cells that express Ser constructs. Notch activity was monitored by Cut expression localized to the nucleus (Blochlinger et al., 1988). All clones shown are located on the ventral side and within ten cell diameters of the margin. (**A**,**D**) Single cells expressing either wild-type Ser (A) or SerDel6 (D) (green). (**B**,**E**) Cells expressing Cut (red) to monitor Notch activation. (**C**,**F**) Merged images that include DAPI staining (blue) to indicate locations of nuclei. Neither wild-type (A-C) nor SerDel6 (D-F) expression shows activation of Notch (Cut localization in the nucleus) within Ser-expressing cells. Asterisks indicate approximate location of cell nuclei. Scale bars: 10 μm.

usually – the case that each cell initially expresses both the receptor and a ligand simultaneously. Therefore, what defines whether a cell becomes the receiving versus the signaling partner is pivotal. Elegant genetic data first postulated the possible functional significance of cis interactions and also indicated that it is the ratio between receptor and ligand expressed on a given cell that guides its developmental fate (Heitzler and Simpson, 1991; Heitzler and Simpson, 1993). Early molecular indications (Fehon et al., 1990) pointed to trans as well as cis interactions between Notch and its ligands on the cell surface, but the significance of the cis interactions and their relation to the trans mode of interactions remained unclear. Only recently, through the development of imaging approaches, have we started to gain insight into how the receptor and ligand interact on the cell surface (Sprinzak et al., 2010) (reviewed by D'Souza et al., 2010).

The importance of cis versus trans interactions and how these relationships serve the developmental logic of the Notch signaling pathway has been the central theme of the current study. Existing data most strongly implicate the ligand Ser in cis-interactions (Klein et al., 1997; Li and Baker, 2004). Our findings have allowed us to define a region in the extracellular domain of Ser that is essential for cis-inhibitory signaling interaction with the Notch receptor. We show that cis interactions clearly inhibit the ability of the Notch receptor on the same cell to signal and that this property is dependent on the presence of the NIR that encompasses Ser ELRs 4, 5 and 6. Importantly, we find that the capacity of the ligand to inhibit in cis can be separated from its ability to activate in trans. Our results support the model in which the interplay between cis and trans interactions between the ligand and the receptor on the same cell determines whether this cell will be capable of receiving or transmitting the Notch signal.

Definition of the NIR

Our results indicate a noteworthy modularity involving the ELRs in the extracellular domain of Ser. It is the ensemble of ELRs 4, 5 and 6 that is required for cis-inhibition of Notch, yet removal of any of the individual repeats ELR 4 through 7 has no discernible effect on Notch transactivation in our assays. This outcome does not strictly hold true for the SerhydroΔ6 construct. This construct, which removes only the hydrophobic, non-EGF-like sequences within ELR 6, cis-inhibits Notch but appears to have reduced Notch transactivation capabilities as judged by the absence of anterior border staining when expressed via *Gal4ptc*. This reduction in Notch activation is not observed when the entirety of ELR 6 is deleted. We believe, therefore, that the reduction in Notch activation by SerhydroΔ6 is unlikely to be physiologically significant.

Although the cis-inhibitory properties of Ser seem to be determined by the NIR, we must conclude that sequences mapping N-terminal to the NIR also contribute to some degree. The first two ELRs of Ser constitute the DOS domain, a region known to be necessary for Notch activation in other systems (Komatsu et al., 2008). Additionally, the N-terminal portion of human jagged 1, which includes the DOS domain plus the third ELR of that ligand, has been implicated in both cis- and trans-interactions with Notch (Cordle et al., 2008). Furthermore, the requirement for the DSL domain in both transactivation and cis-inhibition has been clearly demonstrated in the *Drosophila* wing (Glittenberg et al., 2006). ELR 3 is clearly required for Notch transactivation *in vivo* and behaves accordingly in the cell aggregation assays. The lack of inhibition with the NIRtom construct demonstrates a requirement for at least some of these domains in addition to the NIR for Notch inhibition. Given that the loss of any ELR N-terminal to the NIR also affects Notch transactivation, assessing the contribution to cis-inhibition becomes increasingly cumbersome based on the assays that we use.

We find that NIR-deficient forms of Ser, as with the wild-type counterparts that are capable of transactivation, still require endocytosis to activate Notch on the adjacent cell. Thus, if the endocytosis-dependent step necessary to produce a ligand form that can activate Notch modifies the ligand in some key region (Wang and Struhl, 2004), this modification must reside outside of the NIR. Moreover, signal directionality from Ser does not depend on the NIR. Individual cells expressing either wild-type or NIR-deleted Ser forms remain incapable of activating Notch on their own cell surfaces. We therefore presume that cis-inhibition does not reflect a need for signal-sending cells to block auto-activation. It is likely that the requirement for endocytosis of ligand in signal-sending cells accounts for the directionality of the Notch signal.

Analysis of the Ser secreted forms implicates the NIR in the dominant-negative effects of these molecules. We cannot however distinguish whether the inhibitory properties of secreted dominant-negative ligand forms that lack the IC and transmembrane domains are limited to cis-interactions with Notch. Since they are secreted,

it might be that these forms can interact with Notch on neighboring cells (in trans) as well as on their own cells (in cis) to produce Notch inhibition. Although the site of action of these secreted forms is unknown, we clearly attribute the dominant-negative properties of these mutant forms to the same ELR region as cis-inhibition.

Cis-inhibition and cell aggregation

We extended the *in vivo* analyses by utilizing the classic Notch-ligand aggregation assays (Fehon et al., 1990; Rebay et al., 1991) to examine whether ligand-induced cis-inhibition interferes with cellular aggregation. The fact that wild-type *Ser* DNA, when transfected into a Notch-expressing cell, inhibits the ability of this cell to aggregate with ligand-expressing cells demonstrates that cis-expression of Ser on the Notch cell effectively competes with Notch-Ser interactions in trans. By contrast, the cis-inhibition-deficient SerDel6 form fails to inhibit aggregation of Ser- and Notch-expressing cells, clearly indicating that the loss of cis-inhibition results from a failure of interaction between Ser and Notch on the same cell. Importantly, as the distribution of SerDel6 appears indistinguishable from that of wild-type Ser, the NIR is unlikely to be involved in the cellular distribution of the protein but rather is more likely involved in Notch interactions. The NIR does not appear to extend N-terminal to ELR 4 since, in this assay, SerDel3, which fails to activate Notch in trans but retains its cis-inhibition properties, can inhibit aggregation and hence appears to retain the ability to compete for Notch interaction in cis.

The aggregation assays therefore support the notion that the activation and inhibition roles of Ser are separable properties of the ligand. They also raise the possibility that two levels of Notch-ligand interaction exist. Aggregation is likely to depend on the most N-terminal regions of the ligands, including the DSL and perhaps DOS domains (Cordle et al., 2008; Glittenberg et al., 2006; Komatsu et al., 2008). The possibility that both cis- and trans-interactions might be mediated by the N-terminal regions of the ligand in either of two orientations (Cordle et al., 2008; Glittenberg et al., 2006) could explain the cis/trans competition for binding observed in these assays. The first level of Notch-ligand interaction might simply mediate the association of ligand and receptor in either the cis or trans orientation. This interaction would be independent of either Notch activation (as demonstrated by SerDel3 competition) or Notch inhibition (as demonstrated by SerDel6 competition).

The second level of Notch-ligand interaction would entail association of these ligands and receptors following binding. When associated in the trans-conformation, the alignment of ligand and Notch leads to dynamic Notch activation (requiring at least ELR 3 of Ser). By contrast, when associated in the cis-conformation, the association of these molecules appears to generate a purposeful Notch inhibition that requires the NIR. This dual-type interaction could explain the residual inhibition associated with secreted forms of Ser that lack NIR sequences (Ser^{sec}Del6 and Nterm6Del4). Clearly, these secreted forms cannot activate Notch, yet they must retain the ability to bind with Notch in order to produce their inhibitory effects. Both the Ser^{sec}Del6 and Nterm6Del4 constructs retain some degree of negative interaction with Notch even though parts of the NIR (ELR 6 and ELR 4, respectively) have been removed. It is likely that the remaining inhibitory activity associated with these molecules results from simple competition for Notch binding between these non-activating (and non-inhibiting) forms and the endogenously expressed Notch ligands.

NIR conservation in Ser but not in Delta homologs

Cross-species comparisons showed that ELRs 4 through 6 of Ser are conserved, consistent with the fact that the vertebrate ligands have been shown to cis-inhibit Notch in a manner similar to that seen in *Drosophila* (Chitnis et al., 1995; Franklin et al., 1999; Itoh et al., 2003). Thus, we postulate that NIR function is also likely to be conserved. These sequence comparisons failed to identify a comparable region in the Delta ligands, even though Delta displays both Notch transactivation and cis-inhibition (Jacobsen et al., 1998; Klein et al., 1997). Whether this reflects that a different region of Delta is functionally equivalent to the Ser NIR or that the folded Delta protein has a structure that mimics the Ser NIR and adopts its function, remains to be determined. In either scenario, the non-conserved nature of the NIR between Ser and Delta ligands is not necessarily surprising. The majority of the Notch extracellular domain, including most of the ELRs, has not been functionally defined. Therefore, even if one assumes that the NIR directly contacts Notch in order to mediate inhibition, it remains

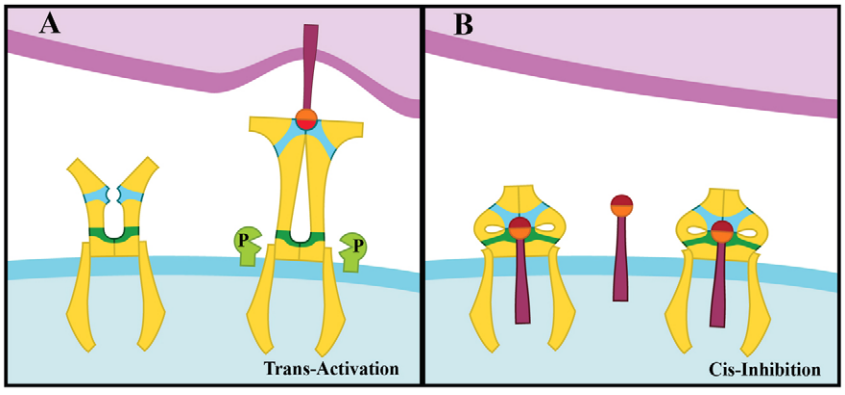

Fig. 7. Cis/trans interactions of Notch and Ser. Interactions between the Notch receptor (yellow) and the Ser ligand (purple) are illustrated. Notch is represented as a dimer, but could be a monomer or a multimer. An unoccupied receptor is illustrated to the left in A. The demonstrated binding region of Notch (ELRs 11 and 12) is illustrated in light blue and a region controlling negative regulation is depicted in green. (**A**) Transactivation. When Ser binds to Notch in trans, the NIR (orange) is not involved but the N-terminal region through ELR 3 (red) is used (Cordle et al., 2008; Glittenberg et al., 2006). Trans Notch-Ser engagement induces a critical signal leading to cleavage by metalloproteases (P) and Notch activation (reviewed by Kopan and Ilagan, 2009). (**B**) Cis-inhibition. When Ser and Notch are co-expressed on the same cell, Ser interacts with Notch using both the DSL and DOS regions that interact with Notch ELRs 11 and 12 (Cordle et al., 2008) and the NIR that might also interact with Notch. This interaction stabilizes the Notch dimer in an inactive state, leading to signal inhibition.

possible that negative regulation of Notch could involve interactions between Ser or Delta with different regions of the receptor. This hypothesis is supported by the finding that a specific ELR of Notch is used for Ser-Notch but not Delta-Notch interactions (Yamamoto et al., 2012).

Our findings allow us to propose a simple model that explains our observations and is consistent with the notion that the balance between cis and trans ligand interactions may ultimately provide directionality to the Notch signal. We interpret our findings to indicate differential interactions between Ser and Notch in cis and in trans (Fig. 7). When Ser is expressed in trans to Notch, the interaction does not require the NIR and results in activation. By contrast, given that the NIR requires the DSL and N-terminal regions to mediate cis-inhibition, we propose that the N-terminal DSL region of Ser interacts with Notch (depicted as a dimer, although it could be mono- or multimeric) in cis and that this interaction is stabilized by further interaction of the NIR with Notch. This stabilized cis-interaction is hypothesized to maintain Notch in an inactive state, recalcitrant to crucial signal-producing proteolytic cleavages. Further examination of Notch-Ser interactions will be necessary to determine whether the NIR does indeed directly contact Notch and to locate where that interaction occurs.

Acknowledgements
We thank the Bloomington stock center for *Drosophila* stocks used in this study. The 2B10 anti-Cut antibody and the anti-β-tubulin antibody were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. We acknowledge the Nikon Imaging Center at Harvard Medical School for assistance with imaging and thank Christopher M. Fleming for the illustration of Notch-Ser interactions.

Funding
Partial funding for this work by R.J.F., G.V.F. and A.C.M.-B. was provided by the National Science Foundation and the Faculty Research Council of Trinity College, Hartford, CT, USA; work performed in the S.A.-T. laboratory was supported by the National Institutes of Health. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.087916/-/DC1

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