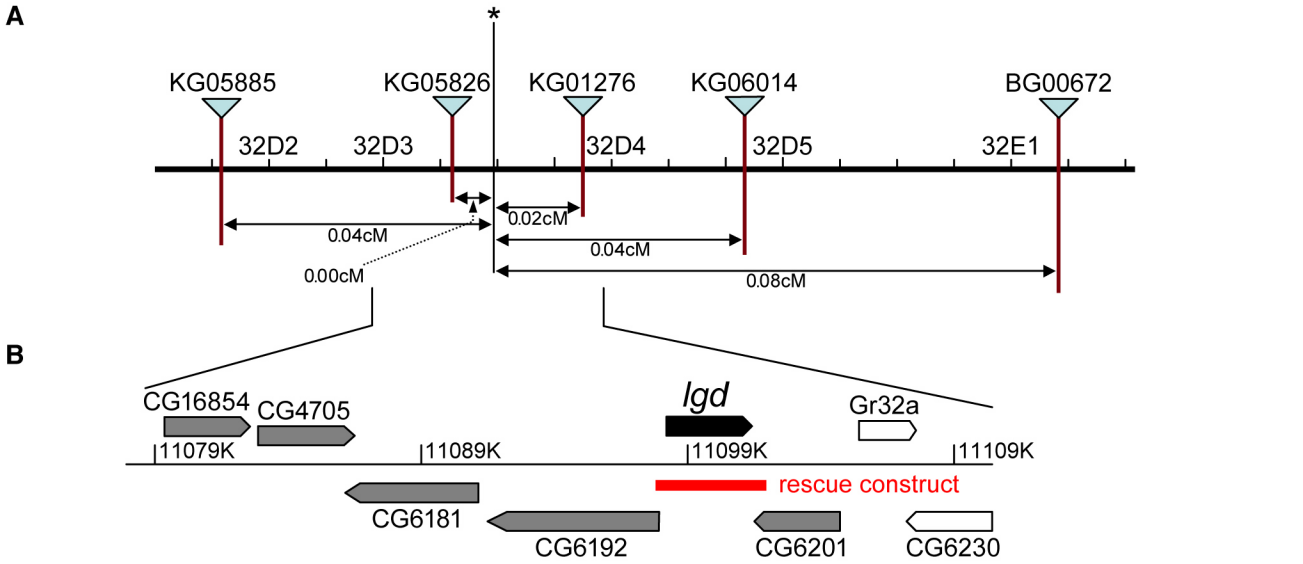

C

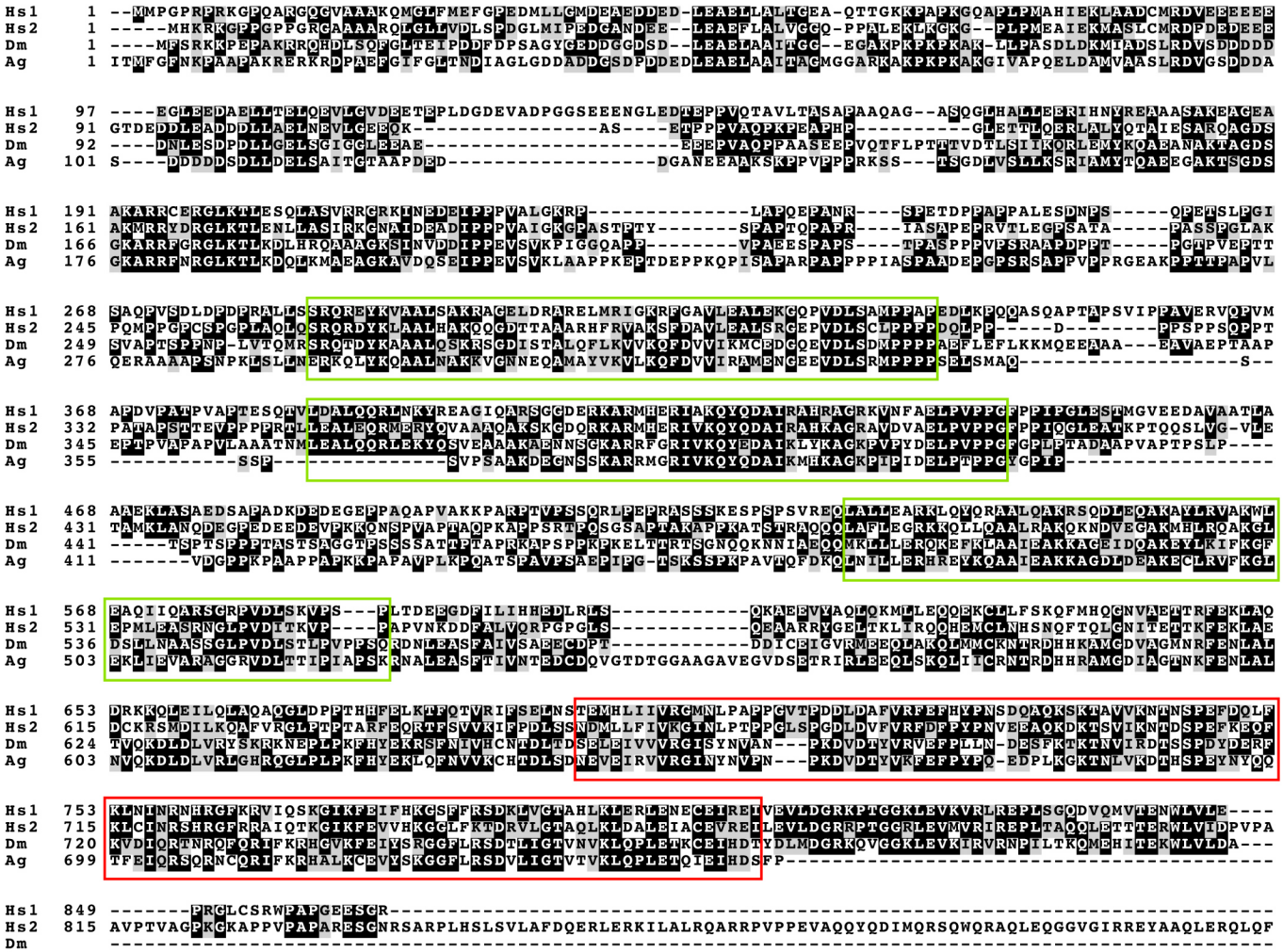

Figure S1 (Childress et. al.)

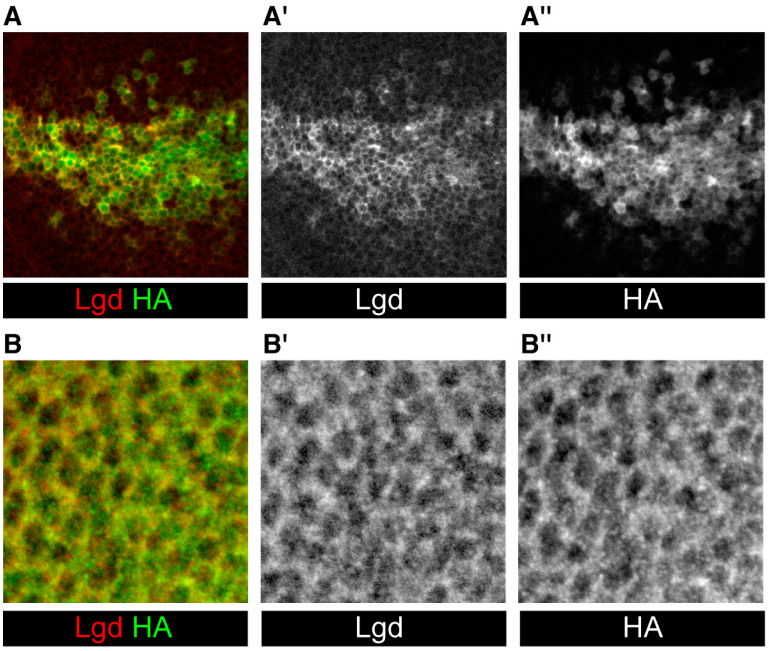

Figure S2 (Childress et. al.)

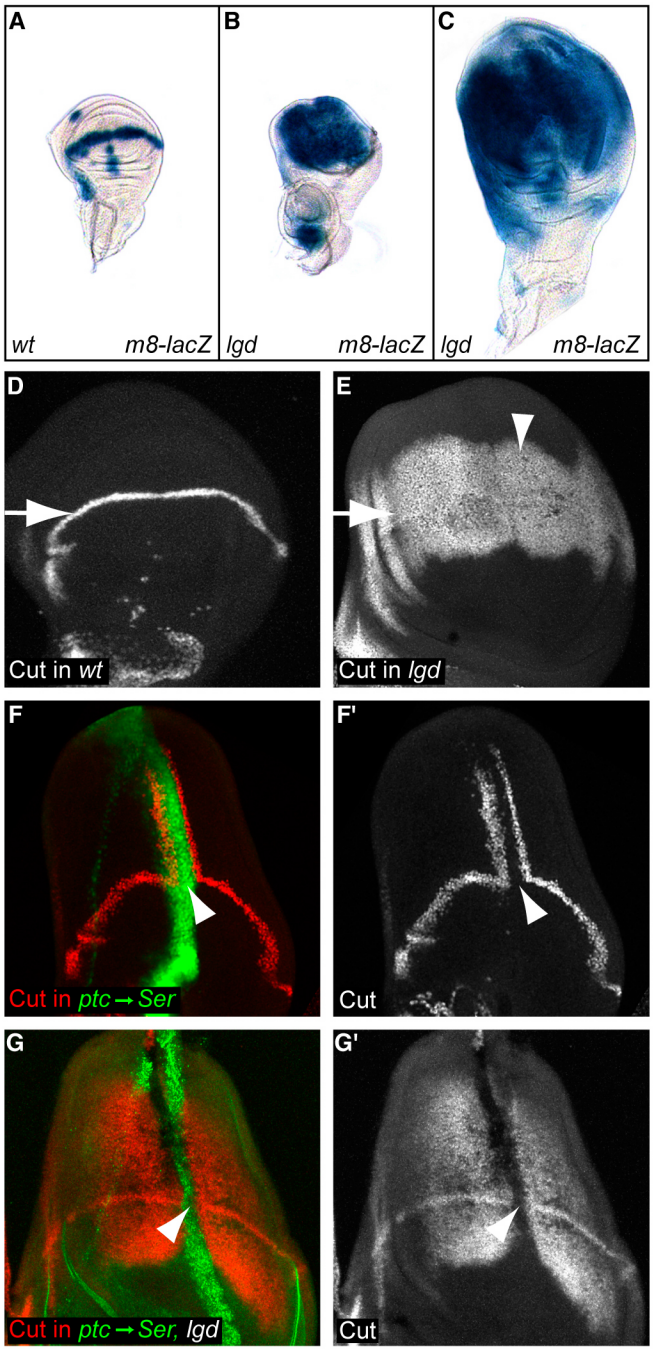

Figure S3 (Childress et. al.)

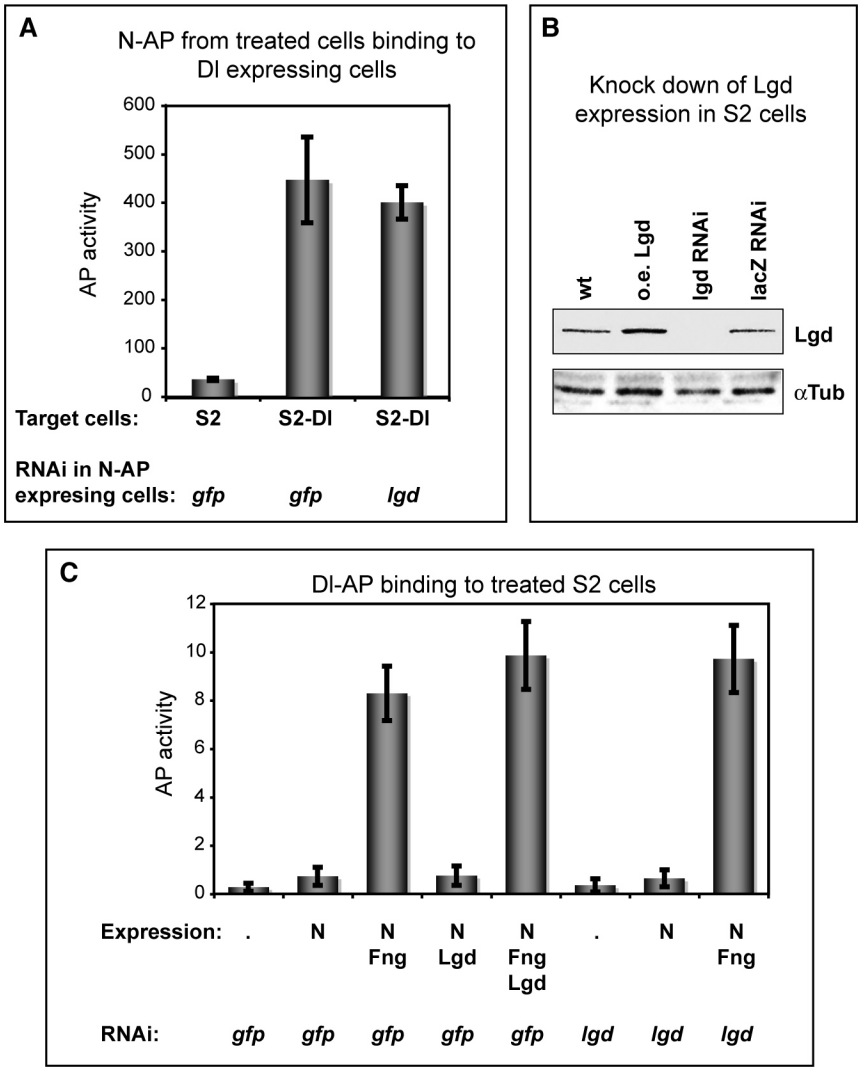

Figure S4 (Childress et. al.)

Supplementary Material

Experimental Procedures

Drosophila stocks and genetics

Crosses were performed at 25°C. Genetic mosaics were obtained by heat shock induction of FLP recombinase as described, using recombinant chromosomes carrying p[FRT] insertions *FRT40A*, *FRT42D* or *FRT82B* as appropriate [S1]. To generate *lgd, hrs, or Dl, Ser* mutant clones, the following alleles were flipped against corresponding *ubiGFP* marked *FRT* chromosomes: lgd^{1} and lgd^{d7} [S2, S3]; hrs^{D28} [S4]; lgd^{1},hrs^{D28}; and Dl^{rev10},Ser^{RX106} [S5]. Other fly strains used were *E(spl)m8 2.61-lacZ* [S6]; P-elements KG05885, KG05826, KG01276, KG06014, and BG00672; *Vps 25[N55], UAS-P35* [S7] (UAS-p35 was a kind gift from B. Hay); *ptcGAL4*; *UAS-Serrate*; *C96 Gal4* [S8]; and *FRT42D* MARCM [S9].

The mutations in *lgd* were mapped by recombination mapping [S10]. For sequence analysis, DNA was isolated from homozygous mutant larvae identified by the absence of a GFP marker on the balancer chromosome, and the *lgd* coding region was analyzed by PCR amplification of all exons and subsequent sequencing.

For the EMS screen, isogenized *yw* males were starved for 12 hours and subsequently fed 25mM EMS (0.001% Bromophenol Blue and 5% sucrose) for 24 hours. They were mated to *2πm FRT 40A* lgd^{1} */ CyO GMRhid* virgins. F1 single male *CyO GMRhid* flies were then mated to *2πm FRT 40A* lgd^{1} */ CyO GMRhid* virgins. The F2 progeny were scored for lack of wild-type eyes. New mutant lines were collected and the *lgd* gene was sequenced.

Immunohistochemistry and Western Blotting

Antibody stainings of third instar imaginal discs were performed as described previously [S11]. S2 cells were grown in Schneider's media and affixed to Concanavalin A- covered slides, then fixed in 4% Paraformaldehyde in KNC buffer, pH7 (7.5gNaCl, 0.35gKCl, 0.21gCaCl in 1000mL H2O), and blocked and stained with 1^0 and 2^0 antibodies in heat-inactivated 5% milk. The following antibodies were used (dilutions and source in parentheses): mouse α-Cut (1:200; DSHB); mouse α-N^{ICD} C17.9C6 (1:200; DSHB); mouse α-N^{ECD} C458.2 (1:50; DSHB); guinea-pig α-Hrs (1:100; [S4]); guinea-pig α-Lgd (1:1000; generated for this work by Bethyl Laboratories); guinea-pig α-Sens (1:1000 [S12]); mouse α-HA (1:100). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

To extract protein for western blots, S2 cells were washed with PBS and lysed in 1x Passive Lysis Buffer (Dual-Luciferase Assay; Promega). Larvae were dissected in cold PBS and homogenized in SDS buffer with the addition of protease inhibitors. Western blots were performed according to standard protocols and detected with the SuperSignal West Femto Detection reagents (Pierce). Antibodies were used on western blots as follows: guinea-pig α-Lgd (1:2000), and mouse α–αTub T-5168 (1:10,000; Sigma).

S2 cell culture and binding assays

S2 cell transfections were performed using Fugene-HD (Roche) and the manufacturer's protocol. Constructs expressing N-AP, Dl-AP, full length Notch and Fringe ([S13], gifts of Ken Irvine and Steve Cohen), UAS-Lgd, and Gal4-full length (gift of Duoja Pan) were used for transfections. The *pRL-tk* vector was used as an internal control to normalize the transfection efficiencies.

Renilla Luciferase levels were measured using Renilla Luciferase Assay system (Promega) and TD20/20 luminometer from Turner Biosystems. RNAi was performed using the protocol published in [S14]. DNA transfections using RNAi treated cells were performed on the third day of the RNAi treatment. Induction of transfected cells was performed using 0.7 mM CuSO4 one day after transfection. N-AP secretion was collected for three days after induction. S2-Dl cells were obtained from DGRC and Dl expression was induced using 0.7 mM $CuSO_4$ for two days before the binding assay. N-AP levels from different cells were equilibrated using conditioned medium. The N-AP binding assay was performed using the protocol published in [S13]. Detection of AP was performed using Chemiluminescent AP Ultra Sensitive reagent from BioFX Laboratories and TD20/20 luminometer from Turner Biosystems.

Molecular Biology

For protein knockdown, dsRNAs were synthesized using the MEGAscript RNAi kit (Ambion) from PCR products containing the T7 promoter (taatacgactcactataggg). Primer pairs were:

Lgd dsRNA:

5’-primer: GAATTAATACGACTCACTATAGGGAGATGCCTCTGAGGAACCCGTCCAG

3’-primer: GAATTAATACGACTCACTATAGGGAGAGTGTGGGTTCTGGGGCAGCAGT

GFP dsRNA primers:

5'-primer: TAATACGACTCACTATAGGGAGCTGGACGGCGACGTAAAC

3'-primer: TAATACGACTCACTATAGGGATGGGGGTGTTCTGCTGGTAG

For the genomic rescue construct, genomic DNA was extracted from wild-type flies, and the whole region around and containing *CG4713* (from 548 base pairs before the start codon to 553 base pairs after the stop codon) was PCR amplified and inserted into the *pCaSpeR4* vector. To construct *UAS-HA-Lgd*, *CG4713* cDNA was inserted into an HA-tagged *pUAST* vector.

Supplementary Figure S1: Mapping and Sequence of Lgd.

(A) Mapping of *lgd* relative to five P-elements located in the 32D2 to 32E1 region on 2L. Triangles show P-elements with their names above. Recombination distances between *lgd* (vertical line with star) and each P-element are shown in centiMorgans (cM). **(B)** The genomic region around *lgd*. Predicted open reading frames are depicted as arrowed boxes. The genes depicted in the five gray boxes, as well as *lgd* (black box), were sequenced. The genomic region included in the *lgd* genomic rescue construct is shown in red. **(C)** Sequence alignment of Human HsLgd1 (Hs1- formerly called CC2D1B), Human HsLgd2 (Hs2- formerly called CC2D1A), *Drosophila lgd* (Dm), and *Anopheles* Lgd (ENSANGP00000020521) (Ag). Identical residues are shown on a black background, and similar residues are shaded in gray. The conserved Lgd homology domains are boxed in green and the conserved C2 domains are boxed in orange.

Supplementary Figure S2: Lgd is cytoplasmically localized

(A-A'') C96 Gal4-driven UAS-HA-Lgd along the wing margin of third instar imaginal discs, detected by α-Lgd (red) and α-HA (green). **(B-B'')** C5 Gal4 driven UAS-HA-Lgd along the wing margin of third instar imaginal discs, detected by α-Lgd (red) and α-HA (green). The HA-Lgd fusion protein was cytoplasmic.

Supplementary Figure S3: Lgd restricts Notch activation to target cells and is not required for Cis-inhibition.

(A-C) *Drosophila* wing imaginal discs stained to show the expression pattern of the *E(spl)m8 2.61-lacZ* reporter gene. Anterior is to the left and Dorsal is down in all panels. (A) A late third instar *wild-type* wing disc. E(spl)m8 2.61-LacZ was expressed in a stripe along the wing margin. (B) An early third instar *lgd* mutant wing disc. The *E(spl)m8 2.61-lacZ* reporter was expressed throughout the wing pouch even though the disc was not yet overgrown. (C) A late third instar lgd^{d10} mutant wing disc. The disc was overgrown and the E(spl)m8 2.61-LacZ reporter expression extended throughout the wing pouch. **(D)** Cut expression in a wild-type third instar imaginal wing disc. The arrow indicates the expression along the wing margin. **(E)** Cut expression in an lgd^{1} mutant wing disc. Cut expression was no longer restricted to the D/V boundary. **(F-G')** Lgd is not required for 'cis-inhibition' of Notch signaling, as detected by suppression of Cut expression at the wing margin. Anterior is to the left and Dorsal is down in all panels. (F-F') Cut expression (red in F and gray in F') in a patched-Gal4, UAS-Serrate, UAS-GFP wing disc. GFP expression is shown in green. The arrowhead indicates the interrupted wing margin due to cis-inhibition. The ectopic Serrate expression also induced two stripes of ectopic Cut expression on either side of the Serrate expression domain. This ectopic Notch activation was limited to the ventral side of the wing pouch, because Fringe suppresses Serrate function in the dorsal compartment. (G-G') Cut expression (red in G and gray in G') in an $lgd^{1/d7}$ mutant wing disc with patched-Gal4, UAS-Serrate. Serrate expression is shown in

green. The arrowhead indicates the interrupted wing margin due to cis-inhibition. The high levels of Serrate cell-autonomously inhibited the expression of Cut in the wing margin as well as the ectopic Cut normally found in the wing pouch in *lgd* mutant wing discs. However, outside of the Patched expression domain, ectopic expression of Cut was still present in these discs.

Supplementary Figure S4: Lgd does not affect binding affinities between Notch and Delta. **(A)** Knock-down of Lgd does not affect the binding affinity of extracellular Notch-Alkaline Phosphatase (N-AP) to S2-Delta cells. Graph of AP activity of secreted N-AP from treated cells bound to Delta-expressing cells. Secreted Notch-AP fusion protein was purified from S2 cells treated with either *lgd* or *gfp* dsRNA (as a wild-type control). AP levels were normalized and the resulting N-AP was allowed to bind to either S2 cells or S2 cells stably expressing Delta (S2-Dl). After N-AP bound to the S2-Dl cells, unbound N-AP was washed away, and the amount of bound N-AP was determined by measuring the amount of AP activity. We found that the amount of AP activity bound to S2-Dl cells in the *lgd* RNAi preparation was equivalent to the AP activity bound to S2-Dl cells in the *gfp* RNAi preparation **(B)** Western blot showing efficiency of Lgd knockdown. Protein extracts from S2 cells alone (first lane), transfected with UAS-Lgd driven by full-length Gal4 (second lane), treated with *lgd* dsRNA (third lane), and treated with *lacZ* dsRNA as a control (fourth lane) are shown. The blots were probed with αLgd and α-αTub as a loading control. **(C)** Neither knock-down nor overexpression of Lgd affects the binding levels of Delta-AP (Dl-AP) to full-length Notch. Graph of AP activity of secreted Dl-AP from wild-type cells bound to S2 cells expressing the indicated constructs and treated with the indicated dsRNA. We expressed full-length Notch in normal S2 cells and in S2 cells

containing *lgd* dsRNA or *gfp* dsRNA as a control. We then incubated these cells with Dl-AP secreted from wild-type cells. We performed this experiment in the presence or absence of Fringe, which is a glycosyltransferase that modifies the extracellular domain of Notch, reducing the ability of Serrate to bind and activate Notch, while enhancing binding and activation by Delta. We included Fringe in these experiments both to have a positive control for increased binding affinity as well as to accommodate the possibility that Lgd affects only Fringe-modified Notch protein. To account for the possibility that Lgd function or levels were limiting in S2 cells, we also overexpressed Lgd protein (UAS-Lgd driven by full-length Gal4) together with full length Notch and Fringe in S2 cells and incubated them with Dl-AP. The expression of Lgd indicates transfection with UAS-HA-Lgd and full-length Gal4. While Fringe had a strong effect on the amount of Dl-AP bound to the S2 cells, neither overexpression nor knockdown of Lgd had an effect.

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