Echinoid synergizes with the Notch signaling pathway in *Drosophila* mesothorax bristle patterning

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Accepted 9 September 2003

Development 130, 6305-6316
Published by The Company of Biologists 2003
doi:10.1242/dev.00869

Summary

***echinoid* (*ed*) encodes an immunoglobulin domain-containing cell adhesion molecule that negatively regulates the Egfr signaling pathway during *Drosophila* photoreceptor development. We show a novel function of Ed, i.e. the restriction of the number of notum bristles that arise from a proneural cluster. Thus, loss-of-function conditions for *ed* give rise to the development of extra macrochaetae near the extant ones and increase the density of microchaetae. Analysis of *ed* mosaics indicates that extra sensory organ precursors (SOPs) arise from proneural clusters of *achaete-scute* expression in a cell-autonomous way. *ed* embryos also exhibit a neurogenic phenotype. These phenotypes suggest a functional relation between *ed* and the Notch (N) pathway. Indeed, loss-of-function of *ed* reduces the expression of the N pathway effector *E(spl)m8* in proneural clusters. Moreover, combinations of moderate loss-of-function conditions for *ed* and for different components of the N pathway show clear synergistic interactions manifested as strong neurogenic bristle phenotypes. We conclude that Ed is not essential for, but it facilitates, N signaling. It is known that the N and Egfr pathways act antagonistically in bristle development. Consistently, we find that Ed also antagonizes the bristle-promoting activity of the Egfr pathway, either by the enhancement of N signalling or, similar to the eye, by a more direct action on the Egfr pathway.**

Key words: *echinoid*, *Notch*, EGF receptor, Cell adhesion, Signaling, Bristle patterning

Introduction

The dorsal mesothorax of *Drosophila* is a classical model with which to study pattern formation (reviewed by Jan and Jan, 1994). On each heminota, 11 macrochaetae develop in precise positions and over 100 microchaetae appear in a characteristic density pattern. Each of these external sensory organs (SOs) comprises five cells (hair, socket, neuron, sheath cell and glial cell) that are generated through three asymmetric cell divisions of a SO precursor (SOP) (Gho et al., 1999; Reddy and Rodrigues, 1999). During third instar larval and early pupal stages, SOPs are selected from small groups (20-30 cells) of wing imaginal disc cells, known as proneural clusters, that express the proneural genes *achaete* (*ac*) and *scute* (*sc*), two members of the *achaete-scute* complex (AS-C) (reviewed by Campuzano and Modolell, 1992). Proneural genes encode basic helix-loop-helix (bHLH) transcriptional factors and confer to cells the ability to become SOPs (reviewed by Bertrand et al., 2002).

In the notum territory of the imaginal wing disc, the pattern of proneural clusters prefigures the adult pattern of chaetae (Cubas et al., 1991; Skeath and Carroll, 1991). Although many cells within a proneural cluster are competent to become SOPs, they are prevented from doing so by the mechanism of lateral inhibition mediated by the receptor Notch (N) and its ligand Delta (Dl) (reviewed by Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Simpson, 1997). According to current thinking, proneural genes activate Dl, which upon interaction with N, triggers the proteolytic cleavage of the extracellular domain of N by a Kuzbanian/ADAM family protease and produces N^{ECN} (Lieber et al., 2002). Then, a g-secretase complex, including at least Presenilin, Niscatrin, Aph1 and Pen2 mediates another cleavage within the transmembrane domain to release the intracellular domain of N (N^{ICD}) (Chung and Struhl, 2001; Hu et al., 2002; López-Schier and St Johnston, 2002; Struhl and Greenwald, 1999; Ye et al., 1999). N^{ICD} translocates to the nucleus where it displaces Hairless (H) and acts in association with Suppressor of Hairless [Su(H)] and Mastermind to activate transcription of the Enhancer of split complex [E(spl)-C] (Bailey and Posakony, 1995; Barolo et al., 2002; Fryer et al., 2002; Lecourtois and Schweisguth, 1995). Members of the E(spl)-C in turn prevent, in the signal-receiving cells, self-stimulation of proneural genes, and this leads to suppression of SOP cell fate (Culí and Modolell, 1998; Giagtzoglou et al., 2003; Heitzler et al., 1996). Conversely, the future SOP, which becomes insensitive to lateral inhibition and does not express E(spl)-C genes (Jennings et al., 1995), continues to accumulate AS-C proneural proteins by this self-stimulation mechanism that

involves the binding and activation of Ac, Sc and Asense to SOP-specific enhancers of the proneural genes (Culí and Modolell, 1998; Giagtzoglou et al., 2003).

In contrast to the lateral inhibition mediated by N, which prevents SOP cell fate, the Egfr signaling pathway favors the SOP fate – lateral stimulation – by promoting the proneural gene self-stimulatory loops (Culí et al., 2001). Egfr signaling is mediated by the conserved Ras/Raf/MAPK signaling cassette. Excess Egfr signaling promotes ectopic *sc* expression and the production of extra SOPs, while reduced Egfr signaling results in decreased *sc* expression and the loss of SOPs (Culí et al., 2001; Díaz-Benjumea and García-Bellido, 1990). Thus, the Egfr and N pathways act antagonistically in bristle development. Interestingly, this Egfr activity is important for the SOPs of the notum macrochaetae, but much less so for microchaetae or for the tergite bristles (Culí et al., 2001; Díaz-Benjumea and García-Bellido, 1990).

echinoid (*ed*) encodes a cell adhesion protein with seven Ig domains, two fibronectin type III (Fn III) domains and a transmembrane (TM) domain, followed by a 315 amino acid intracellular domain with no identifiable functional motif (Bai et al., 2001). *ed* mutant flies exhibit extra photoreceptor and cone cells in the eye. Conversely, overexpression of *ed* in the eye leads to a decrease in photoreceptor cell number. In addition, *ed* genetically interacts with several components of the Egfr pathway. These results have suggested that *ed* is a negative regulator of the Egfr pathway. Based on genetic mosaic and epistatic analyses, it has been proposed that Ed, via homotypic interactions, activates a novel pathway that antagonizes Egfr signaling by regulating the activity of the TTK88 transcriptional repressor, the most downstream component of the Egfr pathway (Bai et al., 2001). However, it has been shown very recently that during R8 cell selection, Ed negatively interacts with the Egfr pathway at a step upstream from the phosphorylation of the MAP kinase (Rawlins et al., 2003; Spencer and Cagan, 2003). This and other evidence obtained mostly with cell culture assays have allowed them to propose an alternative model in which Ed antagonizes Egfr function by direct interaction between the Ed and Egfr molecules.

In addition to the homophilic adhesive activity, Ed also exhibits a heterophilic trans-interaction with Neuroglian (Nrg), an L1-type CAM. L1-type proteins are composed of six Ig domains, three to five Fn III repeats and a cytoplasmic domain with a conserved ankyrin binding site. Co-expression of *ed* and *nrg* in the eye exhibits a strong genetic synergy in inhibiting Egfr signaling and this effect requires the intracellular domain of Ed, but not that of Nrg. Together, these results suggest a model in which Ed functions as a receptor and is activated by either its own homophilic interaction or by an heterophilic ligand like Nrg (Islam et al., 2003).

In addition to the eye phenotype, we noticed the presence of ectopic bristles over the body parts of *ed* mutant flies. In this study, we use the development of mesothoracic bristles – macrochaetae and microchaetae – as an experimental model with which to explore the interactions between Ed, Notch, and Egfr pathways. We show that loss-of-function mutations at the *ed* locus or overexpression of a dominant-negative form of Ed in proneural clusters promote development of extra macrochaetae near the extant ones and increase the density of microchaetae. These effects are due to *ed* mutant cells within proneural clusters giving rise to extra SOPs. Our genetic data suggest that Ed participates in lateral inhibition within proneural clusters and facilitates N signaling. It also antagonizes the Egfr pathway, either by the enhancement of N signaling or by a more direct interaction. In a parallel study, Ahmed et al. have shown the interaction between *ed* and *N* in the embryonic CNS, and in bristle and wing vein patterning (Ahmed et al., 2003).

Materials and methods

The *Drosophila* stocks used in this study were: ed^{1x5}, ed^{slH8}, ed^{lF20}, *Df(2l)ed-dp*, *UAS-ed* (Bai et al., 2001); *UAS-*N^{ECD}, *UAS-*N^{ICD} (de Celis and Bray, 1997); *UAS-*Dl^{DN} (Huppert et al., 1997); *UAS-*raf^{gof} (Brand and Perrimon, 1994); *UAS-Egfr*, *UAS-*$Egfr^{DN}$ (Buff et al., 1998); *hs-*N^{ICD} (Lieber et al., 1993); *hs-*N^{ECN} (Rebay et al., 1993); N^{55e11} (Brennan et al., 1997); Ax^{M1} (Díaz-Benjumea and García-Bellido, 1990); N^{mcd1} (Ramain et al., 2001); H^{2} (Bang et al., 1991); Dl^{rev10} (Haenlin et al., 1990); *ap-Gal4* (Calleja et al., 1996); *sca-Gal4* (Hinz et al., 1994); *C765-Gal4* (Gómez-Skarmeta et al., 1996); and *C253-Gal4* (Culí et al., 2001).

Molecular biology

The *UAS-*ed^{intra} was made by subcloning the intracellular domain of Ed into the *pUAS* vector (Brand and Perrimon, 1993). The *UAS-*$ed^{\Delta ECD}$, *UAS-*$ed^{\Delta ECD\text{-}48}$ and *UAS-*$ed^{\Delta ECD\text{-}124}$ were generated by subcloning either the transmembrane plus the entire intracellular domain of Ed, or deleting the last C-terminal 48 and 124 amino acids, respectively, into the *pUAS* vector.

To identify molecular lesions in *ed* mutants, genomic DNA, prepared from homozygous *ed* mutant larvae, was used as template in PCR reactions to amplify the entire *ed* sequence. Multiple PCR reactions were pooled and sequenced for each allele.

Mosaic analysis

To generate clones of cells mutant for *ed*, either *yw hs-FLP122* f^{36a}*; ck Pf[+]30B FRT40/CyO* or *yw hs-FLP122; P[ubi-GFP] FRT40/CyO* females (stocks described in FlyBase) were crossed with *w;* ed^{1x5} *FRT40/CyO* males. To generate M^{+} clones we either crossed *w hs-FLP122; P[arm lacZ] M(2)z, FRT40/CyO* females with *w;* ed^{1x5} *FRT40/CyO* males or *y w f36a hs-FLP122;* ed^{1x5} *FRT40/CyO* females with f^{36a}*; M(2)z Pf[+]30B FRT40/CyO* males (*M(2)z* stocks were from the collection of A. García-Bellido). Recombination was induced by heat treatment at 72-96 hours after egg laying for 1 hour at 37°C (Xu and Rubin, 1993).

To produce germline clone embryos deficient for *ed*, the FRT/FLP/DFS technique was used (Chou and Perrimon, 1996). Maternal and zygotic mutant embryos were identified by mating germline clone-bearing virgin females with males carrying ed^{lF20}*/Cyo, wg-lacZ*, and selecting the non-*lacZ* embryos.

Histochemistry

Antibody staining was performed as described [Anti-Sc, anti-Sens and anti-β-galactosidase (Cubas et al., 1991); mAb22C10 (Hartenstein and Posakony, 1990); anti-ELAV (Islam et al., 2003); anti-N^{ICD} (mAb9C6) (Parks et al., 2000)]. Polyclonal rabbit anti-Ed antibodies were generated against a synthetic peptide, corresponding to the C-terminal region of Ed (GEYSTTPNARNRRVIREIIV) and were used at a dilution of 1:200. Secondary antibodies were from Jackson and Amersham. Hybridizations in situ to detect *E(spl)m8* mRNA were performed as described (González-Crespo and Levine, 1993) using an antisense DIG-labeled RNA probe. Discs from control wild-type and overexpressing larvae were hybridized and processed in parallel to allow comparison.

Table 1. Number of macrochaetae/heminotum in *ed* mutant conditions

	ed^{1x5}/ed^{slH8}	*Df(2l)ed-dp/* ed^{slH8}	ed^{lF20}/ed^{slH8}	*apGAL4; UAS-*$ed^{\Delta ECD}$	*C253; UAS-*$ed^{\Delta ECD}$	*C253; UAS-*ed^{III}	*C765; UAS-*ed^{X}	*apGAL4; UAS-*$Egfr^{DN}$	ed^{1X5}/ed^{slH8} *apGAL4;UAS-*$Egfr^{DN}$
ANP+PNP	2.10	1.80	2.04	2.68	3.73	1.23*	2.25	1.61	2.15
PS	1.28	1.27	1.19	1.60	0.97	0.90	1.25	0.70	1.05
ASA	1.13	1.06	1.07	1.48	1.00	0.95	1.15	0.50	0.80
PSA	1.05	1.01	1.06	1.15	1.07	0.88	1.03	0	0
APA	1.80	1.87	1.87	1.05	1.00	0.98	1.20	0	0.60
PPA	1.85	2.11	2.09	1.00	0.92	1.00	1.03	0	0
ADC+PDC	2.80	3.00	2.54	3.60	2.10	1.58	2.85	0.78	1.30
ASC+PSC	2.63	2.24	3.24	3.48	2.50	0.58	3.70	1.55	2.25

*At this position, there were flies with missing macrochaetae and others with macrochaetae duplications (0.65 duplications/heminotum). Extra macrochaetae at other positions in these flies was <0.10 per heminotum.

Results are averages of at least 40 heminota examined.

Results

Loss-of-function mutations at the *ed* locus promote development of extra bristles

Previously, we observed the presence of extra photoreceptor and cone cells in the eyes of *ed* mutant flies (Bai et al., 2001). These observations lead us to uncover the interaction between Ed and Egfr signaling pathway. In addition to the eye phenotype, we noticed the presence of ectopic bristles on the body of these animals. Hence, we examined the function of *ed* in bristle formation. We used three mutant alleles whose associated lesions were molecularly analyzed. ed^{slH8}, an hypomorphic mutation, contains a mis-sense codon that changes the absolutely conserved cysteine (amino acid 618) of the sixth Ig domain into a serine. This should disrupt the characteristic disulfide bond, and therefore the overall structure of the Ig domain (Walsh and Doherty, 1997), and might lead to weaker homo- and/or heterophilic interactions of the *ed* extracellular domain. ed^{lF20} and ed^{1X5}, two homozygous lethal alleles, have stop codons in the first (amino acid 63) and fifth (amino acid 524) Ig domains, respectively. As both alleles lack the intracellular and transmembrane domains and only encode part of the extracellular domain, they should be at least strong hypomorphs or probably null alleles. The combination of either of these alleles with ed^{slH8} permits viability to adulthood. The notum of the resulting flies displayed an increased density of microchaetae and extra macrochaetae (Table 1 and Fig. 1A,B). The latter always arose very near to the position of the wild-type macrochaetae. Extra bristles also appeared in other parts of the fly, as on the head, legs, abdominal regions and at the wing margin (not shown). This phenotype is very similar to that caused by a failure of lateral inhibition, which permits extra SOPs to arise from a proneural cluster of *ac/sc* expression (Simpson and Carteret, 1990). The phenotype of the two combinations and that of the viable ed^{slH8} over the deficiency of the locus were very similar (Table 1), consistent with the amorphic condition of ed^{lF20} and ed^{1X5}.

We examined the phenotype of the homozygous condition for ed^{1X5} in mitotic recombination clones induced by the FRT method. The clones had poor viability. In imaginal wing discs, the precursor epithelia of the notum and wings of the fly, they were much smaller than the wild-type twins, and many twins had no associated mutant clone (Fig. 1D). This occurred all over the wing disc, which indicated a generalized requirement for the function of *ed*, consistent with its ubiquitous expression (not shown). Induction of ed^{1X5} homozygous clones in a background of ubiquitous forced expression of full-length Ed protein (Gal4 system) (Brand and Perrimon, 1993) permitted viability of many clones (Fig. 1K,L). This indicated that their poor survival was indeed due to the absence of the Ed protein. Moreover, the presence of this protein rescued the smooth contours of the ed^{1X5} clones (Fig. 1K), and they became uneven, like those of the wild-type clones (Fig. 1L). This suggests that Ed participates in the regulation of cell affinity.

Within the ed^{1X5} clones, at positions near extant SOPs for the notum macrochaetae, extra SOPs were detected by staining with an anti Senseless (Sens) antibody (Nolo et al., 2000). Often, these SOPs corresponded to homozygous ed^{1X5} clones comprising just a single cell (Fig. 1D,E). This suggests that reaching the SOP state improved the viability of the mutant cells. Adults bearing these clones displayed single extra macrochaeta of the mutant phenotype (f^- marker; Fig. 1F) congruent with the very small size of the clones.

To improve the recovery of the homozygous ed^{1X5} cells, we used the M^+ technique (Morata and Ripoll, 1975). ed^{1X5}; M^+ clones were viable and they contained extra SOPs when they included regions from where the extant SOPs arose (Fig. 1G). No ectopic SOPs were observed at positions far from these regions. Moreover, clones never contained clusters of SOPs, suggesting that the mechanism of lateral inhibition was still active in the clones. As expected from these observations, on the adult cuticle, the ed^{1X5} M^+ could give rise to groups of macrochaetae (Fig. 1H) or areas of increased density of microchaetae (Fig. 1I,J). In both cases, the bristles were separated by epidermal cells. In the discs, extra SOPs always appeared within the clones, indicating that the *ed* phenotype was cell autonomous. Similar results were obtained with the ed^{lF20} allele.

Overexpression of *ed*

We assessed the effect of *ed* overexpression on bristle patterning using two *UAS-ed* lines (*UAS-*ed^{X} and *UAS-*ed^{III}, names refer to their chromosomal positions). *UAS-*ed^{III} driven by the *C253-Gal4* line, which is expressed in proneural clusters relatively late in development (third instar larvae and early pupa) (Culí et al., 2001), caused a mild suppression of notum macrochaetae and the appearance of some extra macrochaetae at the notopleural position (Table 1). With another driver also expressed in proneural clusters (*sca-Gal4*), these effects became more pronounced (Fig. 1C). With Gal4 lines promoting earlier and generalized expression at the notum

[*C765-Gal4* (Gómez-Skarmeta et al., 1996) or *MS1096-Gal4* (Milán et al., 1998)] there was little effect with *UAS-ed*^{III}, but with the *ap-Gal4* driver (Calleja et al., 1996) at 20°C macrochaetae were removed from some positions and extra bristles were generated in others (not shown). With the *UAS-ed*^{X} line and with the generalized drivers *C765-Gal4* and *MS1096-Gal4* extra macrochaetae appeared in all notum positions (Table 1 and not shown). Hence, the overexpression of full-length Ed can cause phenotypes similar to those of the loss-of-function mutations of *ed* and suggest that an excess of full-length Ed can act as a dominant negative.

Generation of a dominant-negative form of Ed

A form of the Ed protein with a deletion of its extracellular domain (*UAS-ed*$^{\Delta ECD}$, Fig. 2F) was overexpressed either early in the whole dorsal compartment of the wing disc (*ap-Gal4* driver) or late in the proneural clusters (*C253-Gal4* driver) using either one or two copies of *UAS-ed*$^{\Delta ECD}$ (Table 1, Fig. 2E). In all cases, phenotypes similar to those of the loss-of-function *ed* mutant combinations were observed, except that the PA positions seemed more insensitive to the overexpression of *UAS-ed*$^{\Delta ECD}$ than to the *ed* hypomorphic combinations (Table 1). More extra macrochaetae developed in the presence of two copies of *UAS-ed*$^{\Delta ECD}$ (Fig. 2E) than in flies with only one copy (Fig. 3A), while microchaetae density was not further increased. With stronger drivers (*sca-Gal4* and *MS248-Gal4*) (Cavodeassi et al., 2002; Sánchez et al., 1997) more macrochaetae or even tufts of macrochaetae developed, but always occurred at or near the wild-type macrochaetae positions (Fig. 3G and not shown). These and other data indicated that Ed$^{\Delta ECD}$ behaves as a dominant-negative form of Ed. Indeed, *UAS-ed*$^{\Delta ECD}$ driven by *ap-Gal4* and *GMR-Gal4* produce flies with extra wing veins and rough eyes, respectively, phenotypes similar to the *ed* hypomorphic combinations (not shown) (Bai et al., 2001). Moreover, the removal of one wild-type copy of *ed* increased the number of extra macrochaetae generated by *UAS-ed*$^{\Delta ECD}$ (*C253-Gal4* driver) (not shown). The additional deletion of either the 48 C-terminal amino acids or the transmembrane domain of Ed$^{\Delta ECD}$ rendered the construct ineffective (Fig. 2F), suggesting that this

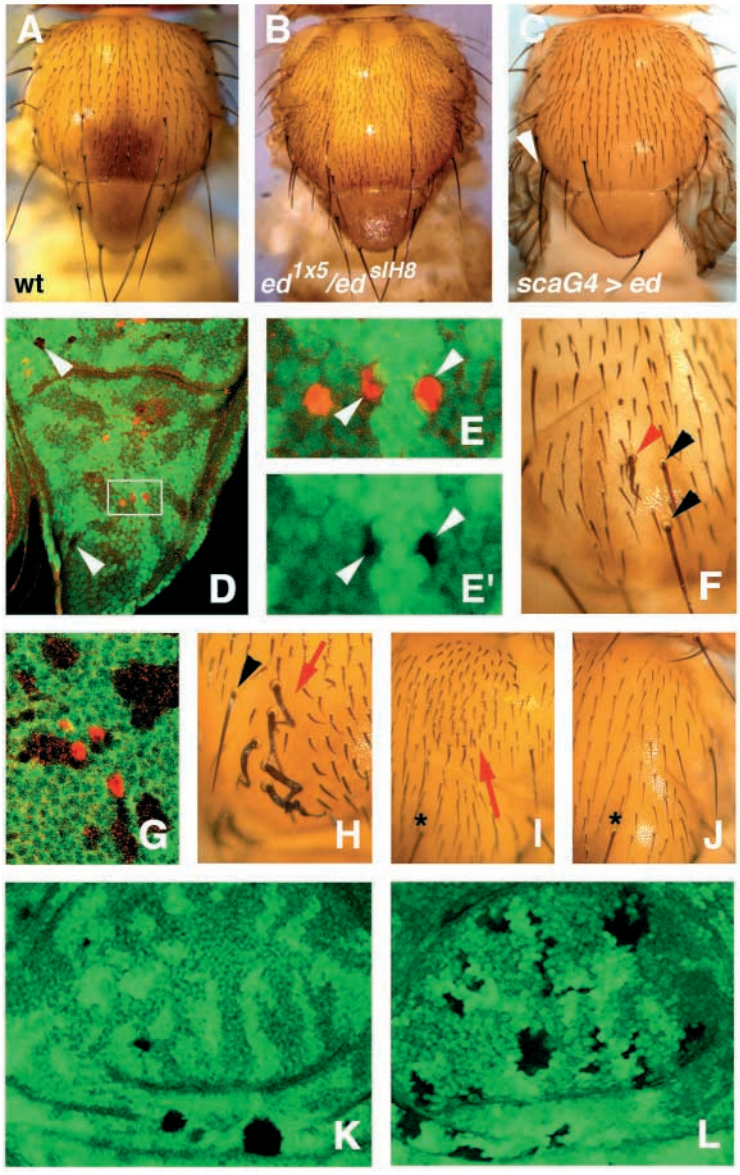

Fig. 1. *ed* mutations promote generation of extra bristles in the notum of *Drosophila*. (A-C) Nota from wild-type, *ed*^{1X5}/*ed*^{slH8} and *sca-Gal4; UAS-ed*^{III} flies, respectively. On B, note the extra macrochaetae arising near the extant ones and the increased density of microchaetae with respect to A. (C) Expression of *UAS-ed*^{III} partially suppressed macrochaetae (such as the dorsocentrals and scutellar, and some microchaetae) and also caused occasional duplication of some macrochaetae (arrowhead). (D,E,E′) Prospective notum of a third instar wing disc harbouring *ed*^{1x5} homozygous clones (absence of the GFP green fluorescence, arrowheads). Boxed area in A is shown at greater magnification in E and E′ (E′, green channel only). Note the very small size of the clones, which in some cases consist of a single cell (arrowheads in D,E,E′) that has been singled out as an SOP (red, Sens marker). One of the two *ed*^{1x5} SOPs corresponds to an ectopic SO, because only two SOPs arise from a wild-type DC proneural cluster. The twin wild-type clones (bright green) consist of many cells, indicating the poor viabilty of the *ed*^{1x5} homozygous cells. (F) An extra DC *f*^{36a} *ed*^{1x5} macrochaeta (red arrowhead) that may have arisen from a clone similar to those in E, but which probably consisted of more than a single cell, as two mutant microchaetae have developed adjacent to the extra macrochaeta. Black arrowheads indicate the ADC and PDC macrochaetae. (G) *ed*^{1x5} *M*$^{+}$ clones, induced at 72-96 hours after egg laying (absence of green marker) survive well in a *M*$^{+/-}$ background and promote development of extra SOPs (red, Sens marker) when they include cells of a proneural cluster, in this case the DC one. No extra SOPs were observed outside the *ed*^{1x5} *M*$^{+}$ clones. (H,I) Groups of extra bristles (labeled with *f*^{36a}) develop within *ed*^{1x5} *M*$^{+}$ clones. Five *f*^{36a} DC macrochaetae are shown in H (red arrow) and a patch of *f*^{36a} microchaetae in I (red arrow; clones were induced at 48-72 hours after egg laying). The increased density of microchaetae can be seen by comparing with the same region of a wild-type notum (J). Asterisks indicate the ADC macrochaeta. Black arrowhead in H indicates a *f*$^{+}$ DC macrochaeta displaced from its position by the *ed*^{1x5} extra macrochaetae. (K,L) Prospective wing of third instar discs harbouring *ed*^{1x5} homozygous clones (absence of the GFP green fluorescence) in a wild-type or *UAS-ed*^{III}/*C765-Gal4* background, respectively. The expression of *UAS-ed*^{III} largely increased the number of surviving clones and that changed their contours from smooth to uneven.

terminus of the protein and its attachment to the membrane are necessary for the dominant-negative effect.

Effects on *ac/sc* and *E(spl)m8* expression

We examined whether the generation of extra chaetae in positions near the extant ones that occurs in *ed* loss-of-function conditions was due to an increase in the levels of *ac/sc* expression in proneural clusters. This was not the case. Accumulation of Sc protein was not appreciably modified in discs expressing two copies of *UAS-ed$^{\Delta ECD}$* driven by *C253-Gal4* (Fig. 2A,B), except in some individual cells, which did accumulate high levels of Sc protein. This is a characteristic of SOPs (Cubas et al., 1991; Culí and Modolell, 1998; Skeath and Carroll, 1991), and their nature was verified by their accumulation of Sens protein, a marker of SOP identity (Nolo et al., 2000) (Fig. 2B). Thus, at least part of these cells should correspond to the precursors of the extra bristles generated by Ed$^{\Delta ECD}$.

Extra bristles also arise from proneural clusters under conditions of decreased N signaling (de Celis et al., 1991a; Heitzler and Simpson, 1991; Simpson and Carteret, 1990). As the bHLH genes of the E(spl)-C are targets of this signaling pathway (reviewed by Artavanis-Tsakonas et al., 1995), we examined the expression of the *E(spl)-m8* gene, which is known to mediate lateral inhibition in proneural clusters (de Celis et al., 1996; Jennings et al., 1995). The levels of *E(spl)-m8* mRNA were clearly decreased in discs expressing *UAS-ed$^{\Delta ECD}$* (Fig. 2C,D). This suggested that interference with *ed* function somehow reduced N signaling.

Interactions between *ed* and N signaling in bristle development

Prompted by the above results, we searched for genetic interactions between *ed* and members of the N signaling pathway. Halving the gene dose of *N*, by using the null *N^{55e11}* allele in heterozygous condition, had a minimal effect on notum chaetae, as it only slightly increased the density of microchaetae (compare Fig. 1A with Fig. 3E). However, the combination *N^{55e11}/+; ed^{1x5}/ed^{slH8}* showed a strong effect, as microchaetae were almost totally suppressed (Fig. 3F). The number of extra macrochaetae was only slightly increased over that of the *ed^{1x5}/ed^{slH8}* flies, but often they had double shafts. As *ed^{1x5}/ed^{slH8}* is a relatively week *ed* loss-of-function condition, we examined the phenotypes of other genetic combinations. Expression in proneural clusters (*C253-Gal4* driver) of either *UAS-ed$^{\Delta ECD}$* or *UAS-N^{ECD}*, a dominant-negative form of N that lacks most of the intracellular domain (Jacobsen et al., 1998), had relatively mild effects (Fig. 3A,B). By contrast, expression of both transgenes together removed most microchaetae and either eliminated macrochaetae or replaced them with tufts of bristles (Fig. 3C). This is a strong neurogenic phenotype. The tufts of bristles result from breakdown of lateral inhibition in proneural clusters, whereas

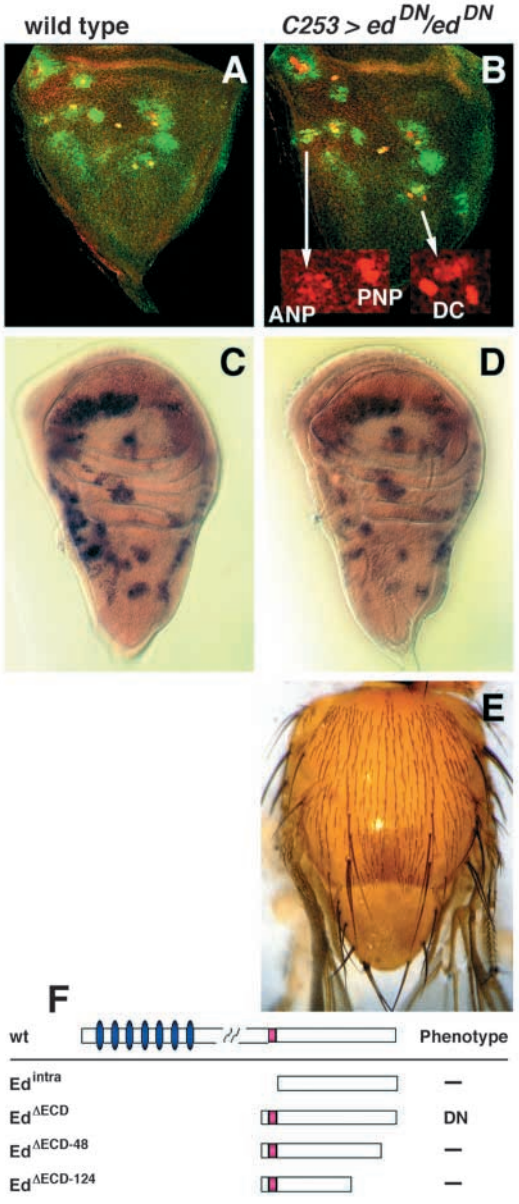

Fig. 2. Accumulation of Sc, Sens and *E(spl)-m8* mRNA in wild-type (A,C) and *C253-Gal4; UAS-ed$^{\Delta ECD}$/UAS-ed$^{\Delta ECD}$* (B,D) wing discs. (A,B) Notum regions showing that the levels of Sc protein in proneural clusters (green) are not significantly modified, except for the presence of extra SOPs in the disc expressing *UAS-ed$^{\Delta ECD}$* (B). These also accumulate Sens protein (red channel), as shown in magnified images (insets) of the anterior and posterior notopleural (ANP, PNP) and dorsocentral (DC) proneural clusters. As many extra SOPs develop late, their accumulation of Sens is lower than that of earlier emerging SOPs. (C,D) Expression of *E(spl)-m8* is decreased in the proneural clusters of discs expressing the Ed dominant-negative protein. Images show representative discs from a sample of 41 wild-type and 22 *UAS-ed$^{\Delta ECD}$*-expressing discs. (E) Notum from a fly with the same genotype (*C253-Gal4; UAS-ed$^{\Delta ECD}$/UAS-ed$^{\Delta ECD}$*) of the discs shown in B and D. Note the increased number of macrochaetae arising from proneural clusters (compare with Fig. 1A). (F) Physical structure of Ed derivatives overexpressed in UAS constructs. Ed contains the extracellular and transmembrane (TM, red) domains, followed by the 315 amino acid intracellular domain. Ed^{intra} only contains the intracellular domain. Ed$^{\Delta ECD}$ lacks the extracellular domain but contains the TM and intracellular domain. Ed$^{\Delta ECD-48}$ and Ed$^{\Delta ECD-124}$ are similar to Ed$^{\Delta ECD}$ but lack the C-terminal 48 and 124 amino acids, respectively. As overexpressed with either *ap-Gal4*, *sca-Gal4* or *C253-Gal4*, only *UAS-ed$^{\Delta ECD}$* exhibited ectopic macrochaetae and increased density of microchaetae, and is referred as a dominant negative (DN). Overexpression of the rest of UAS constructs had no effect on bristle pattern (–).

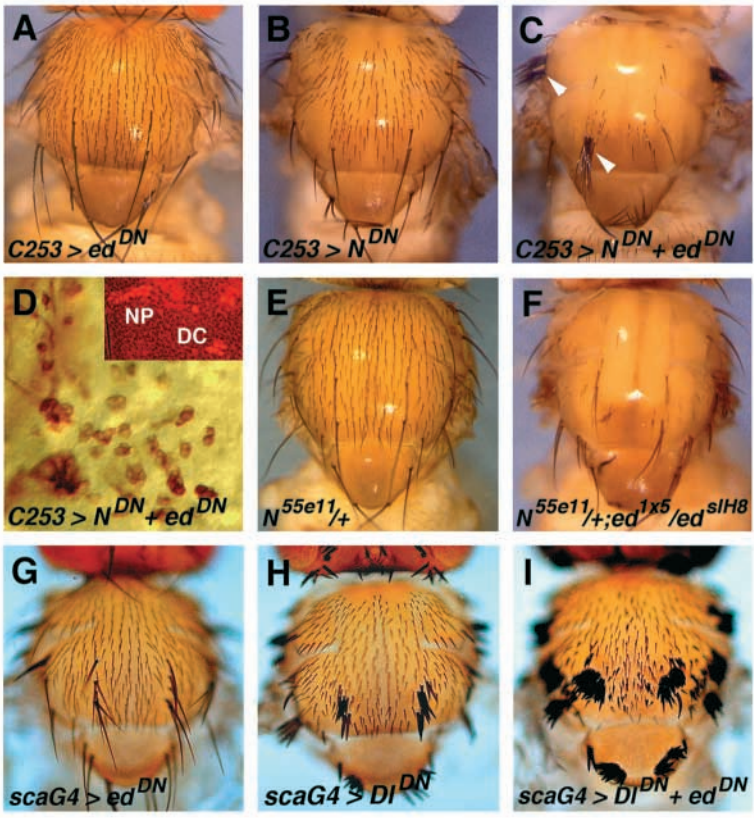

Fig. 3. Synergistic interaction between *ed* and the *N* pathway. (A-C) Nota of flies expressing in proneural clusters (*C253-Gal4* driver) either *UAS-ed*$^{\Delta ECD}$ (abbreviated *ed*^{DN}) (A), *UAS-N*^{DN} (B), or *UAS-N*^{DN} plus *UAS-ed*$^{\Delta ECD}$ (C). Note the strongly enhanced neurogenic phenotype in C: the replacement of extant macrochaetae by tufts of bristles (arrowheads) and the loss of many macro and most microchaetae. (D) Notal dorsocentral region of a *C253*; *UAS-N*^{DN}; *UAS-ed*$^{\Delta ECD}$ pupa stained with 22C10 antibody. Clusters of neurons appear at the sites of the developing sensory organs, as a result of the loss of N signaling, which leads to the differentiation of the descendants of the pIIa precursor cells as extra neurons. In the wild type, only a single neuron innervates each notum bristle. Inset depicts part of the third instar notum region of a *C253*; *UAS-N*^{DN}; *UAS-ed*$^{\Delta ECD}$ larva stained with anti Sens antibody. Note the large clusters of SOPs at the DC and NP positions. (E,F) Nota of *N*^{55e11} (E) and *N*^{55e11}; *ed*^{1x5}/*ed*^{slH8} flies (F). Similar to C, the simultaneous decrease of *N* and *ed* functions increases the neurogenic phenotype manifested by the almost complete absence of microchaetae (compare with *ed*^{1x5}/*ed*^{slH8} notum, Fig. 1B). (G-I) Nota of flies expressing in proneural clusters (*sca-Gal4* driver) either *UAS-ed*$^{\Delta ECD}$ (G), *UAS-Dl*^{DN} (H) or *UAS-Dl*^{DN} plus *UAS-ed*$^{\Delta ECD}$ (I). The neurogenic phenotype caused by a decrease of *Dl* function is potentiated by the simultaneous decrease of *ed* function, resulting in large tufts of macrochaetae and increased density of microchaetae.

the absence of micro and macrochaetae is normally caused by the precursors of the epidermal constituents of the SO (basal cell and shaft) differentiating as extra neurons because of the absence of N signaling (Hartenstein and Posakony, 1990). This occurred under our experimental conditions. mAb 22C10 staining of pupal nota revealed groups of neurons (Fig. 3D) instead of the single, well-separated neurons, each one innervating an individual chaeta, typical of the wild-type notum (not shown) (Hartenstein and Posakony, 1990). As expected, groups of contiguous SOPs were detected in the imaginal discs of these flies (Fig. 3D, inset). Synergistic interactions were also found by overexpressing *UAS-ed*$^{\Delta ECD}$ and *UAS-Dl*^{DN} (Huppert et al., 1997) with the *sca-Gal4* driver (Fig. 3G-I). Moreover, the halving of the genetic dose of *Dl* (*Dl*^{rev10}/+) did not affect the notum macrochaetae (not shown), but it did increase the number of extra macrochaetae promoted by *ed*^{1x5}/*ed*^{slH8} (Table 2).

We conducted genetic epistasis experiments to help characterize the interaction between *ed* and the N signaling pathway. *Hairless* (*H*) is a negative regulator of the effector of the pathway, the Su(H) transcription factor (Barolo et al., 2002; Mumm and Kopan, 2000). Hence, decreasing the dose of H is equivalent to increasing Su(H) activity and, thereby, N signaling. *H*^{2}/+ flies displayed a weak suppression of macrochaetae and an essentially normal pattern of microchaetae (Table 2 and data not shown). Still, the *H*^{2}/+; *ed*^{1x5}/*ed*^{slH8} combination showed that this relatively weak increase of N signaling almost completely eliminated the extra macrochaetae promoted by the decrease of *ed* function (Table 2). (*H*^{2}/+ did not reduce the high density of microchaetae typical of *ed*^{1x5}/*ed*^{slH8}, indicating again that *H*^{2}/+ has little if any effect on the microchetae pattern; not shown.) As the phenotype of macrochaetae suppression was epistatic over that of macrochaetae duplication, *ed* seemed to function in steps of the N signaling pathway upstream of the H/Su(H) interaction (reviewed by Artavanis-Tsakonas et al., 1999).

Next, we examined the effect of constitutive activation of the pathway. Overexpression of the intracellular domain of the N protein (*UAS-N*^{ICD}) (Mumm and Kopan, 2000) with the *C253-Gal4* driver removed essentially all bristles, a phenotype unmodified by reduction of *ed* function (*UAS-ed*$^{\Delta ECD}$) (not shown). Although this is consistent with *ed* acting upstream of the release of N^{ICD} into the cytoplasm, it could also result from the strong activation of the pathway, which might make

Table 2. Genetic interactions between *ed* and N pathway mutations

	SOs	Extra SOs		
	H^{2}/+	*ed*^{1x5}/*ed*^{slH8}	*ed*^{1x5}/*ed*^{slH8}; *H*^{2}/+	*ed*^{1x5}/*ed*^{slH8}; *Dl*^{rev10}/+
ANP+PNP	2.00	0.10	0	0.53
PS	0.97	0.28	0	0.58
ASA	1.00	0.13	0	0.23
PSA	1.00	0.05	0.11	0
APA	1.00	0.80	0	1.33
PPA	0.90	0.85	0.18	2.30
ADC+PDC	1.60	0.80	0.11	0.76
ASC+PSC	1.92	0.63	0	1.00

Figures show number of sensory organs (SO) or extra SOs on the distinct positions of the heminotum. Extra SOs are scored only when more than one SO appear on each position. Results are averages of 28-40 heminota examined.

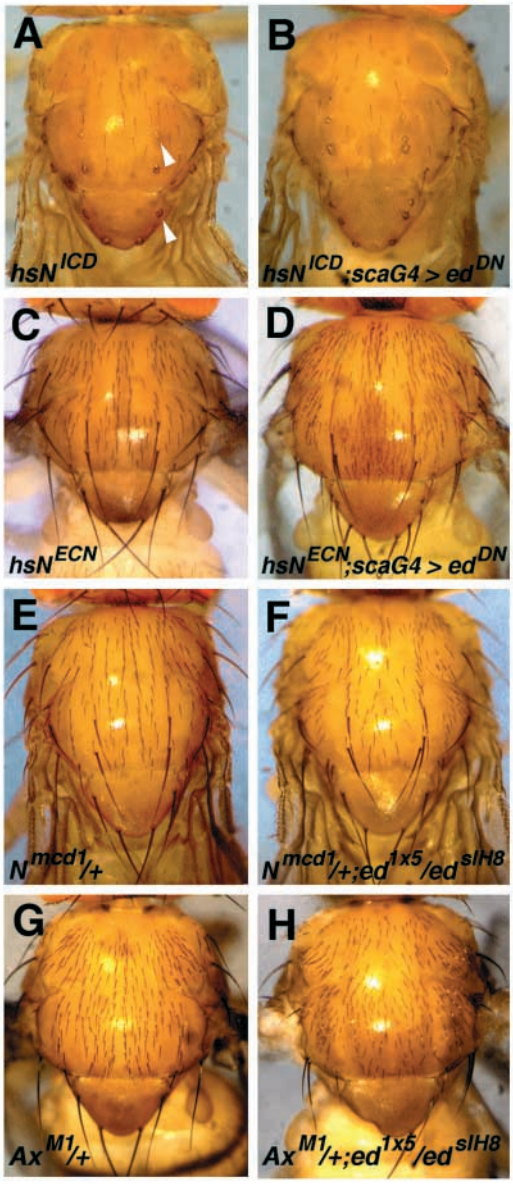

Fig. 4. *ed* acts in the canonic N pathway, before the cytoplasmic release of N^{ICD}. (A,B) Removal of most microchaetae by expression of *hs-N*^{icd} in 0-8 hours-old pupae (A) is not rescued by *UAS-ed*$^{\Delta ECD}$ driven by *sca-Gal4* (B). The control shown in Fig. 3G indicates that *UAS-ed*$^{\Delta ECD}$ driven by *sca-Gal4* was active during microchaetae determination, as it increased microchaetae density. The transformation of macrochaetae to double sockets, owing to the excess of N signaling during differentiation, is clearly visible in some cases (arrowheads). (C,D) In a similar experiment, the weak removal of microchaetae by *hs-N*^{ECN} (C) is not rescued by *UAS-ed*$^{\Delta ECD}$ (D). (E,F) The low density of microchaetae typical of *N*^{mcd1}/+ (E) is not rescued by decreasing *ed* function (F, *ed*^{1X5}/*ed*^{slH8} combination). (G,H) The absence of macrochaetae characteristic of *Ax*^{M1}/+ (G) is not rescued by *ed*^{1X5}/*ed*^{slH8} (H).

ineffective the antagonizing effect of the loss-of-function of *ed*. Thus, we examined the effect of milder activations of the pathway. We resorted to transient activations and administered 1.5 hour heat shocks (37°C; 0-8 hours after puparium formation) to individuals harboring a *hs-N*^{ICD} transgene (Lieber et al., 1993; Struhl et al., 1993). This eliminated most microchaetae (Fig. 4A), as their SOPs emerge during or just after the heat shock (Rodríguez et al., 1990; Usui and Kimura, 1993). By contrast, it did not prevent macrochaetae determination, which occurred before the heat shock treatment [Fig. 4A; note that most macrochaetae were converted to double sockets due to the excess of N signaling during differentiation (Schweisguth and Posakony, 1994)]. Under these conditions, expression of *UAS-ed*$^{\Delta ECD}$ (*sca-Gal4* driver) gave rise, as expected, to extra macrochaetae (extra 'double sockets'), but did not rescue the loss of microchaetae (Fig. 4B), again suggesting that *ed* functions previously to N^{ICD} release into the cytoplasm. An even milder overactivation of the N pathway was accomplished by a similar heat treatment of individuals carrying an *hs-N*^{ECN} transgene (Rebay et al., 1993). N^{ECN} has the N^{ICD} fragment bound to the transmembrane domain of N and this only permits a slow release of N^{ICD}. Heat-treated *hs-N*^{ECN} flies lost microchaetae on only a relatively small region of the notum (Fig. 4C). Still, expression of the *UAS-ed*$^{\Delta ECD}$ could not rescue this weak phenotype, although as expected it promoted the emergence of extra macrochaetae (Fig. 4D). All these data suggest that *ed* may interact with the N pathway in processes previous to the release of the N^{ICD}.

N activity also participates in a pathway independent of Su(H) which affects neural competence (reviewed by Martínez-Arias et al., 2002). Gain-of-function *N* alleles that affect this Su(H)-independent pathway prevent SOP emergence by interfering with formation of proneural clusters. We examined whether *ed* functioned in this alternative pathway. *N*^{mcd1} is a modification of the intracellular domain of N that decreases the number and density of microchaetae of the notum (Martínez-Arias et al., 2002; Ramain et al., 2001). *ed*^{1x5}/*ed*^{slH8} was unable to rescue the loss of microchaetae (Fig. 4E,F). Consistently, *ed*^{1x5}/*ed*^{slH8} did not affect the loss of macrochaetae that occurs in *Ax*^{M1}/+ flies (Fig. 4G,H), another GOF mutation that affects expression of *ac/sc* in proneural clusters (Martínez-Arias et al., 2002). These results, together with the absence of effect of *ed* loss-of-function conditions on *sc* expression (Fig. 2A,B), suggests that *ed* mainly interacts with N-dependent lateral inhibition. However, we cannot rule out that *ed* may affect both lateral inhibition and neural competence, if Ed interacts with the N pathway prior to the separation of these two functions.

Ed colocalizes with N at the zonula adherens of wing imaginal disc cells

Our epistatic and clonal analyses are compatible with Ed facilitating N signaling by acting at a step previous to the release of the N^{ICD}. Accordingly, we tested the possibility that Ed might physically interact with N. First, we examined the subcellular localization of both proteins in the wing imaginal disc. Using antibodies that recognize the C terminus of Ed and the zonula adherens marker Armadillo (Arm), we observed that Ed mainly, if not exclusively, accumulates at the zonula adherens where it colocalizes with Arm (Fig. 5A-C). This is in sharp contrast to the eye disc, where Ed resides throughout the cell membrane of all cells (Islam et al., 2003). Using N^{ICD}-specific antibodies, we further observed that N is mainly colocalized with Ed (Fig. 5D-F). Similar colocalization with Ed at zonula adherens can also be detected with N^{ECN}-specific antibodies, but Ed is not present in the N^{ECN}-containing

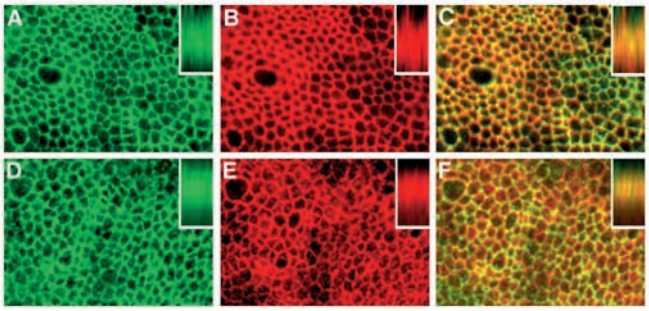

Fig. 5. Ed colocalizes with N at the zonula adherens. (A-C) Third instar larval wing discs were double-labeled with anti-Ed antibodies (green) and anti-Arm antibodies (red). Both Ed and Arm are colocalized at the zonula adherens. (D-F) Wing discs were double labeled with anti-Ed antibodies (green) and anti-N^{ICD} antibodies (red). N colocalizes with Ed to the zonula adherens. Insets in A-F show the corresponding *z* sections along the apicobasal axis of the epithelium; apical is towards the top.

internalized vesicles (data not shown) (Pavlopoulos et al., 2001).

The colocalization of Ed and N at zonula adherens and the observation that the intracellular domain of Ed is required for the dominant-negative effect prompted us to determine whether the intracellular domain of both proteins might also physically interact with each other. We performed both GST pull-down and yeast two-hybrid assays. We did not observe detectable binding between the intracellular domain of N and either the entire intracellular domain or the last 50 amino acids of Ed (data not shown). This suggests that the functional interaction between Ed and N is not mediated by a direct interaction between both proteins, although the possibility still remains that a physical interaction might occur via their extracellular domains.

ed produces a moderate neurogenic phenotype in the embryo

As *ed* promotes development of extra bristles by affecting N signaling, we examined whether *ed* also affects early neural development. Removal of N signaling causes all the neuroectodermal cells to develop as neuroblasts (de la Concha et al., 1988; Lehmann et al., 1981). Eighty percent (n=59) of ed^{lF20} (null) mutant germline clone-derived embryos lacking both maternal and zygotic *ed* expression exhibited ventral holes in the cuticle (Fig. 6A,B), while the rest of embryos (20%) displayed only fusion of ventral denticle belts (data not shown). Both effects indicate a dearth of epidermal precursors. Furthermore, we detected a moderate hyperplasia of the embryonic nervous system, as revealed by the increase in the number of ELAV-positive cells in stage 14 embryos (Fig. 6C,D). Clearly, *ed* embryos exhibit a N-like phenotype, although weaker than those of mutations at the neurogenic genes (de la Concha et al., 1988; Lehmann et al., 1981).

Antagonistic activities between Ed and Egfr pathway

Thus far, our results indicate that Ed cooperates with the N pathway to control the determination of notum macrochaetae. Because Egfr and N pathways act antagonistically in macrochaetae development (Culí et al., 2001), we examined

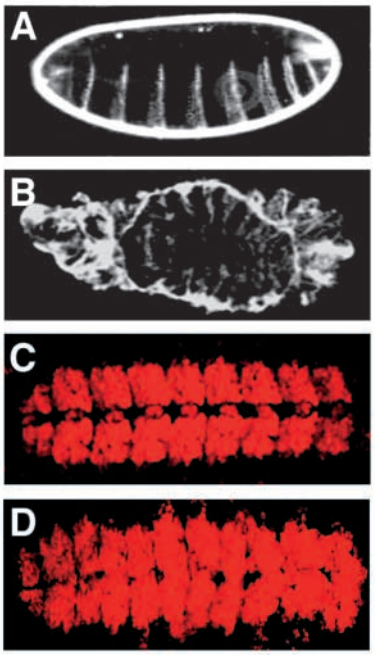

Fig. 6. *ed* produces a neurogenic phenotype in the *Drosophila* embryo. (A) Cuticle of a wild-type embryo showing the characteristic ventral denticle belts. (B) In *ed* germline clone embryos, lacking both maternal and zygotic *ed* expression, there is a ventral hole in the cuticle. (C) A wild-type embryo stained for the neuronal marker ELAV exhibits the condensed central nervous system. (D) An *ed* germline clone embryo displays a disorganized central nervous system with increased number of ELAV-positive cells, a phenotype typical of reduced N signaling.

the genetic interactions between *ed* and members of the Egfr signaling pathway. Overexpression of wild-type Egfr (*UAS-Egfr*) alone by *sca-Gal4*, had a very weak effect on the number of notum bristles (Fig. 7A). However, the co-expression of both *UAS-*$ed^{\Delta ECD}$ and *UAS-Egfr* resulted in a severe tufting phenotype (Fig. 7B). Similar results were obtained when $ed^{\Delta ECD}$ and a constitutively activated form of Raf (*UAS-*raf^{gof}) were co-expressed (Fig. 7C,D). As expected, increased number of SOPs were observed in proneural clusters, as detected with anti-Sens antibody (not shown). The interaction between Ed and Egfr pathways was verified by observing that a decrease of Egfr activity (overexpression of a dominant-negative form of Egfr, *UAS-*$Egfr^{DN}$) partially suppressed the extra bristle phenotype caused by ed^{1x5}/ed^{slH8} (Fig. 7E,F and Table 1). Together, these results demonstrated an antagonism between Ed and Egfr signaling pathways in bristle development. However, considering the known antagonism between the Egfr and N pathways in macrochaetae development (Culí et al., 2001), these results opened the possibility that the Egfr pathway might mediate, at least in part, the interaction between *ed* and the N pathway. If this were the case, one would expect that modifications of the activity of the Egfr pathway would affect the activity of the N pathway. Apparently, this did not occur. The levels of *E(spl)m8* mRNA accumulation in proneural clusters were essentially unmodified by overexpressing either a constitutively activated form of Ras (*UAS-*$ras1^{V12}$) (Karim and Rubin, 1998) or the Egfr-negative ligand Argos (*UAS-aos*) (Schweitzer et al., 1995). These

conditions mimicked a strong stimulation and an inhibition of the pathway, as they respectively lead to formation of many ectopic SOPs or to the removal of most macro and microchaetae (Culí et al., 2001). We conclude that it is unlikely that the interaction of Ed and N is mediated by the Egfr pathway,

Discussion

ed synergizes with N signaling

The present work indicates that development of the pattern of chaetae on the notum of *Drosophila* requires the cell adhesion molecule Ed to limit the number of SOPs that arise from a proneural cluster. Our data futher suggests that *ed* helps to provide cells of proneural clusters with levels of N signaling activity sufficient for effective lateral inhibition. This suggestion is based on the following observations. The loss of *ed* permits generation of extra macrochaetae from proneural clusters and the increase of the density of microchaetae. The loss of *ed* function does not significantly modify the size of proneural clusters or the levels of Sc protein in their cells. Extra SOPs arise from proneural clusters, but not outside of them, consistent with the essentially unmodified pattern of Sc in proneural clusters. By contrast, the loss of *ed* function decreases accumulation in proneural clusters of *E(spl)-m8* mRNA, one of the downstream genes of the N signaling pathway responsible for lateral inhibition. Moreover, combinations of moderate loss-of-function conditions for *ed* and for different components of the N pathway show clear synergistic interactions manifested as strong neurogenic phenotypes, including both the appearance of tufts of bristles in positions corresponding to wild-type macrochaetae and the differentiation of the external components of the SOs as extra neurons. However, even in mitotic recombination clones null for *ed*, lateral inhibition is not completely abolished, as shown by the failure of many proneural cluster cells to differentiate as SOPs and by the presence of epidermal cells in between the extra macro or microchaetae that arise from mutant proneural clusters. In fact, the phenotypes are very similar to those of partial reduction of N signalling observed with N^{ts1} and *N* hypomorphic alleles (de Celis et al., 1991b; Heitzler and Simpson, 1991). Consistent with this positive ed-N interaction, in a screen for components downstream of *ed* signaling, we have isolated *E(spl)-m7*, which when overexpressed suppresses the rough eye phenotype caused by *GMR-Gal4* driven *UAS-*$ed^{\Delta ECD}$ (J.C.H., unpublished).

The main steps of N signaling responsible for lateral inhibition during SO development can be summarized as follows (reviewed by Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000; Simpson, 1997). (1) Activation by the proneural proteins of the Dl ligand in the signal-emitting cell. (2) Interaction of activated Dl with N in the receptor cell, which culminates in the intramembrane proteolytic cleavage of the N molecule. (3) Release of the N^{ICD}, which translocates to the nucleus and, in collaboration with Su(H) and other proteins transcriptionally activates downstream genes. Paramount among these are the bHLH repressors of the E(spl)-C, which interact with specific AS-C enhancers and prevent the proneural gene self-stimulation necessary for cells to reach the SOP state (Culí and Modolell, 1998; Giagtzoglou et al., 2003). Our genetic epistasis experiments, together with the data summarized above, suggest that *ed* may facilitate N signaling by acting previously to the translocation of the N^{ICD} into the nucleus. Hence, Ed might facilitate steps of N signaling that occur at or near the membrane of the receptor cell, like N activation, N proteolytic processing or its membrane release. This is consistent with the colocalization of Ed and N at the zonula adherens and with the apparent absolute requirement for this localization of the Ed intracellular domain to exert its dominant-negative effect. However, at present little can be said of the molecular mechanism underlying the Ed-N interaction. We have been unable to demonstrate, by GST pull-down and two-hybrid assays, a direct physical interaction between the

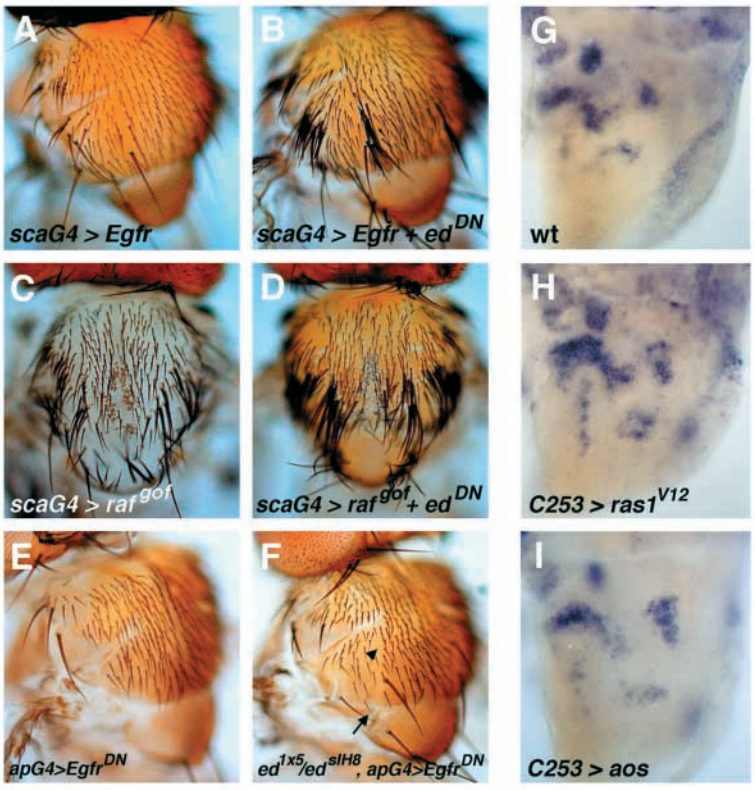

Fig. 7. Egfr acts antagonistically to Ed in bristle patterning, but it does not affect the levels of N signaling in proneural clusters. (A-D) Nota of flies expressing in proneural clusters (*sca-Gal4* driver) either *UAS-Egfr* (A), *UAS-Egfr* plus *UAS-*$ed^{\Delta ECD}$ (B), *UAS-*raf^{gof} (C), or *UAS-*raf^{gof} plus *UAS-*$ed^{\Delta ECD}$ (D). There is a strongly enhanced tufting effect caused by co-expression in B and D. (E,F) Nota of *ap-Gal4;UAS-*$Egfr^{DN}$ (E) and ed^{1x5}/ed^{slH8} *ap-Gal4;UAS-*$Egfr^{DN}$ (F) flies. The ectopic bristle phenotype of ed^{1x5}/ed^{slH8} mutant flies is partially suppressed by the simultaneous decrease of *Egfr* function (compare with Fig. 1B). Arrow and arrowhead in F indicates the positions where ADC and PPA bristles are respectively eliminated. (G-I) Accumulation of *E(spl)-m8* mRNA in the notum region of a wild-type disc (G) and in discs overexpressing either *UAS-*$ras1^{V12}$ (H) or *UAS-aos* (I) in proneural clusters (*C253-Gal4* driver). No significant differences were observed by comparing discs of similar stages from a total of 22 wild-type, 19 *UAS-*$ras1^{V12}$ and 16 *UAS-aos* discs examined.

intracellular domain of Ed and N (S.Y.W., unpublished). However, Ed might indirectly interact with N, probably together with other proteins or through its effects on cell adhesion, and provide an optimal environment for N activation/processing. An excess of either the Ed full-length molecule or the intracellular domain anchored to the membrane might disrupt this environment by displacing other molecules necessary for effective signaling and, therefore, exhibit dominant negative phenotypes. Hibris (Hbs), another Ig domain-containing cell-adhesion molecule, acts as a regulator of myoblast fusion and *hbs* mutant embryos show a partial block of myoblast fusion (Artero et al., 2001). Similar to Ed, overexpression in the mesoderm of either full-length Hbs or of a derivative containing the intracellular domain anchored to the membrane also exhibits *hbs* loss-of-function phenotypes.

The interaction of *ed* with the N pathway does not appear to be limited to the process of bristle development. Wing vein determination is also affected, as the combination of loss-of-function conditions for *ed* and *Dl* results in overly enlarged veins (not shown), a characteristic of reduced N signaling. The neurogenic phenotype of the CNS of ed^{lF20} (null) mutant germline clone-derived embryos is also consistent with reduced N function. However, clones null for *ed* did not disrupt formation of the wing margin (L.M.E., unpublished), another process dependent on N function.

Ed and Egfr signaling

In the eye disc, Ed functions as a receptor and elicits an independent signaling pathway that converges into the nuclei, where it apparently acts upstream of TTK88 to antagonize the Egfr pathway. Ed can be activated either non-autonomously by its own homophilic interaction or autonomously by heterophilic trans-interaction with Nrg from neighboring cells (Bai et al., 2001; Islam et al., 2003). During R8 photoreceptor specification, *ed* also acts both autonomously and non-autonomously to antagonize Egfr function and a model of direct interactions between the Ed and Egfr molecules has been proposed (Rawlins et al., 2003; Spencer and Cagan, 2003). Other work has shown that Egfr signaling is necessary for the emergence of the SOP of the notum macrochaetae (Culí et al., 2001). This function, triggered by *ac-sc* expression in the cells of the proneural cluster, has been denominated 'lateral cooperation', as it appears to be antagonistic to the 'lateral inhibition' promoted by N signaling. In fact, the self-stimulation of proneural genes that occurs in the SOP and which is essential for neural commitment (Culí and Modolell, 1998) appears to be the target of both signals, one stimulatory (Egfr) and the other inhibitory (Dl-N). Our present finding that *ed* not only synergizes with N in lateral inhibition, but it also antagonizes Egfr in lateral cooperation opened the possibility that the effect of *ed* on either the N or the Egfr pathway might result from the action of *ed* on the reciprocal pathway. However, the available data suggests that there is an interaction during chaetae formation with the N pathway. Indeed, evidence has been provided that N signaling downregulates Egfr signaling by inhibiting *rhomboid/veinlet* mRNA accumulation in proneural clusters, a molecule that facilitates Egfr activation (Culí et al., 2001). By contrast, the activity of the N pathway, as measured by the accumulation of a major effector of lateral inhibition, the *E(spl)-m8* mRNA, seems independent of the levels of Egfr signaling (Fig. 7G-I). Moreover, loss-of-function conditions for *ed* decreased the accumulation of *E(spl)-m8* mRNA, while that of *rhomboid/veinlet* mRNA was not detectably affected (L.M.E., unpublished). The independence of the *ed-N* interaction from Egfr is also supported by the neurogenic effect of the null ed^{lF20} allele in the embryo. We conclude that, the reduction of N-dependent lateral inhibition concomitant with the decrease of *ed* function might explain, at least in part, the interaction of *ed* with the Egfr pathway. Still, a more direct interaction between *ed* and this pathway, similar to that which occurs in photoreceptor cell determination, should also be considered.

ed and *fred*

Recently, the presence near *ed* of the structurally related gene *fred* has been reported (Chandra et al., 2003). *ed* and *fred* have been considered paralogous genes, because they have 69% identity in their extracellular domains, although only limited similarity in their intracellular domains. Similarly to *ed*, *fred* has been proposed to act in concert with the N signaling pathway and the absence of either gene decreases cell viability. However, *ed* and *fred* do not completely replace each other, because mutations that affect only *ed* (Bai et al., 2001) (this work) or expression of RNAi constructs specific for *fred* (Chandra et al., 2003) have clear mutant phenotypes. The fact that these genes are not redundant may be related to their largely different intracellular domains (Chandra et al., 2003). The 48 amino acid C-terminal region of Ed necessary for the activity of the intracellular domain (this work) is absent from Fred.

We are grateful to S. Campuzano, J. F. de Celis, J. L. Gómez-Skarmeta, M. Hortsch, S. Sotillos and colleagues of J.M.'s laboratory for advice on the work and constructive criticism of the manuscript; to J. Culí for the imaginal discs shown in Fig. 7; to E. Caminero for excellent technical help; to H. Vaessin for the communication of unpublished results; to C.-Y. Tang (H.Y. Sun's laboratory) for microinjection; to H. Bellen, A. García-Bellido, P. Heitzler and D. St Johnston for providing reagents and stocks. Predoctoral fellowship from Comunidad Autónoma de Madrid to L.M.E. is acknowledged. Grants from National Science Council (91-2311-B-007-027), Taiwan, Republic of China to J.C.H., Dirección General de Investigación Científica y Técnica (PB98-0682, BMC2002-411) to J.M. and an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa are also acknowledged.

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