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Ligand endocytosis drives receptor dissociation and activation in the Notch pathway

Annette L. Parks‡, Kristin M. Klueg, Jane R. Stout and Marc A. T. Muskavitch*

Department of Biology, Indiana University, Bloomington, IN, 47405, USA

*Author for correspondence (e-mail: muskavit@indiana.edu)

‡Present address: Exelixis Pharmaceuticals, Inc., 260 Littlefield Ave., South San Francisco, CA 94080, USA

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SUMMARY

Endocytosis of the ligand Delta is required for activation of the receptor Notch during *Drosophila* development. The Notch extracellular domain (NotchECD) dissociates from the Notch intracellular domain (NotchICD) and is trans-endocytosed into Delta-expressing cells in wild-type imaginal discs. Reduction of dynamin-mediated endocytosis in developing eye and wing imaginal discs reduces Notch dissociation and Notch signalling. Furthermore, dynamin-mediated Delta endocytosis is required for Notch trans-endocytosis in *Drosophila* cultured cell lines. Endocytosis-defective Delta proteins fail to mediate trans-endocytosis of Notch in cultured cells, and exhibit aberrant subcellular trafficking and reduced signalling capacity in *Drosophila*. We suggest that endocytosis into Delta-expressing cells of NotchECD bound to Delta plays a critical role during activation of the Notch receptor and is required to achieve processing and dissociation of the Notch protein.

Key words: Delta, Notch, Signal transduction, Endocytosis, *Drosophila*

INTRODUCTION

The Notch pathway functions during the development of invertebrates and vertebrates to ensure the correct specification of a diverse array of cell types. The Notch signalling pathway appears to be substantially conserved in most organisms examined, and has been implicated in a variety of human diseases, including Alagille Syndrome (reviewed in Artavanis-Tsakonas, 1997) and acute lymphoblastic T cell leukemia (Ellisen et al., 1991). In *Drosophila*, the ‘core’ Notch pathway members include two transmembrane ligands, Delta and Serrate, one transmembrane receptor, Notch, and one primary downstream transducer, Suppressor of Hairless [Su(H)]. While components necessary in signal-generating cells are relatively uncharacterized, many proteins that act in Notch signal-receiving cells have been identified, including transcriptional targets such as the *Enhancer of split-Complex* genes (reviewed in Artavanis-Tsakonas et al., 1999; Bray, 1998; Egan et al., 1998; Greenwald, 1998). Several proteins are thought to impinge on the pathway in specific contexts to regulate signalling by acting at the level of ligand/receptor interactions (Fringe), binding to the Notch intracellular domain (Deltex and Numb), or binding to the downstream transducer Su(H) (Hairless) (Artavanis-Tsakonas et al., 1999; Bray, 1998; Egan et al., 1998; Greenwald, 1998).

The Delta protein is a transmembrane ligand that acts nonautonomously (reviewed in Muskavitch, 1994) and exhibits a complicated subcellular trafficking pattern, the significance of which is not yet fully understood. Delta is detected on cell surfaces and in intracellular vesicles during some developmental stages, but is detected solely in vesicles during others (Huppert et al., 1997; Kooh et al., 1993; Parks et al., 1997, 1995). For instance, wild-type Delta protein is detected only in vesicles throughout most of retinal development (Parks et al., 1995). Examination of Delta protein accumulation during eye development in the endocytic mutants, *hook* (*hk*) and *shibire* (*shi*), suggests that most, if not all, Delta-containing vesicles are endocytic (Krämer and Phistry, 1996, 1999; Parks et al., 1995). This indicates that during many stages of imaginal development, Delta is initially transported to the cell surface, but does not accumulate to detectable levels. Instead, it is taken up very efficiently by endocytosis and appears to accumulate in multivesicular bodies (Krämer and Phistry, 1996, 1999; Parks et al., 1995). We have reported the counterintuitive observation that the temperature-sensitive *Delta* (*Dl*) allele Dl^{RF} encodes a protein that resides persistently on the surfaces of retinal cone cells, yet acts as a loss-of-function mutation (Parks et al., 1995). More recently, we have discovered a set of *Dl* loss-of-function mutations, which we call the ‘trafficking-defective’ alleles, that encode proteins with similar properties (A. A. Dos Santos and M. A. T. Muskavitch, unpublished data). The fact that these trafficking-defective *Dl* alleles are loss-of-function mutations suggests that the removal of Delta from the cell surface may be critical for its function as a signalling molecule.

Many of the phenotypes that result from reductions in *shi* function are strikingly similar to those that result from reductions in Notch pathway signalling (Poodry, 1990; Poodry

et al., 1973; Shellenbarger and Mohler, 1978). The *shi* gene encodes the *Drosophila* dynamin homologue (dynamin reviews include: McNiven, 1998; Schmid et al., 1998). Dynamin, a GTPase, is required for the formation of clathrin-coated vesicles derived from the plasma membrane during endocytosis (McNiven, 1998; Schmid et al., 1998). Seugnet et al. (1997) have shown that *shi* function is necessary in signal-generating (Delta-expressing) cells and signal-receiving (Notch-expressing) cells for proper Notch signalling to occur during *Drosophila* sense organ development. They propose that removal of inactive ligand-receptor complexes is important for continued Notch signalling, possibly to allow the continuing formation of active ligand-receptor complexes.

Receptor and ligand processing have recently come under scrutiny as critical elements in the regulation of Notch signalling. Delta is proteolytically processed to yield at least four isoforms (Klueg et al., 1998); however, the functional significance of this processing is currently unclear. Processing of Delta may be necessary for signal activation (Qi et al., 1999) or for downregulation (Klueg et al., 1998), or may result from protein degradation following clearance of ligand from the cell surface. Notch is processed in a complex manner that is thought to be required for genesis and activation of the receptor (reviewed in Artavanis-Tsakonas et al., 1999; Chan and Jan, 1998; see Fig. 1). First, Notch is cleaved during transport through the Golgi at a site amino-proximal to the transmembrane domain ('site 1' or 'S1') by a furin-like convertase (Logeat et al., 1998). Following this cleavage, Notch is 'reassembled' and transported to the cell surface as a heterodimeric receptor (Blaumueller et al., 1997; Logeat et al., 1998). Another cleavage event (termed 'S3') within the intracellular domain has also been shown to occur. The S3 cleavage of Notch is ligand-dependent and produces a Notch intracellular domain fragment that may act, in conjunction with Su(H), in the nucleus as the primary Notch signal transducer (Jarriault et al., 1995; Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). The mechanism that triggers the intracellular domain cleavage is unknown. However, the Notch/lin-12 repeats (LNRs) within the receptor extracellular domain may contribute to regulation of this cleavage (Kopan et al., 1996; Lieber et al., 1993). When the LNRs are removed, intracellular domain cleavage occurs in a constitutive manner in the absence of ligand (Kopan et al., 1996). This has led to the hypothesis that binding of Delta to Notch may result in a cleavage event (termed 'S2') in the Notch extracellular domain. This cleavage would uncouple the LNRs from the remainder of the receptor, allowing the intracellular domain cleavage (S3) to occur constitutively (reviewed in Bray, 1998; Chan and Jan, 1998; Kimble et al., 1998). Recently, Notch S2 cleavage has been demonstrated in mammalian cells (Mumm et al., 2000). S2 cleavage in these cells occurs in response to ligand binding and blocking S2 cleavage results in loss of S3 cleavage, consistent with a proteolytic cascade model of Notch activation (Mumm et al., 2000).

We present evidence indicating that endocytosis of the ligand Delta, complexed with the Notch extracellular domain (NotchECD), into signal-generating cells is required for separation of the NotchECD from the Notch intracellular domain (NotchICD), possibly by inducing cleavage of the receptor at the S2 site. The separation of NotchECD from NotchICD would relieve LNR-mediated repression of the S3 cleavage, which would then occur constitutively to release a non-membrane bound, activated form of NotchICD. Several predictions of this model are borne out. *Dl* alleles that encode endocytosis-defective ligands are loss-of-function mutations, and these defective ligands fail to support Notch trans-endocytosis in cultured cells (trans-endocytosis is described in Klueg et al., 1998) and Delta-mediated signalling in vivo. We find that dynamin function, which is necessary for Notch signalling, is required for Delta endocytosis and for Notch trans-endocytosis in cultured cells, and for dissociation of NotchECD from NotchICD in developing imaginal tissues. In addition, we demonstrate that the third epidermal growth factor-like repeat within the Delta extracellular domain is required for Delta endocytosis and Notch trans-endocytosis, and for Delta-dependent signalling during development.

MATERIALS AND METHODS

Fly stocks

Flies carrying the enhancer reporter transposon *A101* (*P[lArB]A101.IF3*) (Bellen et al., 1989) balanced with *TM3* were the gift of Hugo Bellen (Baylor College of Medicine, Houston, TX, USA) and were used as control animals throughout this report. The presence of the *A101* transposon in the heterozygous condition has no discernible effects on bristle or eye development (Parks and Muskavitch, 1993; Parks et al., 1995). *Oregon-R*, *ss* e^4 *ro* and *BER-1*, are kept in our laboratory. shi^{ts1} and *hk pr* stocks were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA). Dl^{RF}/*TM6C*, *cu Sb e Tb ca (TM6C)*, *ss* Dl^{6B37} *e/TM6C* and shi^{ts1} stocks were maintained at 18°C. Dl^{CE9}/*TM6C* was isolated from an EMS screen in the *ss* e^4 *ro* background, and Dl^{BE21}/*TM6C* was isolated from an EMS screen in the $E(spl)^D$ *tx* background. The *1348::GAL4* driver (isolated by G. Technau at Universität Mainz; Mainz, Germany) and the *dpp::GAL4/TM6B* driver (Staehling-Hampton et al., 1994) were used to induce ectopic expression of *UAS::DeltaWT-1* (Jacobsen et al., 1998) and *UAS::DeltaC301Y* (see below).

Heat pulses and dissections

To perform temperature shifts, white prepupae were picked and aged at 18°C in a humid chamber. Pupae were raised to the restrictive temperature (32°C) for the times indicated in the figure legends and were dissected and processed for immunohistochemistry immediately following the heat pulse. In experiments not requiring heat pulses, white prepupae were picked and aged at 25°C to the times indicated in the figure legends. We find that 24 hours after puparium formation (APF) at 25°C is equivalent to 48 hours APF at 18°C.

Immunohistochemistry

For production of polyclonal antibodies to the Delta N-terminal domain (N2), the Delta N2 fusion construct was generated by inserting sequences encoding amino acids 33-131 from the Delta extracellular domain (nt236-nt536 of pDl1; Kopczynski et al., 1988) into pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA; construct generated by ATG Laboratories, Inc., Eden Prairie, MN, USA). Expression of these fusion constructs and subsequent fusion protein purification were carried out by ATG Laboratories, Inc. The immunogen was prepared by standard methods and injected into rabbits (Indiana University, Bloomington, IN, USA); this antiserum (DeltaRab-N2) was used at 1:2000 dilution. Other primary antibodies used were: guinea pig polyclonal antiserum against the Delta extracellular domain (GP581; Huppert et al., 1997), used at 1:3000 dilution (imaginal tissue) or 1:5000 (cultured cells); mouse polyclonal antiserum to the Delta extracellular domain (M5; Kooh et al., 1993),

used at 1:2000 dilution; rat polyclonal antiserum to the Notch extracellular domain (NotchECD) (Rat8; Klueg et al., 1998), used at 1:800 dilution; monoclonal antibody ascites to the Notch intracellular domain [C17.9C6 (mAb9C6); Fehon et al., 1990], to the NotchECD [C458.2H (mAbC458); Diederich et al., 1994] and to the Delta extracellular domain [C594.9B (mAb9B, also known as mAb202); Diederich et al., 1994], used at 1:4000-5000 dilution.

We find that long fixation times result in loss of some aspects of the NotchECD pattern in developing wings and retinas. Antibody staining on retinal tissue was carried out as in Parks et al. (1997); fixation times were limited to 40 minutes or less. Immunohistochemistry on *Drosophila* cultured cells was carried out as in Klueg et al. (1998). For antibody staining of pupal wings, pupae were pulled from pupal cases in 4% paraformaldehyde and allowed to fix for approximately 30 minutes before wings were pulled from the cuticle. Cuticle-free wings were refixed in 4% paraformaldehyde for 15-20 minutes before washing and subsequent staining. When a horseradish peroxidase-conjugated secondary antibody was used, the peroxidase color reaction was usually intensified using silver enhancement (Gallyas et al., 1982; Liposits et al., 1984). Micrographs were taken using a Sony DXC-960MD video camera. In some instances, two or more micrographs were combined in a montage to generate a continuous image of the disc epithelium or were overlaid using Adobe Photoshop Layers utility to display multiple focal planes.

Whole-mount microscopy

Wings were removed from adults and mounted using Gary's Magic Mountant (Ashburner, 1989).

Cell culture

Drosophila Schneider line 2 (S2) cells were maintained as described by Fehon et al. (1990). The *Drosophila* EH34A3 (*shi*^{ts1}) cell line (Woods and Poodry, 1983) was maintained in M3 medium supplemented with bacto-peptone (2.5 g/l), yeast extract (2 g/l) and fetal calf serum (12.5%). For transient transfections by calcium phosphate precipitation (Table 1), cells were transfected as described (Klueg and Muskavitch, 1999). Protein expression was induced with 1 mM $CuSO_4$ as populations of cells were mixed for aggregations. Those cells incubated at the restrictive temperature were placed at 31°C at the start of induction/aggregation. For transient transfections by electroporation (Table 2), cells were prepared as described (Klueg and Muskavitch, 1999). Protein expression was induced with 1 mM $CuSO_4$ for 2 hours, after which Delta-expressing cells were mixed 1:1 with Notch-expressing cells. Aggregation (along with continued protein induction) was then carried out overnight. Transient transfections were used to express the following proteins: DeltaWT and NotchWT (Fehon et al., 1990); DeltaC301Y (described below); DeltaC301S (described below); DeltaΔ1-3, DeltaΔ4-5 and DeltaNG3 (Shepard, 1991). For all experiments, cells were allowed to settle onto poly-L-lysine (Sigma, St Louis, MO, USA)-coated slides, fixed and then double-labelled with antibodies against Delta and Notch as in Klueg et al. (1998). Individual experiments for each table were run in parallel, e.g. S2 cell aggregations alongside EH34A3 cell aggregations at permissive and restrictive temperatures. Data were compiled from three separate sets of experiments.

Molecular cloning and sequence analysis

Dl^{CE9}*/TM6C* males were crossed to *BER-1* females, a wild-type strain carrying a *StyI* RFLP within the sixth exon of *Delta* (Haenlin et al., 1990). Genomic DNA was isolated from the Stubble^{+} progeny. The sixth exon of *Delta* was amplified with Taq Polymerase and Taq Extender (Stratagene, La Jolla, CA, USA) using the primers: Dl-A 5′ CAACTGGGCTGGAAGGG 3′ and Dl-0 5′ AGTTTACGAGTT-ATGCC 3′. The 1945 bp amplification products were cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and mutant transformants were identified based on their *StyI* RFLP. Sequence data were generated by standard dideoxynucleotide-termination and cycle sequencing methods using the Sequitherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) and M13 Reverse/IRD41-dye labelled and T7 Promoter/IRD41-dye labelled (LI-COR, Inc., Lincoln, NE, USA) DNA primers.

The single C301Y lesion found in exon 6 of *Dl*^{CE9} was introduced into a full-length *Dl* cDNA under the control of an inducible metallothionein promoter (pMTDl1; Fehon et al., 1990) producing the plasmid pMTDeltaC301Y (J. R. Stout and M. A. T. Muskavitch, unpublished). Identical methods were used to generate pMTDeltaC301S by introducing the single lesion, C301S, found in exon 6 of *Dl*^{BE21} (J. R. S. and M. A. T. M., unpublished). This cysteine at amino acid position 301 lies within the third Epidermal Growth Factor-like repeat of Delta (Kopczynski et al., 1988; Vässin et al., 1987).

Germline transformation and crosses

The C301Y lesion was introduced into a full-length *Dl* cDNA under the control of yeast UAS sequences in the pUAST vector (Brand and Perrimon, 1993) producing UAS::DeltaC301Y (J. R. S. and M. A. T. M., unpublished). Germline transformation and subsequent crosses were carried out as described in Jacobsen et al. (1998). All crosses of UAS responder lines to GAL4 driver lines were performed at 25°C in a *w*^{1118} background.

RESULTS

Notch receptor dissociation correlates with Notch signalling during development

Given the requirement for endocytosis in Notch signalling and the observation that Delta proteins that do not traffic correctly exhibit reduced function, we investigated the subcellular trafficking characteristics of the Notch receptor. Because of the unusual structure of the functional Notch receptor, we examined the accumulation of NotchECD and NotchICD epitopes in developing eye and wing imaginal discs.

We find that NotchECD and NotchICD epitopes reside in different cellular compartments within retinal latticework cells during pupal development (Fig. 2). This latticework is composed of prospective secondary and tertiary pigment cells, cell types in which *Notch* (*N*) is strongly transcribed (data not shown). NotchECD and NotchICD are associated with the surfaces of these cells (Fig. 2B,D). However, NotchECD exhibits substantial accumulation in intracellular vesicles in latticework cells (Fig. 2C), while NotchICD exhibits non-vesicular, diffuse cytoplasmic staining within these same cells (Fig. 2E). Interestingly, NotchECD, but not NotchICD, is found in vesicles in cone cells (Fig. 2C), a retinal cell type in which *N* is not transcribed (data not shown). The NotchECD found in cone cells is probably derived from Notch expressed at low levels in the primary pigment cells that surround the cone cells (data not shown). Loss of Notch function during this developmental interval results in the loss of primary pigment cells (Cagan and Ready, 1989). Previous analysis of *Dl* transcription and Delta protein subcellular accumulation in pupal retinas has revealed that *Dl* is intensely transcribed, and that Delta protein accumulates to high levels in vesicles in anterior and posterior cone cells during these stages (Parks et al., 1995). *Dl* is transcribed less intensely and Delta protein accumulates to much lower levels in vesicles in the two remaining cone cells and in primary pigment cells (Parks et al., 1995). Double-labelling for NotchECD and Delta reveals that NotchECD-containing cone cell vesicles also contain Delta

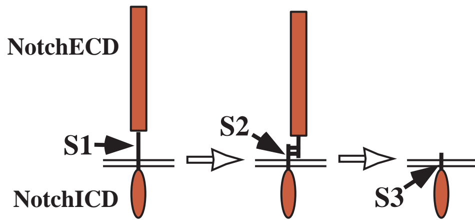

Fig. 1. Notch is proteolytically processed at three distinct sites (see text for details). S1: Notch is cleaved by a furin-like convertase in the trans-Golgi (Logeat et al., 1998). S2: at the cell surface, the heterodimeric form of Notch is cleaved in the extracellular domain in response to ligand (Mumm et al., 2000). S3: the Notch intracellular domain is released via cleavage near or in the transmembrane domain (Schroeter et al., 1998).

protein (Fig. 3A). This suggests that NotchECD is taken up by cone cells in a complex with Delta.

NotchECD and NotchICD epitopes also traffic independently within the provein during wing vein development. The highest levels of NotchECD and NotchICD accumulation are found in the two stripes of lateral provein cells that flank the central provein cells (Fig. 4E,F and I,J), consistent with previous descriptions of NotchICD localization (Huppert et al., 1997) and *N* transcription (de Celis et al., 1997). The adult vein is derived exclusively from central provein cells. Lateral provein cells, while capable of adopting the vein cell fate when Notch signalling is reduced (Lindsley and Zimm, 1992), normally adopt intervein cell fates. NotchECD and NotchICD are found on the surfaces of intervein and lateral provein cells, but NotchECD also accumulates within vesicles in these cells (Fig. 4F), while NotchICD does not (Fig. 4J). Similarly, NotchECD, but not NotchICD, is found prominently in vesicles in central provein cells (Fig. 4F, inset), a cell type that does not transcribe *N*, but does transcribe *Dl* (de Celis et al., 1997). Central provein vesicles that contain NotchECD also contain Delta (Fig. 3D), suggesting that NotchECD is taken up by central provein cells in a complex with Delta, as is the case for cone cells.

These observations suggest that NotchECD and NotchICD dissociate during the signalling that underlies cell fate specification in the retinal latticework and the proveins of the wing, and that different portions of the receptor can be targeted to different compartments following this dissociation. We refer to those instances in which NotchECD appears to be taken up by cells not expressing *N* as NotchECD trans-endocytosis (see Klueg et al., 1998), to reflect the fact that a portion of the receptor is being removed from one cell and taken up by another.

NotchECD localizes to endocytic compartments following receptor dissociation

The NotchECD-containing vesicles that we detect might reflect protein accumulation during processing and trafficking events that occur during secretion. Alternatively, if NotchECD-containing vesicles reflects removal of Notch from the cell

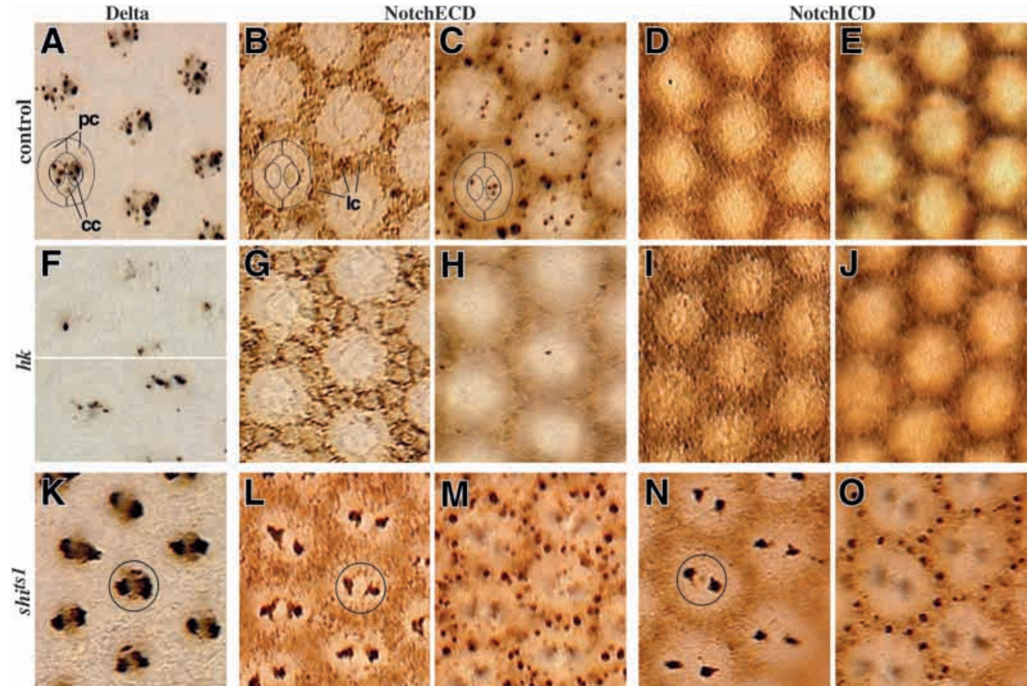

Fig. 2. Notch dissociation observed in control retinas fails to occur following a reduction in dynamin function in shi^{ts1} retinas. (A-E) Retinas from control *A101/TM3* pupae 25 hours after puparium formation (APF) (25°C). (A) Delta is detected primarily in vesicles in anterior and posterior cone cells. pc, primary pigment cell; cc, cone cell (see text). (B,C) NotchECD accumulates on surfaces of latticework cells (lc) in apical focal planes (B) and is detected in vesicles in latticework and cone cells in lower focal planes (C). (D,E) NotchICD accumulates on surfaces of latticework cells in apical focal planes (D) and appears to localize primarily to the cytoplasm in lower focal planes (E). (F-J) Retinas from *hk pr* pupae 25 hours APF (25°C). (F) Delta-containing vesicles decrease in number in *hk pr* retinas (two different retinas are shown). (G,H) NotchECD accumulates primarily on latticework cell surfaces in apical focal planes (G) and few or no NotchECD-containing vesicles can be detected in latticework or cone cells in lower focal planes (H) in *hk pr* retinas. (I,J) NotchICD localization in apical (I) and lower (J) focal planes of *hk pr* retinas resembles that of control retinas. (K-O) Retinas from shi^{ts1} pupae pulsed to 32°C from 41-46 hours APF (K,N,O) or 39-45 hours APF (L,M) (18°C growth). Control retinas from similarly grown and pulsed *A101/TM3* pupae are identical to A-E (data not shown). (K) Delta localizes on cone cell microvillar tufts (circled). (L-O) NotchECD (L) and NotchICD (N) colocalize on cone cell microvillar tufts in apical focal planes (circled). NotchECD (M) and NotchICD (O) are found in vesicles at similar locations within latticework cells in lower focal planes. For reference, single ommatidia are outlined in A, B and C. Circles in K, L and N demarcate sets of four cone cells. Antibodies used: Delta, mAb9B; NotchECD, mAbC458; NotchICD, mAb9C6.

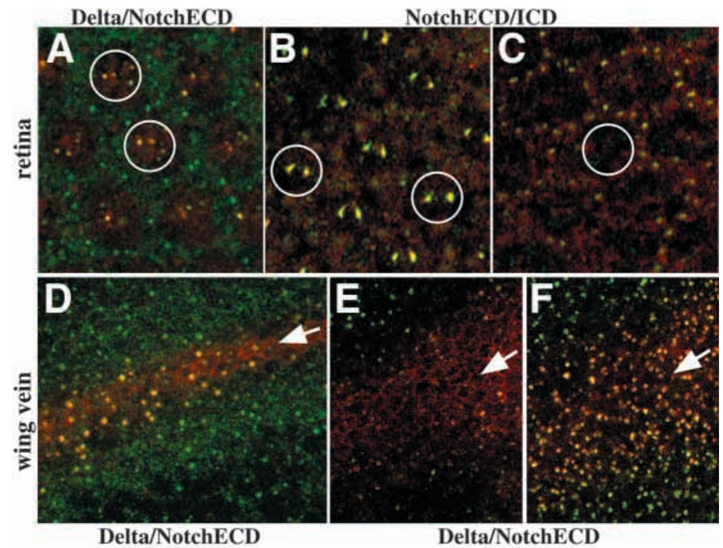

Fig. 3. Dissociated NotchECD colocalizes with Delta in control retinas and wing discs. Receptor dissociation fails and NotchECD colocalizes with NotchICD and Delta following a reduction in dynamin function in shi^{ts1} retinas and wing discs. (A) Double-labelling reveals that Delta (red) and NotchECD (green) colocalize in vesicles (yellow) in control *A101/TM3* retinal cone cells at 24 hours APF (25°C) (circles demarcate sets of four cone cells). (B,C) Double-labelling for NotchECD (green) and NotchICD (red) in retinas from shi^{ts1} pupae pulsed at 32°C from 47-56 hours APF (18°C growth) reveals that NotchECD and NotchICD colocalize on cone cell microvillar tufts (yellow) in higher focal planes (B; cone cell microvillar tufts are circled) and to vesicles in latticework cells in lower focal planes (C; circle demarcates set of four cone cells). (D) Double-labelling for NotchECD (green) and Delta (red) in wings from *A101/TM3* pupae pulsed at 32°C from 40-47 hours APF (18°C growth) reveals that Delta and NotchECD colocalize in vesicles (yellow) in vein cells; Delta expression (red) marks the developing vein. (E,F) Double-labelling for NotchECD (green) and Delta (red) in apical (E) and lower (F) focal planes in shi^{ts1} wing discs treated as in D reveals that NotchECD and Delta colocalize in vesicles (yellow) in abnormally widened developing veins after reduction in dynamin function. NotchICD and NotchECD also colocalize in vesicles following similar pulses, indicating that NotchICD localizes with NotchECD in these Delta-containing vesicles (data not shown). Arrows in D, E and F indicate the center of each vein. Antibodies used: NotchECD, mAbC458; Delta, GP581 (A,D-F); NotchECD, Rat8; NotchICD, mAb9C6 (B,C).

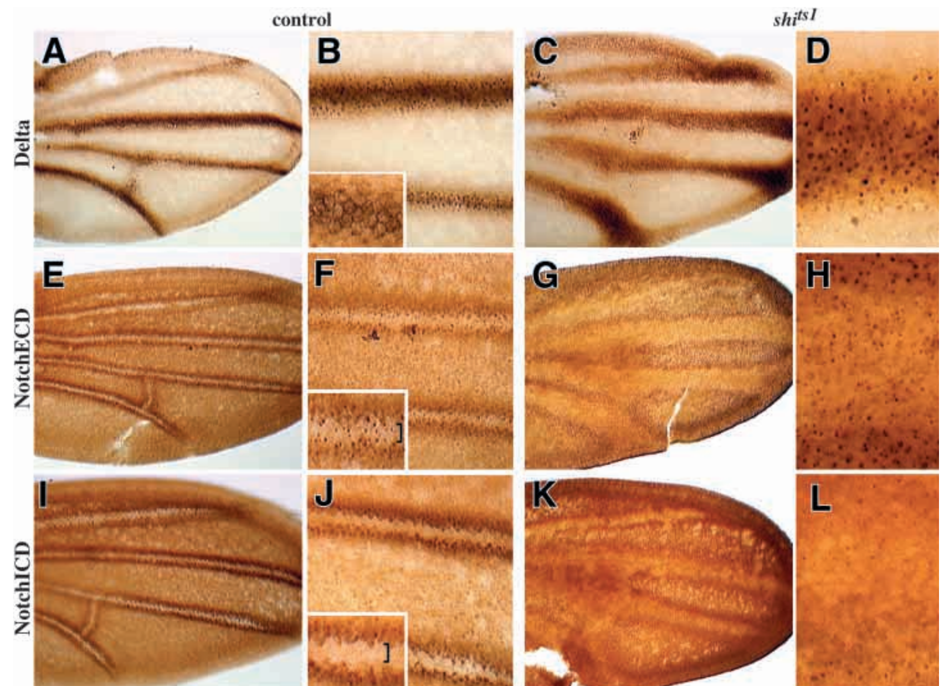

Fig. 4. Notch dissociation observed in control wing discs fails to occur following a reduction in dynamin function in shi^{ts1} wing discs. (A,B) Delta localizes to cell surfaces (B, inset) and in vesicles in provein cells of a wing from an *A101/TM3* pupa pulsed from 39-45 hours APF. This pattern is similar to that previously reported for unpulsed wing discs (Huppert et al., 1997). (C,D) Delta accumulates in intracellular vesicles in a wing from a similarly pulsed shi^{ts1} pupa; vein width has increased significantly compared to that of control veins in A. (E,F) NotchECD accumulates to low levels in vesicles in intervein cells (the cells that lie between provein regions) and to high levels in vesicles in the lateral provein cells that flank each central provein in control wings from *A101/TM3* pupae pulsed from 42.5-50 hours APF. NotchECD localizes to vesicles within central provein cells (cells that do not express *N*) (inset in F; bracket marks the central provein). Similar patterns are observed in unpulsed *A101/TM3* control wings (data not shown). (G,H) NotchECD localizes to cell surfaces and to larger intervein vesicles and smaller provein vesicles in a shi^{ts1} wing pulsed as in D. Note the increased vein width demarcated by lower levels of Notch accumulation. (I,J) NotchICD accumulates to low levels in the cytoplasm and in a perinuclear location within intervein cells, and to high levels in similar subcellular locations in stripes that flank the central provein cells in a wing from a control *A101/TM3* pupa pulsed from 48-53 hours APF (J, inset; bracket marks the central provein). (K,L) NotchICD accumulates to low levels in vesicles in intervein and provein cells in a wing from a shi^{ts1} pupa pulsed from 44-50.5 hours APF. NotchECD appears to accumulate to high levels in provein and intervein vesicles (H), while vesicles stained for NotchICD appear to have much less intense signals (L), perhaps indicating a reduced amount of NotchICD compared to NotchECD in these vesicles. All pupae were grown at 18°C, except during indicated heat-pulses at 32°C. Antibodies used were the same as in Fig. 1.

surface, then vesicular NotchECD should reside in one or more endocytic compartments. To determine whether NotchECD-containing vesicles are indeed endocytic, we examined NotchECD localization in the endocytic mutant, *hook* (*hk*). Krämer and Phistry (1996, 1999) have shown that Delta accumulation in vesicles within retinal cells and Boss accumulation in multivesicular bodies (MVBs) within R7 photoreceptor cells are dependent on *hk* function (see Fig. 2F). These authors suggest that Hook does not play a role in early endocytic events, but is required later within the endocytic

pathway, for MVB formation (Krämer and Phistry, 1996, 1999). We find that NotchECD-containing vesicles in latticework cells and cone cells are eliminated or greatly reduced in number in *hk* mutant retinas at 24 hours APF (compare Fig. 2C,H). NotchICD localization remains unchanged in *hk* mutants (Fig. 2I,J). NotchECD-containing vesicles in intervein cells, and in lateral and central provein cells are also greatly reduced in number in 30 hour APF *hk* mutant wings (data not shown). This implies that most, if not all, NotchECD-containing vesicles in wild-type retinas and wings result from NotchECD accumulation in MVBs following endocytosis. Therefore, the dissociation and independent subcellular trafficking of NotchECD and NotchICD that we observe in developing retinas and wings apparently occurs following transport of heterodimeric Notch to the cell surface.

Dynamin function is required for receptor dissociation in vivo

Given that NotchECD-containing vesicles are endocytic, we examined the dependence of Notch dissociation on *shi* (*Drosophila* dynamin) function. Dynamin is required during endocytosis for formation and pinching off of clathrin-coated vesicles from the plasma membrane (reviewed in McNiven, 1998; Schmid et al., 1998). Heat-pulsed flies that carry the temperature-sensitive *shi* allele shi^{ts1} exhibit many phenotypes that are observed in Notch-signalling pathway loss-of-function mutants, suggesting that endocytosis plays an integral role in Notch signalling (see Introduction).

Reductions in *shi* function from 41-47 hours APF (18°C growth) in developing retinas result in the loss of primary pigment cells and the development of supernumerary cells that resemble secondary pigment cells (data not shown). Identical cellular phenotypes are observed following reductions in Notch function during a similar interval (Cagan and Ready, 1989). Delta subcellular trafficking is aberrant in shi^{ts1} retinas (see Fig. 2K; Parks et al., 1995), indicating that dynamin function is required for correct Delta trafficking in developing cone cells. We find that NotchECD and NotchICD fail to dissociate in shi^{ts1} retinas at the restrictive temperature (Fig. 2L-O). Instead, NotchECD and NotchICD colocalize with Delta on cone cell apical microvillar tufts (Figs 2K,L,N and 3B), which become enlarged with excess membrane when dynamin function is reduced (Parks et al., 1995). This observation suggests that Delta can bind to NotchECD in the absence of dynamin function, and that dynamin-dependent endocytosis is required for dissociation of NotchECD from NotchICD and for trans-endocytosis of NotchECD into Delta-expressing cone cells.

NotchECD and NotchICD also colocalize in vesicles within latticework cells (Figs 2M,O and 3C) under these conditions. The uniform colocalization of NotchECD and NotchICD in latticework cells when dynamin function is reduced suggests that dynamin is required for the dissociation and independent trafficking of NotchECD and NotchICD within latticework cells. The functional significance of latticework cell vesicles that contain NotchECD and NotchICD is not clear. However, the accrual of vesicles in the absence of dynamin function implies that they arise by a dynamin-independent mechanism, and their disappearance in shi^{ts1};*hk* double mutants (data not shown), in which *hk* and *shi* function are both reduced, implies that they lie within the endocytic pathway.

Reductions in *shi* function in the developing wing disc results in widening of the central provein domain implying that Notch signalling is reduced within the pupal provein. This phenotype is easily detected in pupal wings immediately following a heat-pulse of shi^{ts1} animals to the restrictive temperature (compare Fig. 4A,C). The proveins recover from this insult and adult wings exhibit only mild vein thickenings at the marginal termini of longitudinal veins 3, 4 and 5 (data not shown). NotchECD accumulation in heat-pulsed shi^{ts1} pupal wings is strikingly similar to that of NotchICD. Both domains are found on cell surfaces and in vesicles in lateral provein and intervein cells and, to a lesser extent, on cell surfaces and in vesicles in central provein cells (Fig. 4G,H and K,L). Double-labelling for NotchECD and NotchICD reveals that the two domains colocalize in provein and intervein vesicles when dynamin function is reduced (data not shown). This suggests that in wings, as in retinas, NotchECD and NotchICD fail to dissociate, and instead traffic together, when dynamin function is reduced.

Delta localizes to intracellular vesicles, which form in a dynamin-independent manner, in heat-pulsed shi^{ts1} wing discs (Fig. 4C,D). Double-labelling for NotchECD and Delta reveals that Delta-containing vesicles in shi^{ts1} central provein cells also contain NotchECD (Fig. 3F) and by inference, NotchICD (see above). This indicates that, as in the retina, Delta can bind to Notch in wing discs in the absence of dynamin-mediated endocytosis, dissociation of Notch does not occur in this context, and that Delta, NotchECD and NotchICD traffic together under these conditions. The presence of vesicles that contain Delta, NotchECD and NotchICD in central proveins following reductions in dynamin function indicates the existence of a dynamin-independent endocytic pathway that can clear inactive Delta/Notch complexes from cell surfaces.

These findings imply that dynamin function is required for dissociation of Notch, and for independent subcellular trafficking of NotchECD and NotchICD during retinal and wing vein development. Because of the prominent trans-endocytosis of NotchECD into Delta-expressing cells, we suggest it is dynamin-dependent Delta endocytosis that is responsible for receptor dissociation and NotchECD trans-endocytosis.

Reductions in Delta function result in reduced Notch trans-endocytosis

If Delta endocytosis is necessary for Notch dissociation and trans-endocytosis, then reductions in Delta function might be expected to impede these processes. Dl^{RF}/Dl^{6B37} is a *Dl* temperature-sensitive allele combination that exhibits moderate to strong *Dl* hypomorphic phenotypes following pulses at restrictive temperatures (Parks and Muskavitch, 1993; Parody and Muskavitch, 1993). The number of NotchECD-containing vesicles in retinal cells is dramatically reduced in Dl^{RF}/Dl^{6B37} animals heat-pulsed during early pupal development (Fig. 5A,B). In addition, NotchECD trans-endocytosis into Delta-expressing cone cells following heat pulses later in pupal development (Fig. 5C,D) is also reduced. The Delta6B37 protein exhibits normal trafficking in retinas and is detected primarily in MVBs; however, the Dl^{RF} allele encodes a trafficking-defective protein that accumulates on the cell surface of retinal cone cells (see Introduction). Trans-endocytosis of NotchECD into pupal cone cells is also

decreased in heat-pulsed animals homozygous for Dl^{RF}(data not shown). These findings strongly support the hypothesis that Delta function, in general, and Delta endocytosis, specifically, are required for dissociation of NotchECD from NotchICD and for trans-endocytosis of NotchECD into Delta-expressing cells.

Trans-endocytosis of Notch is dependent on cis-endocytosis of Delta

To determine whether intercellular trafficking of Notch requires dynamin-dependent subcellular trafficking of Delta, we examined Notch and Delta endocytosis in control (S2; Schneider, 1972) and *shi* temperature-sensitive mutant (EH34A3; Woods and Poodry, 1983) cell lines. In *Drosophila* S2 cells at normal growth temperature (22°C), Delta and Notch are found on cell surfaces and in intracellular vesicles (Fig. 6A,B), as reported previously (Fehon et al., 1990; Klueg et al., 1998; Rebay et al., 1991). In S2 cells at higher temperatures (31°C), Notch subcellular localization remains unchanged (data not shown), while Delta becomes localized in large intracellular vesicles and exhibits diminished cell surface accumulation (Fig. 6C). In EH34A3 cells at the permissive temperature (22°C), Delta and Notch localization patterns are similar to those in control cells (Fig. 6D and data not shown). However, in EH34A3 cells at the restrictive temperature (31°C), Delta is found only on the cell surface (Fig. 6F), while Notch continues to be found on the cell surface and in vesicles (Fig. 6E), as in control cells. These experiments suggest that endocytosis of Delta into the cell in which it is expressed (referred to as ‘cis-endocytosis’) is dynamin-dependent. Our results also indicate that the export of Delta and Notch to the cell surface in cultured cells is not detectably affected by reduced dynamin function.

We examined intercellular transfer of Delta and Notch in mixed aggregates of Delta- and Notch-expressing EH34A3 cells compared to control S2 cells. The frequencies of trans-endocytosis of Delta and Notch in EH34A3 cells at the permissive temperature are similar to those of control cells (Table 1). At the restrictive temperature, however, the frequency of Notch trans-endocytosis into Delta-expressing cells decreases significantly in the EH34A3 line (Table 1), implying that impairment of Delta cis-endocytosis in EH34A3 cells results in reductions in Notch trans-endocytosis. The frequency of Delta trans-endocytosis into Notch-expressing EH34A3 cells is not affected at the restrictive temperature in either S2 or EH34A3 cells (Table 1). These observations suggest that in EH34A3 cells, Delta cis-endocytosis and Notch trans-endocytosis are dynamin-dependent.

Mutations in Delta ELR3 impede trans-endocytosis of Notch

Our data predict that loss of Delta cis-endocytosis in flies might result in loss of NotchECD trans-endocytosis. In addition, if Notch dissociation and trans-endocytosis are necessary for Notch pathway signalling (as suggested by the loss of Notch signalling observed in *shi* mutants), we would predict that Delta endocytosis mutants would act as hypomorphic mutations. In fact, the trafficking-defective *Dl* alleles comprise a set of hypomorphic mutations (M. Vaskova and M. A. T. Muskavitch, unpublished) which encode proteins that exhibit abnormal trafficking in developing eyes (Fig. 7A,B) and bristles (data not shown). Sequence analysis of major portions of two of these alleles, Dl^{CE9} and Dl^{BE21}, reveals single missense mutations at Delta amino acid residue 301, which resides in the third epidermal growth factor-like repeat (ELR) of the Delta extracellular domain. The Dl^{CE9} allele contains a cysteine to tyrosine mutation (C301Y), while the Dl^{BE21} allele is correlated with a cysteine to serine mutation (C301S).

Table 1. Trans-endocytosis of Notch and Delta

Cultured cell line[a]	Temperature[b] (°C)	Domain detected[c]	% Trans-endocytosis[d] (s.e.m.)[e]
S2	22	NotchECD[f]	22 (4)
EH34A3	22	NotchECD	21 (1)
S2	31	NotchECD	21 (1)
EH34A3	31	NotchECD	1 (0)
S2	22	DeltaECD[g]	25 (1)
EH34A3	22	DeltaECD	30 (1)
S2	31	DeltaECD	23 (5)
EH34A3	31	DeltaECD	22 (3)

[a]*Drosophila* S2 and EH34A3 cultured cells were transfected by calcium phosphate precipitation, as described in Materials and Methods.
[b]Temperature at which overnight aggregations were performed
[c]The domain of Delta or Notch that was scored for trans-endocytosis, detected by immunohistochemistry using Delta (GP581) and Notch (mAbC458) antibodies.
[d]The frequency of trans-endocytosis was recorded as the percentage of cells with internal vesicles that contain protein taken up from an adjacent cell surface.
[e]Values presented are the average for three independent experiments.
[f]Detection of NotchECD in Delta$^+$ cells.
[g]Detection of Delta extracellular domain (DeltaECD) in Notch$^+$ cells.

An analysis in cultured cells of the C301Y and C301S missense mutations reveals that these mutations affect the ability of Delta to mediate Notch trans-endocytosis. S2 cells that express DeltaC301Y or DeltaC301S are able to bind to and aggregate with Notch-expressing cells at significant, albeit reduced, frequencies compared to S2 cells that express wild-type Delta (DeltaWT) (K. Klueg and M. Muskavitch, unpublished data). However, cells that express DeltaC301Y or DeltaC301S exhibit substantial reductions in the ability to mediate trans-endocytosis of Notch (Table 2). In addition, removal of Delta ELRs 1-3 (DeltaΔEGF1-3) results in a nearly complete loss of Notch trans-endocytosis, whereas the loss of ELRs 4-5 (DeltaΔEGF4-5) does not significantly affect Notch trans-endocytosis (Table 2). A chimeric protein (DeltaNG3) that contains the Delta amino terminus and ELRs 1-3 fused to the transmembrane and intracellular domains of *Drosophila* neuroglian can mediate Notch trans-endocytosis, but cannot be taken up by trans-endocytosis into Notch-expressing cells (Table 2). These results imply that the structural integrity of Delta ELR3 is critical for efficient trans-endocytosis of Notch, and suggest that the Delta amino terminus and ELRs 1-3 are sufficient for interaction with the cellular machinery that mediates Notch trans-endocytosis into Delta-expressing cells.

The DeltaC301Y and DeltaC301S variants fail to be taken up into Notch-expressing cells by trans-endocytosis, suggesting that Delta ELR3 is also necessary for Delta trans-endocytosis (Table 2). However, additional Delta sequences carboxy-proximal of ELR3 must be required for efficient endocytosis of Delta into Notch-expressing cells because the Delta sequences contained within DeltaNG3 are not sufficient to support Notch-mediated trans-endocytosis of Delta. While Delta trans-endocytosis has been observed in cultured cells

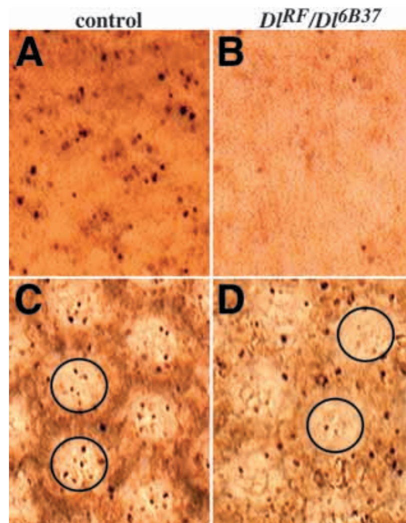

Fig. 5. Reduced *Dl* function diminishes NotchECD trans-endocytosis into retinal cone cells. (A) NotchECD resides in vesicles in lower focal planes in a control *A101/TM3* eye disc pulsed from 0-5 hours APF. (B) NotchECD-containing vesicles are severely reduced in number in Dl^{RF}/Dl^{6B37} eye discs following a similar pulse. (C) NotchECD localizes to vesicles in latticework cells (out of focus) and in cone cells (circles demarcate sets of four cone cells) in an *A101/TM3* retina pulsed from 41-47 hours APF. (D) NotchECD-containing vesicles stain less intensely and are reduced in number in cone cells (circled) in a Dl^{RF}/Dl^{6B37} retina similarly pulsed. Note that Dl^{RF}/Dl^{6B37} retinas still contain numerous NotchECD-positive vesicles in latticework cells. Because the latticework is quite disorganized in these retinas, comparison of vesicle intensities and numbers in control and mutant latticework cells was not undertaken. All pupae were grown at 18°C, except during indicated pulses at 32°C. Antibodies used: NotchECD, mAbC458.

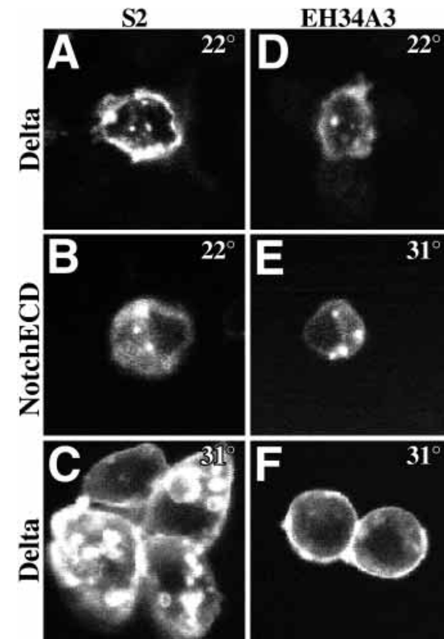

Fig. 6. Reduced dynamin function diminishes Delta cis-endocytosis. (A) Delta accumulates on the cell surface and in vesicles in S2 cells at 22°C. (B) NotchECD accumulates on the cell surface and in vesicles in S2 cells at 22°C. (C) Delta accumulates in large intracellular vesicles in S2 cells at 31°C. (D) Delta localization in EH34A3 cells at 22°C is similar to that seen in S2 cells in A. (E) NotchECD localization in EH34A3 cells at 31°C is similar to that seen in S2 cells in B. (F) Delta is detected exclusively on the cell surface in EH34A3 cells at 31°C. Antibodies used: Delta, GP581; Notch, mAbC458.

(Fehon et al., 1990; Klueg and Muskavitch, 1999; Klueg et al., 1998), we have been unable thus far to detect Delta intercellular transfer in wing and eye imaginal discs (A. Parks and M. Muskavitch, unpublished data).

The C301Y mutation eliminates Delta signalling in flies and yields a trafficking-defective ligand

To examine whether the C301Y mutation correlated with the

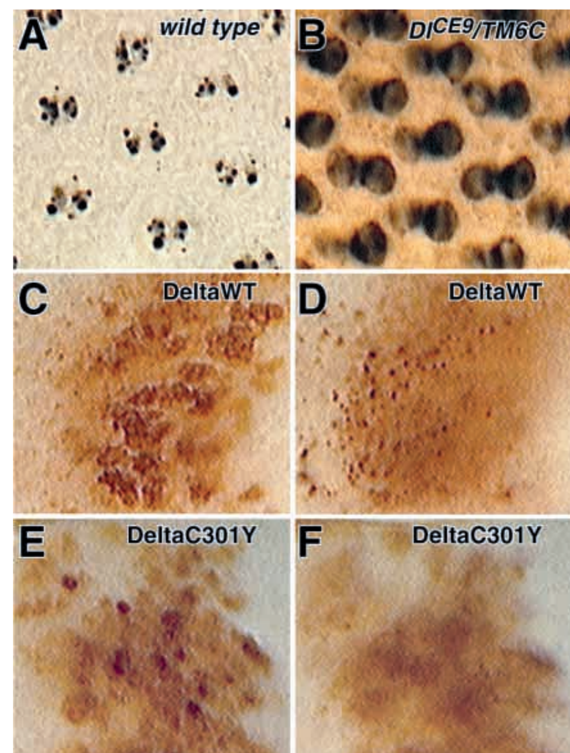

Fig. 7. Delta encoded by Dl^{CE9} is retained on retinal cone cell surfaces and DeltaC301Y, which contains a Dl^{CE9}-correlated mutation, does not enter endocytic vesicles when expressed ectopically in the developing wing. (A) Wild-type Delta is detected solely in vesicles in cone cells in a 48 hour APF (18°C) *Oregon-R* retina. (B) Delta encoded by Dl^{CE9} is retained on cone cell surfaces in a 48 hour APF (18°C) $Dl^{CE9}/TM6C$ retina. In other focal planes, Delta is also found in vesicles (data not shown). These vesicles presumably contain wild-type Delta encoded by the TM6C balancer. Cis-endocytosis of protein encoded by one dose of the wild-type *Dl* gene provides sufficient Notch activation to support normal eye development. (C,D) DeltaWT protein accumulates on cell surfaces (C) and, in lower focal planes, in vesicles (D) when expressed along the anterior-posterior compartment boundary in third larval instar *UAS::DeltaWT/+; dpp::GAL4/+* wing discs. (E,F) DeltaC301Y protein appears to accumulate exclusively on cell surfaces, not in vesicles, when expressed within the same region in third larval instar *UAS::DeltaC301Y/+; dpp::GAL4/+* wing discs. Antibodies used: M5 (A,B); mAb9B (C-F).

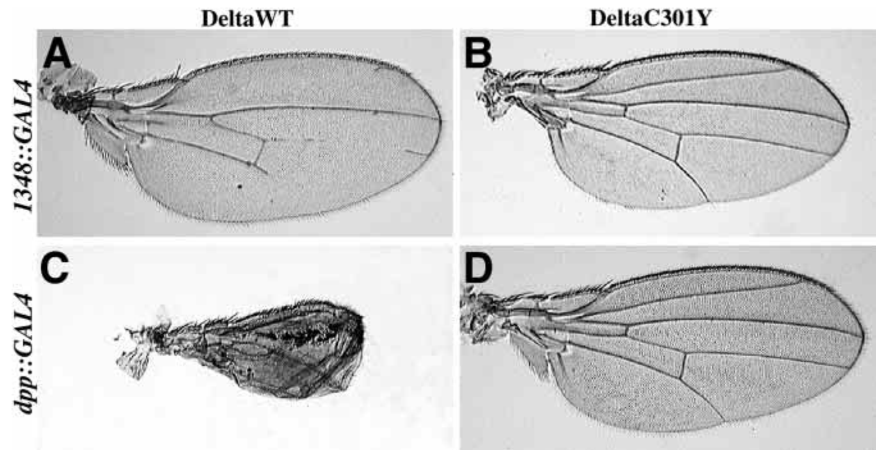

Fig. 8. DeltaC301Y fails to activate Notch signalling when expressed in the developing wing. (A) Wing vein loss is observed following expression of DeltaWT in intervein regions in *UAS::DeltaWT/+; 1348::GAL4/+* wing discs. (B) Veins appear normal following expression of DeltaC301Y in *UAS::DeltaC301Y/1348::GAL4* wing discs. (C) Wings appear small, misshapen and display ectopic wing margins following expression of DeltaWT along the anterior-posterior compartment boundary in *UAS::DeltaWT/+; dpp::GAL4/+* wing discs. (D) Wings develop normally following expression of DeltaC301Y within the same region in *UAS::DeltaC301Y/+; dpp::GAL4/+* wing discs.

Table 2. Trans-endocytosis of Notch and Delta variants in cultured S2 cells

Delta variant[a]	Domain detected[b]	% Trans-endocytosis[c] (s.e.m.)[d]
DeltaWT	NotchECD[e]	23 (1)
DeltaC301Y	NotchECD	2 (0)
DeltaC301S	NotchECD	3 (0)
DeltaΔEGF1-3	NotchECD	0 (0)
DeltaΔEGF4-5	NotchECD	21 (1)
DeltaNG3	NotchECD	25 (4)
DeltaWT	DeltaECD[f]	24 (1)
DeltaC301Y	DeltaECD	4 (0)
DeltaC301S	DeltaECD	3 (0)
DeltaΔEGF1-3	DeltaECD	1 (0)
DeltaΔEGF4-5	DeltaECD	18 (3)
DeltaNG3	DeltaECD	2 (1)

[a]Delta variant expression constructs were introduced by electroporation as described in Materials and Methods.
[b]The domain of Delta or Notch that was scored for trans-endocytosis, detected by immunohistochemistry: GP581 was used to detect DeltaWT and all Delta variants except DeltaNG3; DeltaRab-N2 was used to detect DeltaNG3 and DeltaWT control cells; mAbC458 was used to detect NotchWT.
[c]The frequency of trans-endocytosis was recorded as the percentage of cells with internal vesicles that contain protein taken up from an adjacent cell surface.
[d]Values presented are the average for three independent experiments.
[e]Detection of NotchECD in Delta$^+$ cells.
[f]Detection of Delta extracellular domain (DeltaECD) in Notch$^+$ cells.

Dl^{CE9} allele reduces Delta function in animals, we employed the GAL4-UAS system (Brand and Perrimon, 1993) to compare the signalling capabilities of DeltaC301Y and DeltaWT. Expression of DeltaWT under the control of a wing blade intervein driver (*1348::GAL4*) leads to the loss of vein cells, a Notch pathway gain-of-function phenotype (Fig. 8A; Huppert et al., 1997). Expression of DeltaC301Y under the control of the same driver yields a wild-type wing (Fig. 8B), indicating that DeltaC301Y does not exhibit a detectable level of signalling in this context. Expression of DeltaWT along the anterior-posterior compartment boundary in the developing wing using a *dpp::GAL4* driver (Staehling-Hampton et al., 1994) yields misshapen wings and ectopic wing margins (Fig. 8C), another Notch pathway gain-of-function phenotype (Klein and Arias, 1998). In contrast, DeltaC301Y has no

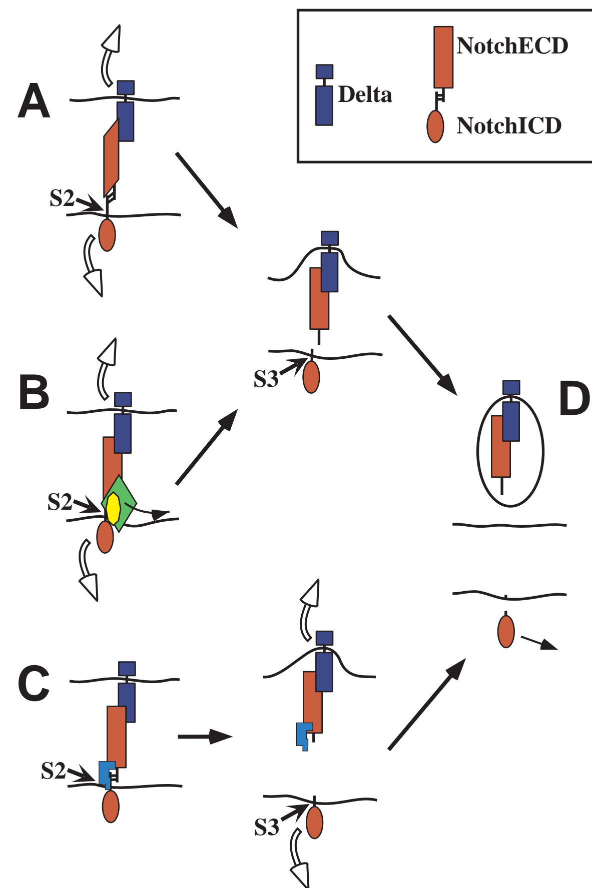

Fig. 9. Endocytosis of Delta and Notch is required to activate the Notch receptor. Endocytosis is indicated by open, curved arrows. Three possible mechanisms are shown. (A) Endocytosis of the Delta/Notch complex results in a conformational change within the Notch protein that allows access by an enzyme(s) to the S2 cleavage site. (B) Endocytosis of Delta/Notch complexes alters the interaction of Notch with other proteins, which unmasks S2 and makes it available for cleavage. (C) Following S2 cleavage, endocytosis is required to remove NotchECD and associated proteins from the remainder of the Notch protein, thus relieving inhibition of S3 cleavage. The final step depicted for each mechanism involves transit of NotchICD to the nucleus following S3 cleavage, where it affects target gene expression (D).

discernible effect when expressed under the control of this driver (Fig. 8D). Examination of DeltaC301Y expression in the larval wing discs of the latter animals reveals substantial protein accumulation (Fig. 7E), indicating that the lack of adult phenotypes is not due to a lack of responder-mediated Delta expression. Results implying that DeltaC301Y is a severe loss-of-function mutation have also been obtained with drivers that direct expression in the eye and in the microchaetae of the notum (data not shown). Six independent DeltaC301Y responder lines have been crossed to these drivers, and none appears to encode a functional Delta protein. These data indicate that the C301Y mutation renders Delta nonfunctional in these contexts during imaginal development, and strongly suggest that this lesion constitutes a severe loss-of-function *Dl* mutation.

We also compared subcellular trafficking of DeltaC301Y and DeltaWT expressed under the control of the *dpp::GAL4* driver in third larval instar wing discs. DeltaWT appears on cell surfaces and in numerous vesicles along the anterior-posterior compartment boundary in these discs (Fig. 7C,D). DeltaC301Y appears to be sequestered primarily, if not exclusively, on cell surfaces along the compartment boundary (Fig. 7E,F). Three independent DeltaC301Y responder lines have been tested in this manner, and the protein encoded by each responder appears to be sequestered on cell surfaces (data not shown). This observation implies that the DeltaC301Y mutation is sufficient to cause the trafficking-defective behavior observed for the Dl^{CE9}-encoded protein in retinas.

DISCUSSION

Ligand endocytosis is required for Notch activation

We propose that endocytosis is essential for Delta-dependent Notch pathway signalling based on several lines of evidence. First, Delta proteins that are cleared inefficiently from cell surfaces exhibit reduced signalling capacity in vivo. Second, reductions in dynamin function, which impede Delta endocytosis, impede Notch signalling in vivo. Third, dissociation of NotchECD and NotchICD during signalling in vivo depends on dynamin function and Delta endocytosis. Fourth, cultured cells with reduced dynamin function exhibit reduced Delta cis-endocytosis and reduced Notch trans-endocytosis. Fifth, a point mutation that reduces Delta-mediated Notch trans-endocytosis in cultured cells blocks Delta endocytosis in vivo and eliminates Delta-dependent Notch signalling in vivo. In addition, research from several groups indicates that Delta proteins lacking the intracellular domain act as dominant-negative proteins in *Drosophila* and in vertebrates (Chitnis et al., 1995; Jen et al., 1997; Parody, 1998; Sun and Artavanis-Tsakonas, 1996). Although a Delta variant (DeltaD) lacking the intracellular domain can still bind to Notch (Klueg et al., 1998), preliminary evidence indicates that DeltaD is not endocytosed efficiently in imaginal discs (J. Stout and M. Muskavitch, unpublished data). The dominant-negative character of such proteins may result from the inability of these ligands to undergo efficient endocytosis, and mediate receptor dissociation and activation, after they have bound to Notch.

Seugnet et al. (1997) demonstrate that phenotypes resulting from the expression of ligand-independent, membrane-tethered, constitutively active Notch proteins are unaffected by reductions in shibire function. This suggests that dynamin does not play a role in S3 cleavage or events downstream of receptor activation. In addition, these authors find that *shi* loss-of-function phenotypes are epistatic to phenotypes resulting from a ligand-dependent gain-of-function N^{Ax} allele. They conclude from this datum that dynamin is either required for some aspect of ligand 'presentation' or is required during ligand-mediated activation of the receptor. These data are consistent with a role for endocytosis at the level of Delta-Notch ligand-receptor interactions.

Seugnet et al. (1997) also find requirements for dynamin-mediated endocytosis in signal-generating (Delta-expressing) and signal-receiving (Notch-expressing) cells during sensory organ precursor specification in the developing notum. Consistent with this, we find that ligand endocytosis in the signal-generating cell is required for Notch signalling and that Notch signalling falters when shibire function is reduced by expressing a dominant-negative form of shibire in signal-receiving cells during wing vein formation (A. Parks and M. Muskavitch, unpublished data). This implies that dynamin function is required in both signal-generating and signal-receiving cells for activation of Notch. We suggest that dynamin-dependent endocytosis of Delta, in combination with Notch endocytosis, triggers events that result in processing and activation of the membrane-bound receptor, Notch.

We suggest three alternative mechanisms by which endocytosis may induce receptor activation (Fig. 9). (A) After binding of Delta to Notch, molecular strain imparted to Notch by endocytosis in the signalling and receiving cells results in a conformational change that permits access by processing enzyme(s) to the S2 site. (B) The S2 site is masked by proteins that interact with Notch to form a complex. Following Delta binding, endocytosis of Delta and Notch alters intramolecular interactions within the complex, unmasking the S2 site and making it available for cleavage. (C) Endocytosis is not required for Delta-induced S2 cleavage, but is instead required to separate NotchECD and associated proteins from the remainder of the Notch protein, thus relieving inhibition of S3 cleavage by NotchECD. These proposed mechanisms for activation of Notch are distinct from other established modes of ligand-dependent receptor activation, such as dimerization and trans-phosphorylation (reviewed in Heldin, 1995). Similar requirements for endocytosis might exist in other instances in which membrane-bound ligands activate signal transduction pathways. We do not know, as yet, whether Serrate-expressing cells in developing flies mediate Notch trans-endocytosis during activation of Notch. However, the fact that Serrate-expressing cultured *Drosophila* cells mediate Notch trans-endocytosis at frequencies similar to those observed for Delta-expressing cells (Klueg and Muskavitch, 1999) suggests that trans-endocytosis of NotchECD is one aspect of the Notch activation mechanism that is common to Notch ligands.

Qi et al. (1998) present data suggesting the active form of Delta is a non-membrane-tethered Delta extracellular domain (DeltaECD), generated by the metalloprotease Kuzbanian (Kuz). This inference might suggest that DeltaECD binds Notch, and Notch S2 and S3 cleavages occur during ligand-receptor endocytosis into the signal-receiving cell. Data presented here and elsewhere suggest that this model may not apply in all instances of Notch signalling. First, NotchECD trans-endocytosis in association with Delta occurs during the

development of at least two imaginal tissues in *Drosophila* and is dependent on dynamin and Delta function in vivo. Second, expression of secreted Delta or Serrate extracellular domains during retinal development yields dominant-negative effects in *Drosophila* (Sun and Artavanis-Tsakonas, 1997). This suggests that secreted forms of Delta may not be capable of activating Notch in *Drosophila*, and may sequester Notch ineffectually, in a manner similar to that suggested for dominant-negative membrane-associated forms of Delta that lack the intracellular domain (see above). Third, evidence from work done on *Drosophila* Kuz (Rooke et al., 1996; Sotillos et al., 1997) and the related *C. elegans* protein Sup-17 (Wen et al., 1997) suggests that Kuz/Sup-17 activity is required in the signal-receiving (Notch/Lin-12-expressing) cell. Fourth, a dominant-negative Delta variant (Parody, 1998) that lacks the Delta intracellular domain is cleaved efficiently in a Kuz-dependent manner in *Drosophila* cultured cells (K. Klueg and M. Muskavitch, unpublished data), suggesting that cleavage of Delta by Kuz is not sufficient to generate an active Delta signal. Despite these contraindications, Kuz function may be required, in some other fashion, for Delta-dependent Notch signalling in vivo. Kuz activity may be a prerequisite for generation of a tethered, activated Delta extracellular domain that is anchored to the cell surface via a membrane-spanning portion of the Delta protein or another membrane-bound protein. Alternatively, Kuz might act on one or more members of the Notch pathway, other than ligand or receptor, in a manner required for Notch-dependent signal transduction.

Delta-dependent Notch trans-endocytosis requires elements of the Delta ELR array

We are only beginning to understand the structural requirements for Delta function within Delta-Notch signalling pathways. Ligands for Notch receptors share the Delta/Serrate/Lag-2 (DSL) motif, a conserved cysteine-rich amino-proximal sequence important for Notch pathway ligand-receptor interactions in invertebrates and vertebrates (Henderson et al., 1997; Jiang et al., 1998; Muskavitch, 1994; Shepard, 1991; Xue et al., 1999). Fleming et al. (1997) have demonstrated that the Delta and Serrate DSL motifs are not functionally interchangeable. This implies that the DSL motifs themselves are not capable of imparting the signal intrinsic to a given ligand and suggests that ligand sequences in addition to the DSL motifs are required to generate ligand-specific signals.

Our data imply that ELR3, which resides within the Delta extracellular domain, is essential for ligand endocytosis and ligand-dependent Notch signalling in a variety of developmental contexts. The fact that mutations in this repeat eliminate Notch trans-endocytosis in cultured cells reinforces the inferred importance of this process for Delta-dependent signalling in vivo. Lieber et al. (1992) found that the point mutation G305N within ELR3 (originally designated as ELR4; Lieber et al., 1992) is correlated with a *Dl* allele (Dl^{sup5}) that suppresses the rough eye phenotype associated with the N^{spl} mutation, and partially impedes ligand-receptor binding in cultured cells. They suggested that the structure of ELR3 might affect quantitative and qualitative characteristics of Delta-dependent signalling. We speculate that alteration of C301 within the Delta ELR array disrupts intrarepeat disulfide bonding, which in turn may induce structural change(s) sufficient to eliminate detectable ligand-dependent signalling without eliminating ligand-receptor binding. Collectively, our findings imply that ELR3, a region outside of the DSL motif, plays a critical role in Delta-dependent Notch signalling.

In *C. elegans*, deletion of the single ELR within Lag-2 does not impede signalling in vivo (Henderson et al., 1997). While removal of subsets of the ELRs in *Drosophila* Delta does not abolish Delta-Notch interactions in cultured cells (Muskavitch, 1994; Shepard, 1991), it is clear that the one or more of the Delta ELRs (e.g. ELR3) is necessary for signalling in vivo. These observations, considered in light of a phylogenetic analysis that provides no evidence for a close relationship of between *C. elegans* and *Drosophila* or vertebrate ELR arrays (Lissemore and Starmer, 1999), suggest that ELR arrays have evolved structurally and functionally since the divergence of the metazoan ancestors of *C. elegans* and *Drosophila*. Phylogenetic analysis of the ELR arrays of *Drosophila* and vertebrate Delta-related proteins indicates that only ELR2 has been conserved among these animals (Lissemore and Starmer, 1999). In addition, other point mutations within the Delta ELR array, which map outside of ELR3, are correlated with trafficking-defective *Dl* alleles (J. Stout and M. Muskavitch, unpublished data). It is likely therefore that future studies will identify ELRs in addition to ELR3 that have functional roles in Delta subcellular trafficking and/or Delta-Notch signalling during development.

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