Echinoid facilitates Notch pathway signalling during *Drosophila* neurogenesis through functional interaction with Delta

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Summary

The Notch intercellular signalling pathway is important throughout development, and its components are modulated by a variety of cellular and molecular mechanisms. Ligand and receptor trafficking are tightly controlled, although context-specific regulation of this is incompletely understood. We show that during sense organ precursor specification in *Drosophila*, the cell adhesion molecule Echinoid colocalises extensively with the Notch ligand, Delta, at the cell membrane and in early endosomes. Echinoid facilitates efficient Notch pathway signalling. Cultured cell experiments suggest that Echinoid is associated with the cis-endocytosis of Delta, and is therefore linked to the signalling events that have been shown to require such Delta trafficking. Consistent with this, overexpression of Echinoid protein causes a reduction in Delta level at the membrane and in endosomes. In vivo and cell culture studies suggest that homophilic interaction of Echinoid on adjacent cells is necessary for its function.

Key words: Notch pathway, *Drosophila*, Delta, Neurogenesis

Introduction

The Notch signalling pathway is highly conserved in the animal kingdom from nematode to mammals (Artavanis-Tsakonas et al., 1995; Greenwald, 1998; Weinmaster, 1997). The basic molecular pathway is well characterised: in essence a selected cell(s) signals via a transmembrane DSL ligand [i.e. Delta (Dl) in *Drosophila* neurogenesis], which activates the transmembrane Notch (N) receptor in surrounding cells. Intramembrane cleavage of activated Notch releases its intracellular domain (N^{ICD}) (Mumm and Kopan, 2000), which enters the nucleus where, in concert with various other proteins, it activates target gene expression (Bray and Furriols, 2001). As would be expected for such a central and widely deployed signalling pathway, this basic process is the target of extensive modulation by regulatory factors at every step, which act to adapt and shape the pathway to suit different developmental contexts.

Much of what is known about Notch signalling stems from studies of sense organ precursor (SOP) fate specification in *Drosophila* (reviewed by Artavanis-Tsakonas and Simpson, 1991; Baker, 2000). For the SOPs of the adult external sense (es) organs, the proneural genes *achaete* (*ac*) and *scute* (*sc*) are expressed in the third larval instar imaginal discs in groups of about 20 epidermal cells – the proneural clusters (PNCs). As development proceeds, proneural expression is reinforced in three or four PNC cells before being vastly upregulated in the future SOP and deactivated in the remainder of the cluster (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). This inhibition results from unidirectional Notch signalling triggered by the SOP, i.e. lateral inhibition. But before the appearance of an SOP, a state is thought to exist where signalling is bidirectional between all PNC cells (mutual inhibition), and this is often equated with prevention of premature SOP formation or maintenance of competence.

Selection of the SOP requires the progression from bidirectional to unidirectional signalling – a process that is incompletely understood. One attractive model is based on the notion that *Dl* transcription is repressed by Notch pathway activity. This model suggests that differences in the initial levels of Dl (and/or N) expression across the PNC results in small differences in levels of inhibitory signalling. These differences will be amplified by positive feedback until signal production has been switched off in all cells except one – the SOP (Muskavitch, 1994). This is supported by genetic dose evidence (Heitzler and Simpson, 1991). However, it seems that the levels of N and Dl at the membrane do not necessarily vary across the PNC (Kooh et al., 1993), and Dl transcription is not necessarily regulated by N activity (Li et al., 2003b). Hence, if signalling is initially bidirectional, it is not clear how the SOP can escape from inhibition from surrounding cells. A number of other mechanisms have been proposed that 'break the symmetry' of signalling within the PNC and allow a future SOP to emerge. For example, it is proposed that N protein on the selected precursor becomes resistant to activation by Dl expressed in the surrounding cells, allowing this cell to inhibit surrounding cells unidirectionally (Li et al., 2003b).

A number of other studies suggest the importance of modulating Dl activity for promoting differential signalling in

the PNC. In particular, post-translational downregulation of Dl activity on non-SOP cells may allow an SOP to escape from inhibition and also render the recipient cells more vulnerable to lateral inhibition (Parks et al., 2000). Kuzbanian-mediated proteolysis of Dl may fulfil this function (Mishra-Gorur et al., 2002). Dynamin-dependent regulated cis-endocytosis of Dl has also been strongly implicated (Parks et al., 2000), triggered by the ubiquitin ligase Neuralized (Neur) (Lai et al., 2001; Le Borgne and Schweisguth, 2003).

We investigate the function of the cell adhesion molecule Echinoid (Ed) in modulating Notch pathway signalling. Ed was originally identified as a negative regulator of Egfr signalling in eye development (Bai et al., 2001), where it restricts R8 precursor specification by preventing inappropriate Egfr-mediated induction of R8 fate (Rawlins et al., 2003; Spencer and Cagan, 2003). *ed* mutant flies also bear additional external sense (es) organs in a pattern reminiscent of mutants of lateral inhibition. Our investigation reveals that in the context of SOP specification, Ed modulates Notch pathway signalling, rather than Egfr signalling. We show that Ed protein associates cytologically with N, and especially Dl, at the membrane and in endosomes, and that Ed may modulate signalling by influencing the trafficking/degradation of Dl protein.

Materials and methods

Fly stocks

The following alleles have been described previously: $ed^{4.12}$, $ed^{4.4}$ and $ed^{6.1}$ (Rawlins et al., 2003); ed^{lH23} (de Belle et al., 1993); $ed^{l(2)k01102}$ (Bai et al., 2001); $aos^{\Delta 7}$ (Freeman et al., 1992); sca^{BP2} (Mlodzik et al., 1990); and Dl^{B2} (Micchelli et al., 1997). N^{55e11}, $Egfr^{IK35}$, $Hairless^{1}$, $hook^{1}$, ase^{1}, $Df(1)sc^{B57}$ and *Df(2L)ed-dp* are described elsewhere (Lindsley and Zimm, 1992). The Gal4 and UAS lines used were *sca-Gal4* (Baker et al., 1996); *dpp-Gal4* (Staehling-Hampton et al., 1994); UAS-*ed* (Bai et al., 2001); *109-68-Gal4*, UAS-*sc* (Jarman and Ahmed, 1998); *109-68-Gal4*, UAS-*ase* (Jarman and Ahmed, 1998); UAS-*fng-DXDmut-Myc* (Munro and Freeman, 2000); UAS-*ed-GFP* (this work). For UAS-*ed-GFP*, the full-length *ed* ORF was fused in frame to a C-terminal GFP tag from pEGFP-N1 (Clontech), cloned into pUAST, and transformant flies produced by microinjection. Fly stocks were maintained on standard cornmeal-yeast-agar medium. Crosses to UAS-*ed* were performed at 29°C to increase Gal4-activity. All other crosses were performed at 25°C.

Generation of mitotic clones

Mutant clones were induced using the FLP/FRT method (Xu and Rubin, 1993). *ed* clones were created in a *Minute* background [*y w FLP122; M(2)Z armLacZ FRT40A/CyO* flies obtained from A. Garcia-Bellido] marked by the absence of β-galactosidase immunoreactivity and created using a heat-shock inducible *FLP* (first instar larvae were heat-shocked for 1 hour at 37°C). The genotype of the flies was *y w FLP122/y w hsp70-FLP;* ed^{lH23} *FRT40A/M(2)Z armLacZ FRT40A.*

Cell culture

Schneider 2 (S2) cells were maintained at 25°C in Schneider's medium (Sigma) with 10% fetal bovine serum (Invitrogen). Transient transfections were performed using Effectene Transfection Reagent (Qiagen) according to the manufacturer's protocol. Protein expression was induced 24 hours after transfection with a 35 minute heatshock at 37°C. Cells were harvested after a further 24 hours. Constructs used were pCaSpeR-hs-N, pCaSpeR-hs-Dl (provided M. Baron) and pCaSpeR-hs-Ed-Myc, pCaSpeR-hs-Ed-FLAG (Spencer and Cagan, 2003). All transient transfection experiments were repeated at least three times with qualitatively identical results. The numbers in the text are from one experiment. For aggregation, cells were washed and resuspended in fresh medium before mixing with gentle rotation in microtiter plates for 4 hours at room temperature and then processed for immunohistochemistry.

Histology

Scanning electron microscopy (SEM) was performed according to standard procedures and all scanning electron micrographs were taken at 150× magnification on a Cambridge Stereoscan 250. Immunohistochemical staining of third instar larvae was carried out as described (Rawlins et al., 2003). For the pupal wing discs, pupae at 24-26 hours after puparium formation (APF) were dissected in cold PBS and fixed for 1 hour in 4% paraformaldehyde on ice. S2 cells were allowed to adhere to poly-L-Lysine slides for 20 minutes and then fixed in 3.7% formaldehyde for 3 minutes. Antibody staining followed standard procedures. Primary antibodies used were guinea-pig anti-Sens (1:5000; provided by H. Bellen), guinea-pig anti-Hrs (1:1000; provided by H. Bellen), mouse anti-Ac (1:10; Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Elav (1:200; DSHB), mouse anti-Sca (1:200; DSHB), mouse anti-Fasciclin 2 1D4 (1:50; DSHB), mouse anti-N^{ECD} F461.3B (1:50; DSHB), mouse anti-N^{ICD} C17.9C6 (1:50; DSHB), mouse anti-Dl^{ECD} C594.9B (1:50; DSHB), rabbit anti-Dl^{ECD} N2 (1:3000; provided by M. Muskavitch), guinea-pig anti-Dl^{ICD} (1:3000; provided by M. Muskavitch), mouse anti-E(Spl) 323-2-G (1:2; provided by S. Bray), mouse anti-Myc (1:300; NEB), rabbit anti-FLAG (1:300; Sigma), mouse anti-β-galactosidase (1:250; Promega), rabbit anti-β-galactosidase (1:10 000; Cappel), rabbit anti-Ase (1:1000) (Brand et al., 1993) and rabbit anti-Ed (1:5000). For the anti-Ed serum, the *ed* intracellular domain reading frame was fused to the GST tag of the pGEX-2T bacterial expression vector. Using this construct, the protein was expressed, isolated from bacteria, excised from an SDS-PAGE gel and used to immunise four rabbits. Serum was preabsorbed and checked for specificity to Ed by western analysis (1:50 000) and by immunohistochemical staining (1:5000). Secondary antibodies (1:1000) were obtained from Jackson Laboratories or Molecular Probes. Confocal images were taken on a Leica TCS SP microscope.

Results

echinoid mutants have additional external sense organs

Null alleles of *ed* are semi-lethal, and the majority of progeny die during pupation. Surviving adults display extra macrochaetae (sensory bristles) on their thoraces (Fig. 1A,B; Table 1). These additional macrochaetae are always in close proximity to existing wild-type macrochaetae, suggesting that they arise from the existing PNCs by a reduction in lateral inhibition. Consistent with this, Ac protein and *sc* mRNA distributions are similar to wild type, and the extra SOPs [marked by Senseless (Sens) expression] arise from within these clusters (Fig. 1D-I; not shown). All types of adult es organs can be affected in *ed* mutants. For example, the hypomorphic viable allele $ed^{4.12}$ has more thoracic microchaetae compared with wild type (358±6.3 versus 225±8.36) and more wing margin chemosensory bristles (29.8±0.7 versus 19.6±0.45) (at least six flies of each genotype were counted).

The phenotype of the hypomorph $ed^{4.12}$ is identical to that seen upon overexpression of *sc* using a PNC-specific Gal4 line (109-68-*Gal4*/UAS-*sc*) (Jarman and Ahmed, 1998) (A.P.J., unpublished). Moreover, one copy of $ed^{4.12}$ is an enhancer of this phenotype (Table 1). Conversely the $ed^{4.12}$ homozygous

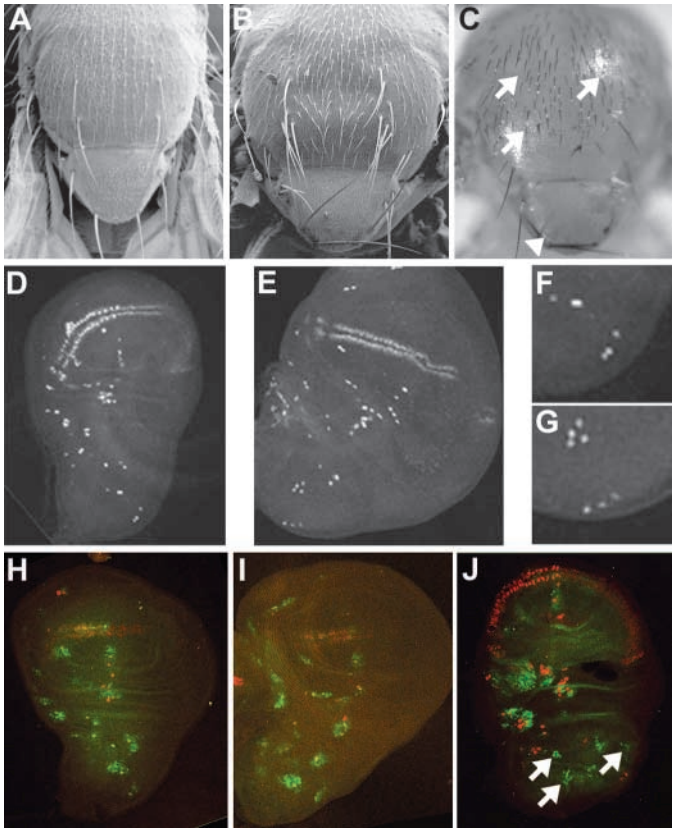

Fig. 1. *echinoid* mutants display additional es organs and SOPs. (A,B) Scanning electron microscopy of the adult thorax. (A) Wild type. (B) $ed^{lH23}/ed^{l(2)k01102}$ showing additional macrochaetae around each wild-type macrochaete location. (C) Light micrograph of *sca-Gal4;* UAS-*ed* thorax. Arrows indicate the positions where macrochaetae should be present. An arrowhead indicates two macrochaetae that have arisen from the same PNC in place of one. (D-J) Confocal microscopy for immunohistochemical detection of (D-G) Sens, and (H-J) Ac (green) and Sens (red) in third larval instar wing discs. (D) Wild type. (E) $ed^{lH23}/ed^{l(2)k01102}$. (F,G) Higher magnification views of scutellar region in (D,E). Note that two SOPs are present at each position (one of the SOPs in F has divided and appears as two adjacent cells). (H) Wild type. (I) $ed^{lH23}/ed^{l(2)k01102}$ showing no difference in Ac level or pattern compared with wild type. (J) *sca-Gal4;* UAS-*ed* wing disc at puparium formation. Arrows indicate proneural clusters that have persisted much later than normal in the absence of SOP specification.

phenotype is suppressed by one copy of a deletion of the entire *ac-sc* complex [$Df(1)sc^{B57}$]. These observations raise the possibility that proneural expression within PNCs is increased in *ed* mutants. However, we found that *sc* mRNA and Ac protein levels do not appear generally increased, except insofar as there are more SOPs (Fig. 1H,I; data not shown). Interestingly, $ed^{4.12}$ is also strongly suppressed by mutation of *asense* (ase^{1}) (Table 1). It is notable that *ase* expression is activated by the transition from PNC to SOP (Brand et al., 1993), suggesting that this transition is facilitated in *ed* mutants.

echinoid interacts genetically with the Notch signalling pathway but not the Egfr signalling pathway

Culí and others have shown that the EGF receptor plays a role in signalling between all the cells of the macrochaetae PNCs, which they term lateral co-operation (Culí et al., 2001). We have previously demonstrated that R8 twinning in *ed* mutants is a consequence of deregulation of Egfr signalling (Rawlins et al., 2003; Spencer and Cagan, 2003). In contrast to the R8 phenotype, the $ed^{4.12}$ bristle phenotype cannot be consistently modified by reducing *Egfr* ($Egfr^{IK35}$/+) or *argos* ($aos^{\Delta 7}$/+) gene dosage (Table 1). Furthermore, we have seen no change in the expression of the Egfr pathway targets *pointed-P1* and dpERK in the wing discs of *ed* mutants (Gabay et al., 1996; Gabay et al., 1997), even though such an effect is seen in the eye (Rawlins et al., 2003) (data not shown). These data suggest that the *ed* bristle phenotype is not due to a direct effect on lateral cooperation. Consistent with this, *ed* mutation affects all thoracic macrochaete locations, whereas only certain proneural clusters have a strong requirement for Egfr signalling (Culí et al., 2001).

In contrast to the above, the $ed^{4.12}$ bristle phenotype interacts strongly with mutations in the Notch pathway (Table 1 and data not shown). This is most marked for *N* and *Dl* themselves.

Table 1. Genetic interactions between *echinoid* and Notch and Egfr pathway mutations

Allelic combination	Average number of DC macrochaetae	Average number of SC macrochaetae	Average number of wing margin recurved bristles
Wild-type (*OrR*)	4.00±0.00, *n*=15	4.00±0.00, *n*=15	19.60±0.45, *n*=15
$ed^{4.12}/ed^{4.12}$	5.40±0.27, *n*=10	5.9±0.31, *n*=10	29.80±0.70, *n*=10
$ed^{LH23}/ed^{l(2)k01102}$	12.0±0.58, *n*=3	7.33±0.88, *n*=3	33.70±1.20, *n*=3
$ed^{l(2)k01102}/Df(2L)ed\text{-}dp$	11.5±1.50, *n*=2	6.50±0.50, *n*=2	ND
$ed^{6.1}/ed^{l(2)k01102}$	12.5±1.60, *n*=2	9.00±1.00, *n*=2	ND
$Df(1)sc^{B57}$/+; $ed^{4.12}/ed^{4.12}$	4.83±0.48, *n*=6	4.17±0.17, *n*=6	ND
ase^{1}/+; $ed^{4.12}/ed^{4.12}$	4.80±0.20, *n*=5	4.40±0.18, *n*=13	ND
109-68Gal4, UAS-*sc* /+	ND	5.40±0.32, *n*=8	ND
109-68Gal4, UAS-$sc/ed^{4.12}$	ND	8.00±0.26, *n*=15	ND
$ed^{4.12}$ $Egfr^{IK35}/ed^{4.12}$ +	5.70±1.42, *n*=18	4.17±0.38, *n*=18	29.30±1.72, *n*=42
$ed^{4.12}/ed^{4.12}$; $aos^{\Delta 7}$/+	6.10±1.08, *n*=15	4.80±0.69, *n*=15	27.70±3.18, *n*=24
$ed^{4.12}/ed^{4.12}$; H^{1}/+	4.0±0, *n*=11	4.0±0, *n*=11	20.5±0.37, *n*=11
sca^{BP2}/sca^{BP2}	5.53±0.19, *n*=28	5.21±0.20, *n*=29	21.82±0.42, *n*=22
$ed^{4.12}$ $sca^{BP2}/ed^{4.12}$ sca^{BP2}	8.42±0.45. *n*=12	4.92±0.25, *n*=12	25.00±0.40, *n*=36

The total number of dorsocentral (DC) and scutellar (SC) macrochaetae and wing margin chemosensory bristles per fly were counted. The mean±s.e.m. is shown. *n*, number of flies that were scored. At least eight of each genotype, with the exception of the null or strongly hypomorphic genotypes $ed^{LH23}/ed^{l(2)k01102}$, $ed^{l(2)k01102}/Df(2L)ed\text{-}dp$ and $ed^{6.1}/ed^{l(2)k01102}$, for which only two or three flies or pharate adults were obtained. ND, categories that were not counted.

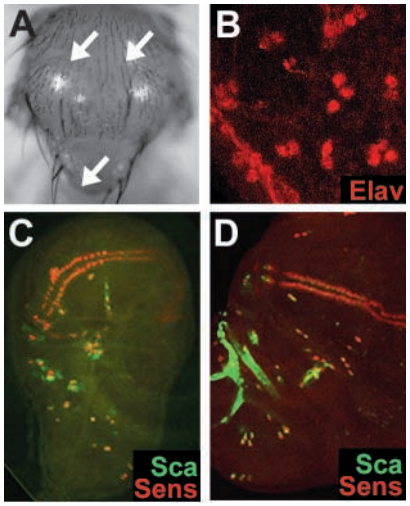

Fig. 2. *echinoid* interaction with *Notch* and *sca.* (A) Light micrograph of $N^{55e11}/+$; $ed^{4.12}/ed^{4.12}$ adult thorax. The arrows indicate the positions that macrochaetae should occupy. (B) Immunohistochemical detection of Elav (neurons) in the $N^{55e11}/+$; $ed^{4.12}/ed^{4.12}$ pupal thorax. Note that the neurons are arranged in groups rather than singly as expected for wild type. (C,D) Immunohistochemical detection of Sca (green) and Sens (red) in third larval instar wing discs. (C) Wild type. (D) $ed^{LH23}/ed^{l(2)k01102}$. Sca expression is unaffected in the *ed* mutant background.

Removing one copy of *N* (N^{55e11}) in an $ed^{4.12}$ background both increases the number of SOPs specified and alters their asymmetric divisions to produce four neurons rather than the wild type es organ (Fig. 2A,B). This is reminiscent of the *N* null phenotype (Guo et al., 1996) and occurs even though null *ed* mutants alone do not display an asymmetric division phenotype. Similarly, $ed^{4.12}$; *Dl*/+ flies have additional shaft cells (data not shown). These genetic interactions suggest that Ed affects the Notch pathway during SOP specification and asymmetric SOP division.

Even in the most severe alleles, which are protein null (e.g. $ed^{6.1}$; E.L.R., unpublished), not all the cells of the proneural cluster become SOPs (unlike *N* mutants for example). Thus *ed* is not essential for lateral inhibition but modulates it. Moreover, no general reduction in *E(spl)* expression could be detected in *ed* mutants (data not shown), suggesting that Ed has a more specific function than a global downregulation of Notch pathway signalling activity. With these characteristics the *ed* mutant resembles *scabrous* (*sca*) null mutants, which display a similar phenotype (Mlodzik et al., 1990). *ed* mutant wing discs shows no loss of Sca protein, so this is not the cause of the *ed* phenotypes (i.e. *ed* is epistatic to *sca*) (Fig. 2C,D). $ed^{4.12}$; sca^{BP2} double mutants have an increase in bristle number that is consistent with the two phenotypes being additive rather than synergistic, suggesting that Ed and Sca are in separate pathways (Table 1).

Ed colocalises with N and Dl at the cell surface and in early endosomes

To address where Ed may function, we raised an antibody to the whole intracellular domain of Ed. The Ed protein detected by this antibody is located at the apical cell membrane of all imaginal disc cells and in the ectoderm of the embryo. It is also present in intracellular vesicles distributed throughout the cytoplasm in all these cells (Fig. 3A,F). A transgene with GFP fused to the C terminus of Ed (UAS-*ed-GFP*) shows an identical distribution when expressed in imaginal discs (Fig. 3B). These vesicles have not been observed in previous reports (Bai et al., 2001; Islam et al., 2003). However, the antibody used in those experiments was raised against the extreme N terminus of the protein, which may be cleaved in vivo and so might not reflect the complete protein distribution (Bai et al., 2001; Spencer and Cagan, 2003). The Ed-containing vesicles are not related to Golgi, because only a small fraction co-label with Fringe-Myc, a Golgi marker (Munro and Freeman, 2000) (Fig. 3C). By contrast, the majority of Ed vesicles in the apical regions of the cells also express the early endosomal marker HRS (Lloyd et al., 2002) (Fig. 3E). Furthermore, in $hook^{1}$ mutants, in which levels of endocytosis are generally decreased (Kramer and Phistry, 1996; Kramer and Phistry, 1999), there was a discernible reduction in the number of Ed-positive vesicles (Fig. 3F,G). These data suggest that the majority of the vesicular Ed visible in the cell is in the endocytic pathway, a large proportion of it being in an early endosomal compartment.

The pattern of Ed is highly reminiscent of the distribution of both Notch and Delta proteins (Klueg et al., 1998; Kooh et al., 1993; Parks et al., 1997; Parks et al., 1995). Indeed, double labelling showed that in the wing discs Ed can colocalise with N^{ECD}, N^{ICD} and Dl both at the cell surface and in vesicles. These vesicles frequently also contain HRS, suggesting that Ed is being endocytosed with N and/or Dl (Fig. 3E; data not shown). In a series of double labelling experiments, most Ed-positive vesicles also contained Dl (94%), or to a somewhat lesser extent N^{ICD} (81%) or N^{ECD} (75%). Such colocalisation was also seen for UAS-*ed-GFP*-containing vesicles (Fig. 3J). Interestingly, Ed does not colocalise as extensively with N and Dl in the eye disc where its function relates to Egfr signalling (48% of Ed vesicles contain Dl in the morphogenetic furrow of the eye) (Fig. 3H,I). Moreover, Ed does not colocalise generally with other endocytosed proteins. The cell surface protein Fasciclin 2 (Garcia-Alonso et al., 1995; Grenningloh et al., 1991), which is also ubiquitously expressed and occasionally endocytosed, is not in the same vesicles as N^{ECD}, Dl or Ed (data not shown). Sca protein is also found in endosomes (Li et al., 2003a), but Sca does not extensively colocalise with Ed (Fig. 3D), presumably because it is located in late endosomes (Li et al., 2003a).

Therefore, the colocalisation of Ed with N and Dl in the wing disc is likely to relate to function rather than being coincidental. Despite this, mutation of *ed* does not affect the frequency of endocytosis of these proteins: the number of N^{ICD}, N^{ECD} and Dl^{ECD} vesicles remains unchanged in *ed* mutant clones (data not shown).

Overexpression of Ed inhibits SOP formation and affects Dl

To determine the effect of overexpression of *ed*, we expressed UAS-*ed* using *sca-GAL4* which drives expression in all proneural clusters. Overexpression of Ed during R8 selection in the eye disc had no effect (Rawlins et al., 2003; Spencer and Cagan, 2003). In notable contrast, *sca-GAL4;*UAS-*ed* flies exhibit many missing bristles (Fig. 1C). Examination of larval

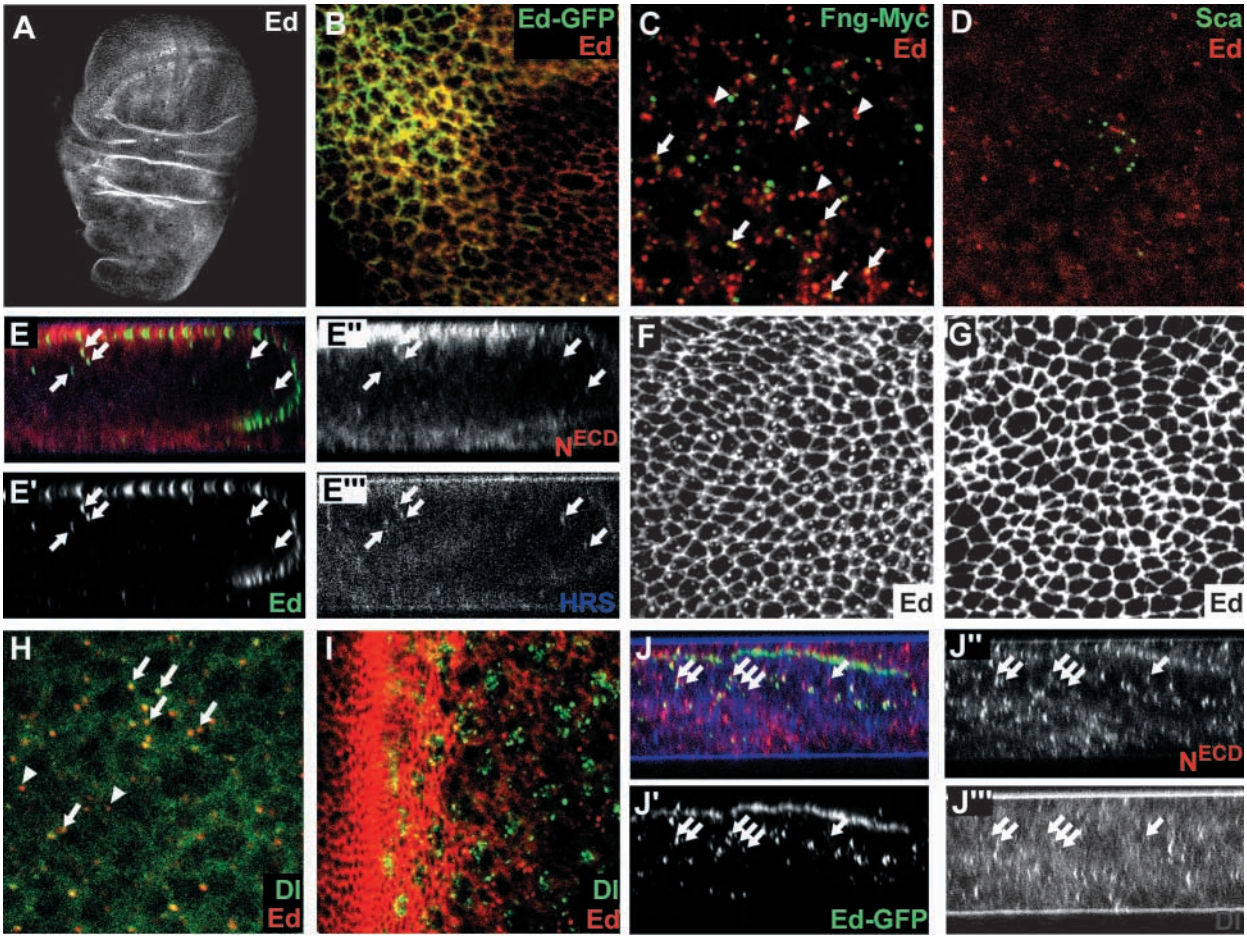

Fig. 3. Echinoid subcellular localisation and colocalisation with Notch and Delta. Third instar larval discs examined by confocal microscopy. (A) Ed protein in wild-type wing disc. (B) *sca-Gal4*/UAS-*ed-GFP*. Detection of Ed-GFP (green) and Ed (red). (C) *sca-Gal4*/UAS-*fng-DXDmut-Myc*. Ed (red) and Golgi represented by Myc (green). Arrows indicate some Ed protein that is in the Golgi. Arrowheads indicate some of the more numerous Ed-only vesicles. (D) Immunohistochemical detection of Sca (green) and Ed (red), showing lack of colocalisation. (A-D) Single confocal sections. (E) Immunohistological detection of Ed (green), HRS (red) and N^{ECD} (blue). Confocal *xzy* projection. Arrows indicate vesicles containing all three proteins. (E′) Green channel (Ed). (E″) Red channel (N^{ECD}). (E‴) Blue channel (HRS). (F,G) Ed expression in confocal projections. (F) Wild type. (G) *hook*^{1} mutant, showing fewer Ed-positive vesicles. (H,I) Immnuohistochemical detection of Dl (green) and Ed (red). Single confocal sections of wild-type discs. (H) Wing disc. Note that the majority of the Ed colocalises with Dl; some of the Ed- and Dl-positive vesicles are marked by arrows. Dl-only vesicles are marked by arrowheads. (I) Eye disc. Ed does not colocalise very strongly with Dl. (J) Immunohistochemical detection of Ed-GFP (green), N^{ECD} (red) and Dl (blue). Confocal *xzy* projection. Arrows indicate vesicles containing all three proteins. (J′) Green channel (Ed-GFP). (J″) Red channel (N^{ECD}). (J‴) Blue channel (Dl).

wing discs at puparium formation revealed that very few SOPs have been selected. In the absence of SOPs, Ac expression persists in many PNCs, owing to lack of lateral inhibition (Fig. 1J). This phenotype is qualitatively similar to those seen upon overexpression of E(Spl)C proteins (Nakao and Campos-Ortega, 1996). Paradoxically, there is also some bristle duplication when Ed is overexpressed: occasionally two SOPs can arise from the edge of the same PNC (Fig. 1C). This may be explained by heterogeneity of misexpression with *sca-Gal4* so that strong UAS-*ed*-induced inhibition of neurogenesis in the centre of a PNC may allow cells at the edge to escape inhibition.

When UAS-*ed* is expressed in the wing disc using *dpp-Gal4*, Ed protein is present in almost all of the Dl-positive vesicles, although there is no change in vesicle number. Strikingly, however, the level of Dl immunofluorescence at the cell surface and in the vesicles is markedly reduced compared with that in adjacent wild-type cells (Fig. 4A,B). By contrast, the levels of N^{ECD} and N^{ICD} are unaffected (Fig. 4C; data not shown). Therefore, inhibition of SOP formation by UAS-*ed* correlates specifically with loss of Dl immunofluorescence, suggesting that Ed function is more closely related to Dl than N, and that Ed plays a role in the trafficking/degradation of Dl.

Homophilic association of Echinoid in culture and in vivo

We used an S2 cell culture assay to explore the cell biology of Ed. It was not possible to detect endogenous Ed in the S2 cells by immunocytochemistry under any circumstances, including manipulation of N and Dl levels (data not shown). Western analysis revealed the presence of endogenous Ed protein, but the relative molecular weight of this protein was higher in

comparison with that of embryo or larval extracts (data not shown). Together, these data suggest that endogenous protein is not processed correctly to form a functional molecule at the cell surface. When S2 cells were transfected with an Ed-expressing plasmid ('transfected with Ed'), exogenous Ed also could not be detected at the membrane. However, such cells can form large aggregates in which Ed protein could be observed at the regions of cell contact (Fig. 5A). This is consistent with the recent conclusion that Ed functions as a homophilic adhesion molecule in S2 cells (Islam et al., 2003). To investigate Ed endocytosis, separate populations of S2 cells were transfected with either FLAG-tagged Ed or Myc-tagged Ed, and allowed to aggregate for 4 hours before inspection. The majority of the Ed protein (FLAG or Myc) remained at the cell membrane, although some cis-endocytosis of Ed did occur (Fig. 5A). Trans-endocytosis of Ed was never observed in these or subsequent experiments. This suggests that the vesicular Ed observed in vivo represents cis-endocytosis.

Clones of homozygous *ed* mutant cells were induced in heterozygous imaginal discs. In $ed^{6.1}$ or $ed^{4.4}$ clones (protein null alleles of *ed*) there was no Ed protein on the membrane of cells within the clone and protein is lost from the membrane

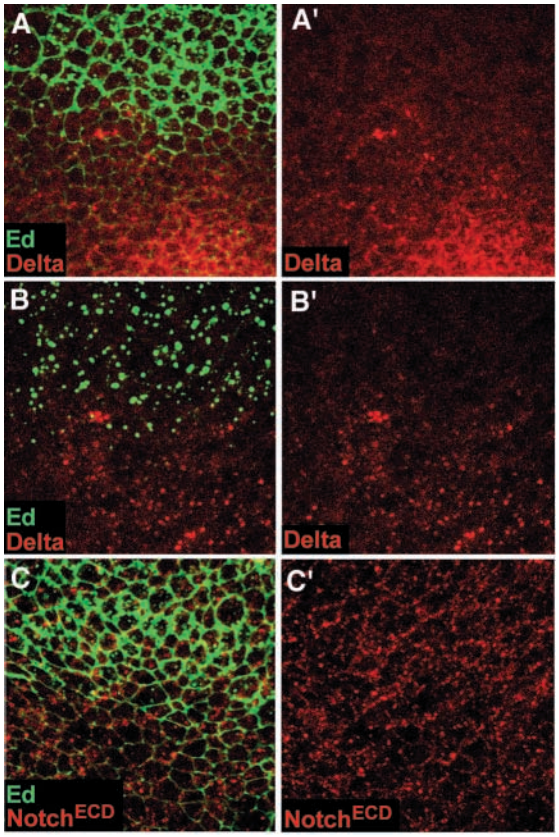

Fig. 4. Overexpression of Echinoid affects Dl levels but not N. Single confocal sections of *dpp-Gal4*/UAS-*ed* wing discs. (A,B) Immunohistochemical detection of Ed (green) and Dl (red). (A) Apical cell surface. (A′) Red channel (Dl). (B) Confocal section taken just below the apical cell surface. (B′) Red channel (Dl). (C) Immunohistochemical detection of Ed (green) and N^{ECD} (red). (C′) Red channel (N^{ECD}). The levels of Dl fluorescence are decreased both at the cell membrane and in intracellular vesicles while the levels of N are unaffected.

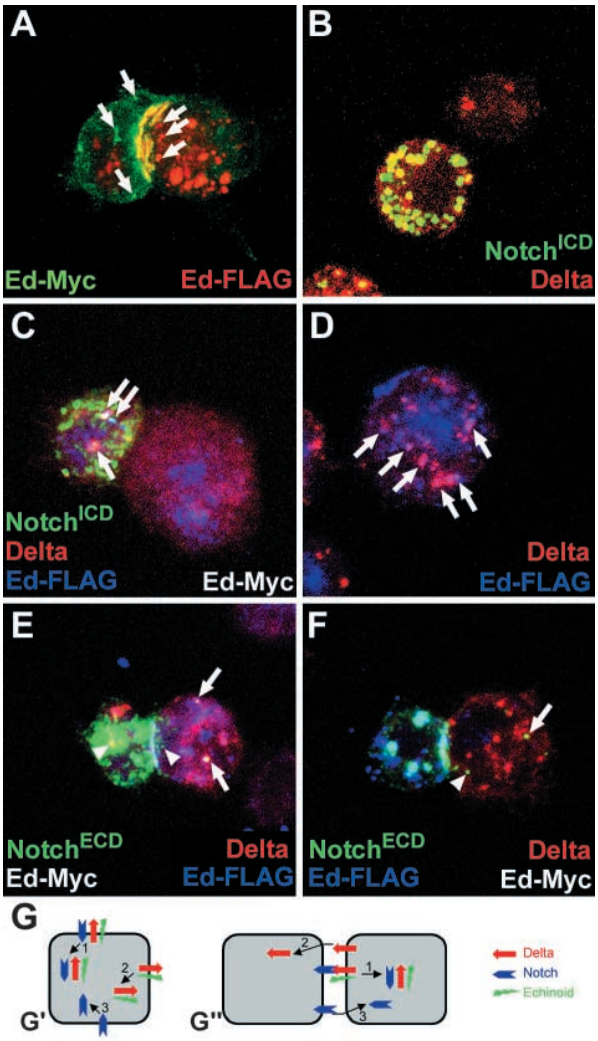

Fig. 5. Echinoid is associated with the cis-endocytosis of Dl in S2 cells. Immunohistochemical detection and confocal microscopy of N, Dl, Ed-Myc and Ed-FLAG in transiently transfected S2 cells. (A) Ed-Myc-expressing cell (green) contacting an Ed-FLAG-expressing cell (red). Ed is visible at the region of contact between the cells and in cis-endocytic vesicles (arrows). (B) Single N- (N^{ICD}, green) and Dl (red)-expressing cell. (C) N- (N^{ICD}, green), Dl- (red) and Ed-FLAG (blue)-expressing cell contacting an Ed-Myc-expressing cell. Arrows indicate cis-endocytic vesicles containing all three proteins. The anti-FLAG antibody also shows some non-specific staining of cell nuclei. (D) Single Dl- (red) and Ed-FLAG (blue)-expressing cell. Dl and Ed are frequently located in the same vesicle (arrows). (E) N- (N^{ECD}, green) and Ed-Myc-expressing cell (not detected) contacting a Dl- (red) and Ed-FLAG (blue)-expressing cell. Ed is cis-endocytosed with Dl as N is trans-endocytosed (arrows). Trans-endocytosis of N or Dl alone is not associated with Ed (arrowheads). (F) N (N^{ECD}, green) and Ed-FLAG (blue)-expressing cell contacting a Dl- (red) and Ed-Myc (not visible)-expressing cell. Ed is not trans-endocytosed with N into the signalling cell. Trans-endocytosis of N or Dl alone is not associated with Ed (arrowheads). (G) Cartoon summary of S2 cell experiments. (G′) In isolated S2 cells Ed is associated with the cis-endocytosis of Dl/N together (1) and Dl alone (2), but not with N alone (3). (G″) In contacting S2 cells Ed is associated with the cis-endocytosis of Dl (1), which is necessary for trans-endocytosis of N^{ECD} during N activation (Parks et al., 2000). It is not associated with other trans-endocytosis events seen in S2 cells that are unrelated to N activation (2, 3).

of wild-type cells immediately adjacent to the mutant cells (not shown). The same is true of clones of a severe loss-of-function allele, ed^{LH23}, except that a small amount of protein remains in this allele (Fig. 6B). The loss of protein from wild-type cell membranes shows that cell contact per se is not sufficient for Ed accumulation at the membrane; Ed must be present on the opposite cell membrane. These results are consistent with a central role for homophilic protein interaction in vivo.

Homophilic association suggests that Ed could be required in either signalling and recipient cells, or both. To assess whether Ed behaves cell autonomously or cell non-autonomously, we examined wing margin SOP specification at the borders of *ed* mutant clones. In 25% of clone borders scored (n=91), adjacent mutant and wild-type cells both become SOPs (Fig. 6A). In these cases, *ed* mutant cells display both a reduction in signalling capacity and a decreased sensitivity to signalling. The mutant cell is becoming an SOP in spite of signalling from the adjacent wild-type SOP, but equally it is incapable of preventing the wild-type cell from becoming an SOP. This autonomous and non-autonomous behaviour suggests that Ed is required in signalling and receiving cells. Such 'twinned SOPs' are not observed in *N* mutant clones (Heitzler and Simpson, 1991) and only rarely in *Dl* clones (Pavlopoulos et al., 2001). These data are consistent with the observations that support homophilic binding and suggest that such binding may be important for function in vivo.

Seventy-five percent of clone borders do not show twinned SOPs, which we interpret as consistent with the fact that significant lateral inhibition still occurs in null *ed* alleles. Of these clone borders, 71% show a mutant SOP adjacent to wild-type non-SOP cells and 39% show a wild-type SOP adjacent to mutant non-SOP cells (n=68). The predominance of the former may reflect the greater density of SOPs within the mutant clone.

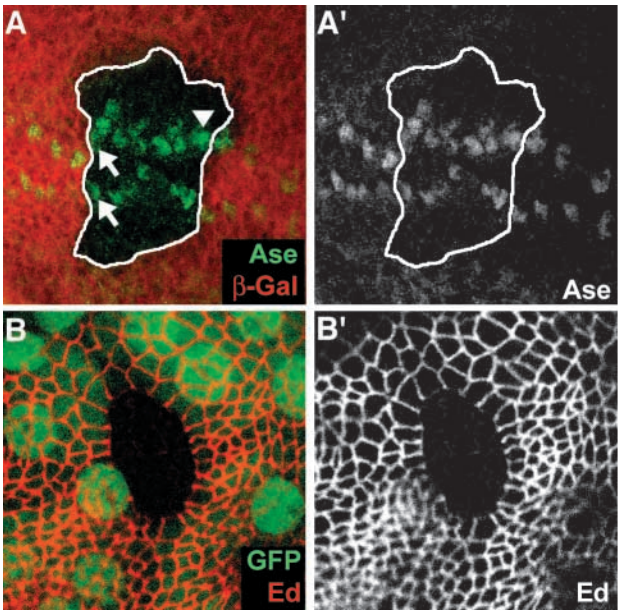

Fig. 6. Genetic mosaic analysis of Echinoid. (A,B) Mosaic analysis of ed^{LH23} in third larval instar imaginal discs examined by confocal microscopy. (A) Immunohistological detection of Ase (green) and β-galactosidase (red) in an ed^{LH23} genetic clone in a wing disc. The ed^{LH23} homozygous region is marked by the absence of β-galactosidase and the clone border has been marked by a white line. The arrows indicate cell-autonomous specification of SOPs. The arrowhead labels a pair of adjacent SOPs; one mutant, one wild type. (A′) The green channel (Ase). (B) Immunohistological detection of Ed (red) in an ed^{LH23} mosaic leg disc. The ed^{LH23} homozygous region is marked by the absence of nlsGFP (green), the remainder of the disc is either wild type or heterozygous for ed^{LH23}. The mutant cells express very little Ed; the wild-type cells at the clone border show a large drop in the amount of Ed on the region of membrane that touches the mutant cells of clone. (B′) The red channel (Ed) from B.

The behaviour of Echinoid in cell culture in relation to N and Dl

In vivo, a number of distinct cellular processes are thought to govern N and Dl signalling and processing (Seto et al., 2002) and it is not easy to determine whether Ed colocalisation correlates with a particular event. The S2 cell assay allows a functional dissection of the various interactions between N and Dl. For example, S2 cells do not endogenously express N and Dl, but transfection with N- and Dl-expressing plasmids causes them to aggregate because of their heterophilic association in trans (Fehon et al., 1990). In isolated (non-contacting) S2 cells co-transfected with both N and Dl, these proteins can be detected at the cell surface and in vesicles, showing that they can undergo cis-endocytosis together (Fig. 5B) (Fehon et al., 1990). Such cis-endocytosis of N and Dl is suggested to be linked to inhibition of trans signal reception in recipient cells (Klueg et al., 1998; Kooh et al., 1993; Parks et al., 2000). When Dl/N expressing cells are also co-transfected with Ed-FLAG, the latter colocalises with the N/Dl-positive vesicles (74% contain Ed-FLAG) and the Dl-positive vesicles (66%) but is rarely seen in vesicles on its own or with N alone (12%) (not shown). This pattern is unchanged upon association (by aggregation) with an Ed-Myc expressing cell (Fig. 5C). This is consistent with the observation in vivo that Ed/N^{ICD}/Dl can be present in the same vesicle (Fig. 3J), and it implies that this in vivo distribution is most likely to reflect cis-endocytosis of N, Dl and Ed together from the cell membrane. Consistent with these results, when Ed-FLAG is co-expressed with Dl alone, the two proteins colocalise well in vesicles (81% of the Dl-positive vesicles also contain Ed), whether the cells are solitary (Fig. 5D) or aggregated (data not shown). This is true to a lesser extent for N and Ed (23% of the N-positive vesicles also contain Ed). Therefore in S2 cells, Ed is cis-endocytosed with Dl, and to a lesser extent N.

Upon association of Dl with N, there is evidence that N^{ECD} must be endocytosed with Dl into the signalling cell in order to trigger N^{ICD} release in recipient cells (Parks et al., 2000). This event can be observed in S2 cells: when N-expressing cells are aggregated with Dl-expressing cells, one can visualise trans-endocytosis of the N^{ECD} and Dl into the signalling cell (Klueg et al., 1998; Parks et al., 2000). To investigate whether Ed is associated with Dl during this event, we aggregated N/Ed-Myc cells with Dl/Ed-FLAG cells and examined vesicle composition for Ed-FLAG epitope. This showed that more than 95% of the N^{ECD}/Dl vesicles in the signalling (i.e. Dl-expressing) cell also contained Ed-FLAG (Fig. 5E). In the reciprocal experiment, N/Ed-FLAG expressing cells were aggregated with Dl/Ed-Myc expressing cells. In this case,

transfer of Ed-FLAG into N^{ECD}/Dl vesicles in the signalling cell was only rarely observed (7%) (Fig. 5F). In these experiments full-length N and Dl are occasionally transferred between cells, but Ed is never associated with them (Fig. 5E,F). These two experiments suggest that, in addition to the cis-endocytosis of Ed with Dl in an isolated cell, Ed is also associated in cis with the cis-endocytosis of Dl that occurs upon N activation. In summary, in S2 cells Ed is not associated with all aspects of N/Dl trafficking, rather it seems to be involved specifically with Dl cis-endocytosis (Fig. 5G).

In all of the above experiments in S2 cells, the amount of N or Dl endocytosis or N activation was never consistently altered by the presence of Ed and vice versa (not shown). Moreover, in multiple experiments, we found no evidence that colocalisation of Ed and Dl/N represents a direct molecular interaction. Cells transfected with Ed-FLAG could not aggregate with cells transfected with N or Dl. Moreover, association between N-expressing and Dl-expressing cells did not redistribute Ed-FLAG to the cell contact when it was co-transfected in one or other population. Therefore in these assays, Ed does not behave as though binding heterophilically to N or Dl. It is conceivable that such a heterophilic interaction may occur in vivo and that it may be contingent on the homophilic binding of Ed.

Discussion

Ed is a modulator of Notch pathway signalling during SOP specification. Furthermore, both in vivo and in culture, Ed protein is strongly associated with Dl at the cell membrane and in the early endosome compartment. Several lines of evidence suggest that Ed self associates in trans. Ed expression promotes the adhesion of cultured cells (Islam et al., 2003), while our genetic clonal analysis shows that in vivo Ed protein cannot accumulate at the cell membrane if it is absent from the adjacent cell. Moreover, this genetic analysis suggested that such a trans interaction might be important for function.

Echinoid is a modulator of SOP singling out

Ed is not essential for Notch signalling but has a modulatory effect. The basis of this effect must be relatively subtle, as we find no strongly visible difference in expression pattern, level, or subcellular localisation of Dl, N or E(spl) in *ed* mutant clones. We favour the idea that Ed influences PNC resolution as part of the specific process that drives the singling out of individual SOPs. In other words it is a part of a 'symmetry breaking' apparatus (Li et al., 2003b). There are two lines of evidence to suggest that Ed functions to inhibit the transition from PNC cell to SOP. First, no more than four SOPs are selected from each PNC even in null *ed* alleles. Second, *ed* interacts particularly strongly with *ase*, which is expressed on the transition from PNC to SOP. We suggest that the role of *ed* is analogous to that proposed for *sca* (Li et al., 2003a). Based on analysis in the eye, it is envisaged that singling out causes several cells to begin to become resistant to Dl ('pre-SOPs'), but a specific genetic mechanism involving *sca* and *gp150* causes all but one of these unwanted SOPs to revert and once again become responsive to Dl from the selected precursor (Li et al., 2003a). Our hypothesis is that, like *sca*, *ed* functions to promote N receptor activation in these pre-SOPs. Despite these similarities between *sca* and *ed* function, our genetic evidence suggests that they take part in parallel processes. Moreover, Sca and Gp150 are located in late endosomes, whereas Ed is located at the membrane and in early endosomes.

Echinoid is closely associated with Delta

In vivo and in cultured cells, Ed protein colocalises very strongly with Dl in cis, both at the membrane and in early endosomes. It is possible that there is a direct molecular interaction between the two proteins, but we have no evidence so far for this. Such an association may require Ed-Ed homophilic binding.

Nevertheless, colocalisation suggests a close and specific association with Dl-N signalling. One possibility is that Ed promotes Dl function in the 'true' SOP, leading to more efficient suppression of the emergence of unwanted SOPs. Cis-endocytosis of Dl into the signalling cell is apparently required for activation of the Notch receptor (Parks et al., 2000), and one could envisage that *ed* may enhance this process in the SOP. This is supported by the colocalisation of Ed with N and Dl during N activation as observed in our cell culture analysis.

An alternative is that *ed* may inhibit Dl activity in recipient (non-SOP) cells. There is evidence that such reduction of Dl activity may promote unidirectional signalling in two ways. First, it would free an SOP from inhibition by surrounding cells. Second, it has been suggested that Dl in recipient cells antagonises their response to trans signalling, perhaps by cis association of Dl and N (Jacobsen et al., 1998; Sakamoto et al., 2002). Therefore, *ed* inhibition of this antagonistic function of Dl would make non-SOP cells more vulnerable to signalling from the SOP. We see no difference in Dl distribution and level in *ed* mutant clones, but suspect that this might only be apparent in the pre-SOPs. However, after overexpression of Ed, we observe a striking and specific decrease in Dl both at the membrane and in vesicles. Remarkably, this correlates with SOP loss, which the opposite phenotype to that normally expected for loss of Dl. Thus, Ed function may be connected to the downregulation of Dl in recipient cells. Proteolysis and endocytosis of Dl have both been implicated as causing its downregulation (Lai et al., 2001; Mishra-Gorur et al., 2002). It is feasible that Ed promotes one of these processes, for example by helping to present Dl to Kuzbanian for cleavage.

Ed affects Notch and EGFR pathways independently

ed mutants have twinned R8 photoreceptors in the eye and additional es organ SOPs everywhere. A priori one would imagine these phenotypes to have the same genetic and mechanistic basis. They appear, however, to indicate the interaction of *ed* interaction with two different signalling pathways. We, and others, showed that Ed negatively regulates Egfr signalling (through direct interaction with pathway components) during R8 specification (Rawlins et al., 2003; Spencer and Cagan, 2003). This is in contrast to the role of Ed during es organ specification, where it modulates Notch pathway signalling. There are several other reasons for concluding that the R8 and SOP phenotypes of *ed* mutants, although superficially similar, have different origins. The latter, but not the former, is sensitive to overexpression of Ed protein. For R8, this is explained because Ed is regulated by EGFR post-translationally and so absolute protein levels are unimportant (Spencer and Cagan, 2003). Sensitivity of SOP

singling out to Ed protein levels suggests a different mechanism is at play. Most strikingly, Ed protein is colocalised extensively with N and Dl in the wing disc cells, but not in the eye disc, where interestingly there appears to be very little N and Dl on the cell surfaces (Kooh et al., 1993). Therefore, all this suggests the conclusion that the two phenotypes do indeed have different origins, and moreover that there are significant differences in SOP singling out compared with R8 precursor selection.

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