echinoid mutants exhibit neurogenic phenotypes and show synergistic interactions with the Notch signaling pathway

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Summary

During neurogenesis in *Drosophila*, groups of ectodermal cells are endowed with the capacity to become neuronal precursors. The Notch signaling pathway is required to limit the neuronal potential to a single cell within each group. Loss of genes of the Notch signaling pathway results in a neurogenic phenotype: hyperplasia of the nervous system accompanied by a parallel loss of epidermis. Echinoid (Ed), a cell membrane associated Immunoglobulin C2-type protein, has previously been shown to be a negative regulator of the EGFR pathway during eye and wing vein development. Using in situ hybridization and antibody staining of whole-mount embryos, we show that Ed has a dynamic expression pattern during embryogenesis. Embryonic lethal alleles of *ed* reveal a role of Ed in restricting neurogenic potential during embryonic neurogenesis, and result in a phenotype similar to that of loss-of-function mutations of Notch signaling pathway genes. In this process Ed interacts closely with the Notch signaling pathway. Loss of *ed* suppresses the loss of neuronal elements caused by ectopic activation of the Notch signaling pathway. Using a temperature-sensitive allele of *ed* we show, furthermore, that Ed is required to suppress sensory bristles and for proper wing vein specification during adult development. In these processes also, *ed* acts in close concert with genes of the Notch signaling pathway. Thus the extra wing vein phenotype of *ed* is enhanced upon reduction of Delta (Dl) or Enhancer of split [E(spl)] proteins. Overexpression of the membrane-tethered extracellular region of Ed results in a dominant-negative phenotype. This phenotype is suppressed by overexpression of E(spl)m7 and enhanced by overexpression of Dl. Our work establishes a role of Ed during embryonic nervous system development, as well as adult sensory bristle specification and shows that Ed interacts synergistically with the Notch signaling pathway.

Key words: IgC2 domain, Echinoid, Notch, Neurogenesis, *Drosophila*

Introduction

Initiation of embryonic neurogenesis in *Drosophila melanogaster* involves the selection of specific groups of neuroectodermal cells in the ventral and procephalic neurogenic regions of the embryo. These groups of neuroectodermal cells or proneural clusters have the potential to develop into neural precursors or neuroblasts. Within such individual proneural clusters, normally only one cell will differentiate into a neuroblast, whereas the remaining cells will develop as epidermoblasts (Campos-Ortega, 1993). The proneural basic helix-loop-helix (bHLH) transcription factors, which include the *Achaete-scute* Complex (*ASC*) and *atonal* gene products, endow the cells of the proneural cluster with neurogenic potential (Bertrand et al., 2002; Modolell and Campuzano, 1998). Initially, all cells of the proneural clusters express these proneural transcription factors, but later on their expression becomes restricted to the future neuroblast. A process of lateral inhibition limits proneural gene expression and hence the neurogenic potential to a single cell within an individual proneural cluster. Lateral inhibition is mediated by the genes of the Notch signaling pathway (Artavanis-Tsakonas et al., 1999). Disruption of this lateral inhibition process allows many more or all proneural cluster cells to differentiate into neuroblasts. The increase in the number of neuroblasts, and parallel loss of epidermal precursors, results in neuronal hyperplasia, which is accompanied by a loss of epidermal structures (Hartenstein and Posakony, 1990; Lehmann et al., 1983).

The neurogenic genes *Notch* (*N*), *Delta* (*Dl*), *Presenilin* (*Psn*), *Suppressor of Hairless* [*Su(H)*], *mastermind* (*mam*) and *Enhancer of split complex* [*E(spl)C*] have been shown to interact genetically and to function in the process of lateral inhibition (Campos-Ortega, 1993; Artavanis-Tsakonas et al., 1999). Proneural genes positively regulate the levels of the N

ligand, Dl (Haenlin et al., 1994). Upon binding its ligand, N undergoes Psn-dependent processing which results in the release of the N intracellular domain (N^{icd}) from the membrane (De Strooper et al., 1999; Struhl and Adachi, 1998; Struhl and Greenwald, 1999). N^{icd} translocates to the nucleus where it forms a complex with the sequence-specific DNA-binding protein, Su (H) (Fortini and Artavanis-Tsakonas, 1994; Furukawa et al., 1992; Schweisguth and Posakony, 1992; Tamura et al., 1995) and Mam (Petcherski and Kimble, 2000), a transcriptional co-activator, to activate the expression of multiple genes of the *E(spl)C* (Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995). This gene complex [*E(spl)m8, E(spl)m7, E(spl)m5, E(spl)m3, E(spl)mδ, E(spl)mγ* and *E(spl)mβ*] encodes seven closely related proteins of the bHLH family of transcription factors (Delidakis and Artavanis-Tsakonas, 1992; Klämbt et al., 1989; Knust et al., 1992). E(spl) bHLH proteins act as transcriptional repressors in a complex with the co-repressor protein Groucho (Delidakis and Artavanis-Tsakonas, 1992; Fisher and Caudy, 1998) and downregulate *achaete* and *scute* expression leading to suppression of neural cell fate in the cell receiving the signal (Heitzler et al., 1996a).

Notch signaling pathway-mediated lateral inhibition is utilized again during the formation of the adult peripheral nervous system (PNS) during larval development. In the larval wing discs, proneural clusters composed of 20-30 cells are established (Campuzano and Modolell, 1992; Modolell, 1997). The Notch signaling pathway limits sensory organ precursor (SOP) cell fate to one or two cells within a proneural cluster (Artavanis-Tsakonas et al., 1995). The SOP gives rise to the adult external sensory organ or bristle, which is composed of four cells (neuron, sheath cell, socket cell and hair cell) (Bodmer et al., 1989; Hartenstein and Posakony, 1989). Disruption of the Notch signaling pathway results in specification of additional SOPs, often resulting in the generation of extra sensory bristles (Dietrich and Campos-Ortega, 1984; Hartenstein and Posakony, 1990; Schweisguth et al., 1996).

Neurogenic gene function is not limited to neural cell fate suppression. Requirement for neurogenic gene function has been shown for the development of other tissues, including eye, segmented appendages such as leg, mesoderm, muscle, somatogastric nervous system, wing and during oogenesis (Artavanis-Tsakonas et al., 1995).

Echinoid (Ed) is a transmembrane (TM) cell adhesion molecule with seven Immunoglobulin (Ig) C2 domains (Williams and Barclay, 1988), two Fibronectin type III domains (Hynes, 1986) and a TM, followed by a 315 amino acid intracellular domain with no identifiable structural or functional domain (Bai et al., 2001). Ed has been shown to act as a negative regulator of the EGFR signaling pathway during photoreceptor development in the larval eye disc (Bai et al., 2001). Neuroglian has been identified as an activating ligand for the antagonistic effect of Ed on the EGFR signaling pathway (Islam et al., 2003). Here we report on the neurogenic phenotype of *echinoid* (*ed*) mutants. Analysis of embryonic lethal alleles of *ed* reveals hyperplasia of the central nervous system (CNS) at the expense of the epidermal structures. Furthermore, we show that *ed* function is required for proper morphogenesis of wing and leg, as well as for the specification of the proper number of adult sensory bristles (macrochaetae and microchaetae). *ed* phenotypes are suppressed by increasing N pathway activity [by overexpressing N^{act} or E(spl)m7] and are enhanced by mutations in *Dl* or reduction of *E(spl)C* activity. Thus *ed* functions synergistically with the Notch signaling pathway during the processes of neural cell fate specification and wing development.

Materials and methods

Fly stocks and genetics

Df(2L)ed1, *Df(2L)M24F11*, ed^{k01102}, $E(spl)^{8D06}$*/TM3,Sb* and *T80 GAL4* were obtained from the Bloomington Drosophila Stock Center. ed^{2B8}, ed^{3C2} (Bier et al., 1989) and ed^{k01102} are P-element insertions in the *ed* locus. ed^{m1} and ed^{ts} are EMS-induced *ed* mutant alleles (this study). *69B GAL4* was kindly provided by A. Garcia-Belido (Universidad Autonoma de Madrid, Spain). For genetic interaction studies the following stocks were used: *UAS-*N^{act}*:* ed^{ts}*/UAS-*N^{act}*:* ed^{ts} (the *UAS-*N^{act}*:* ed^{ts} chromosome was generated by standard recombination), ed^{2B8}*/P(actin5C-lacZ)CyO; Kr-GAL4/P(actin5C-lacZ) TM3Sb, UAS-*N^{act}*/CyO* [E. Giniger (FHCRC, Seattle, WA) and B. Yedvobnick (Emory University, Atlanta, GA)], Dl^{via1}*/TM3,Sb* (Vaessin and Campos-Ortega, 1987), $E(spl)^{8D06}$*/TM3,Sb* (Klämbt et al., 1989), *UAS-EdExt* (this study), pnr^{MD237}*-GAL4* (Heitzler et al., 1996b), Eq-GAL4 [gift from C. T. Chein (Academia Sinica, Taipei, Taiwan)], *UAS-E(spl)m7* (Ligoxygakis et al., 1999) and *UAS-Dl* (Doherty et al., 1996). Flies were raised at 25°C unless otherwise mentioned.

EMS mutagenesis

EMS mutagenesis was performed as described in Vaessin and Campos-Ortega (Vaessin and Campos-Ortega, 1987). *ed* alleles were isolated in a *cn bw* background based on lethality in transheterozygosity over ed^{2B8} at 29°C.

Molecular biology

The *ed* cDNA clone *LD2669* was sequenced to confirm that it contains the entire predicted *ed* ORF. *EdExtra* was generated via PCR, using *LD2669* as template. EdExtra contains the entire Ed extracellular region, the entire predicted TM region and an additional 11 amino acids after the predicted TM domain. Thus, EdExtra contains amino acids 1-1028. *EdExtra* coding region was inserted into the *pUAST* transformation vector (Brand and Perrimon, 1993). *pUAST-EdExtra* construct was sequenced and transgenic lines were generated by P-element-mediated transformation (Spradling and Rubin, 1982). Thirteen independent *UAS-EdExtra* fly lines were obtained. Five lines were further characterized. All five lines produce comparable ectopic expression phenotypes.

The ORF of the ed^{m1} and ed^{ts} alleles were analyzed by direct sequencing of PCR products. Genomic DNA was isolated as described in Schlag and Wassarman (Schlag and Wassarman, 1999). Automated sequencing was performed at the Center for Molecular Neurobiology DNA Sequencing Facility (Ohio State University).

Immunohistochemistry and histology

In situ hybridization to whole-mount embryos was performed as described previously (Vaessin et al., 1991), using digoxigenin-labeled antisense and sense RNA probes. The following antibodies were used for immunohistochemistry: Rabbit-anti-HRP (1:3000, Jackson Laboratories), Goat-anti-rabbit (1:3000, Jackson Laboratories), Rabbit-anti-deadpan (Bier et al., 1992) and monoclonal Mouse-anti-beta-Galactosidase (Promega). Two different anti-Ed sera were generated in this study. Polyclonal antibodies were raised (AnimalPharm Services, Healdsburg, CA) against a His-tag fusion protein corresponding to the N-terminal 60 amino acids (in Rabbits) and against a His-tag fusion protein corresponding to the C-terminal 200 amino acids (in guinea pigs). Both anti-Ed Extracellular and anti-

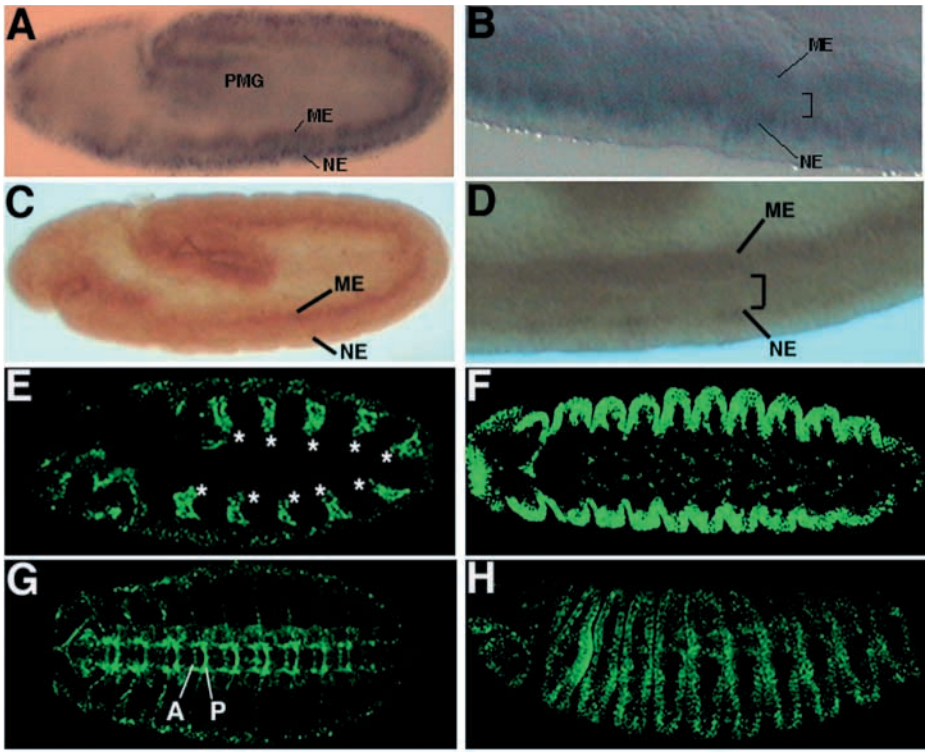

Fig. 1. *ed* expression pattern during embryogenesis. *ed* RNA (A,B) and protein (C-H) expression in wild-type embryos. In stage 9 embryos (A-D), differential expression of *ed* is evident. Whereas *ed* is expressed in the neuroectodermal (NE) and mesodermal (ME) cell layer, no expression is detected in the delaminated neuroblasts. (B,D) Enlarged lateral views of the ventral regions of embryos in A and C, respectively. The NE and ME cell layer are indicated and the bracket spans the layer where delaminated neuroblasts are located. (E) By stage 11, Ed expression ceases in the ventral neurogenic region and becomes restricted to the tracheal pits, marked by the asterisk, and the epidermis. At late stage 13, Ed can be detected in a subset of cells in the developing CNS and also in the epidermis (F). During stage 14, Ed expression is limited to the anterior and posterior epidermal stripes (H). At stage 16, Ed is highly localized to the axons with a higher concentration in the posterior commissures (G). A and P refer to anterior and posterior commissure, respectively.

Ed Intracellular give a similar embryonic expression pattern for Ed. The specificity of both anti-Ed sera was verified in homozygous *Df(2L)ed1* and ed^{2B8} embryos. Anti-Ed intracellular antibody signal was amplified using biotin-conjugated secondary antibody and Fluorescein Avidin D (Vectashield). Both light microscopy and confocal microscopy (BioRad MRC 1024) was employed for immunohistological analysis. Cuticle preparations were performed as described in Vaessin and Campos-Ortega (Vaessin and Campos-Ortega, 1987). Scanning electron micrographs (SEM) as described in Kimmel et al. (Kimmel et al., 1990) as well as standard stereo dissection microscopy were utilized for analysis and documentation of adult phenotypes.

Results

Ed shows a dynamic expression pattern during embryogenesis

ed has previously been shown to be expressed uniformly in the developing larval wing and eye discs (Bai et al., 2001). In contrast, our analysis of both *ed* RNA and protein expression during embryogenesis revealed a dynamic spatial and temporal expression pattern of *ed* gene products. At stage 5, *ed* is expressed in all cells except the pole cells (data not shown). During stage 9, both *ed* RNA and protein expression is eliminated from the delaminated neuroblasts, whereas it is still present in the mesodermal and ectodermal cells (Fig. 1A,C). In addition, subcellular localization of the *ed* transcript becomes evident during this stage. Thus, *ed* RNA appears to be highly concentrated in the basal region of the ectodermal cells, whereas no or only low levels of *ed* RNA is detectable in the apical portions of the ectodermal cells (Fig. 1B). A similar localization of *ed* protein can be observed during this stage (Fig. 1D). During stage 11 *ed* expression becomes more dynamic and a reduction of *ed* expression in the ventral neurogenic region becomes evident. In parallel, high levels of Ed expression is visible in the tracheal pits (Fig. 1E). At late stage 11/early stage 12, *ed* expression in the ventral neurogenic region is terminated. Later in development, *ed* expression is detected in a variety of developing tissues. These include muscle, epidermis, the hindgut and foregut (Fig. 1H and data not shown). In addition, *ed* expression is evident in a subset of cells in the CNS (Fig. 1F). At stage 16 *ed* protein becomes predominantly localized to axons and appears particularly concentrated in the posterior commissures (Fig. 1G).

Embryonic lethal alleles of *ed*

The observation of embryonic *ed* expression suggests an embryonic requirement for *ed* gene function. In agreement with this prediction, three recessive embryonic lethal *ed* mutations, ed^{2B8}, ed^{3C2} and ed^{m1}, were identified and analyzed. Two of these alleles, ed^{2B8} and ed^{3C2}, originated from a P-element screen for recessive lethals (Bier et al., 1989). ed^{m1} and the temperature-sensitive *ed* allele ed^{ts} were isolated as EMS-induced mutations, which are lethal in transheterozygosity with ed^{2B8}. For ed^{2B8}, a single P-element insertion was mapped to the 24D3-4 region on the left arm of the second chromosome by in situ hybridization. The embryonic recessive lethality was mapped to this region using the overlapping deficiencies: *Df(2L)ed1 (24A3-4; 24D3-4)* and *Df(2L)M24F11 (24D3-4; 25A2-3)*. To test whether the phenotype of ed^{2B8} homozygous embryos is associated with the P-element insertion in the *ed* locus, the P-element was remobilized. A complete reversion of the mutant phenotype was observed (data not shown). In situ hybridization and antibody labeling with anti-Ed antisera does not show detectable levels of *ed* gene products in ed^{2B8} homozygous mutant post-blastoderm embryos (data not shown), indicating that ed^{2B8} represents a strong hypomorphic or amorphic *ed*

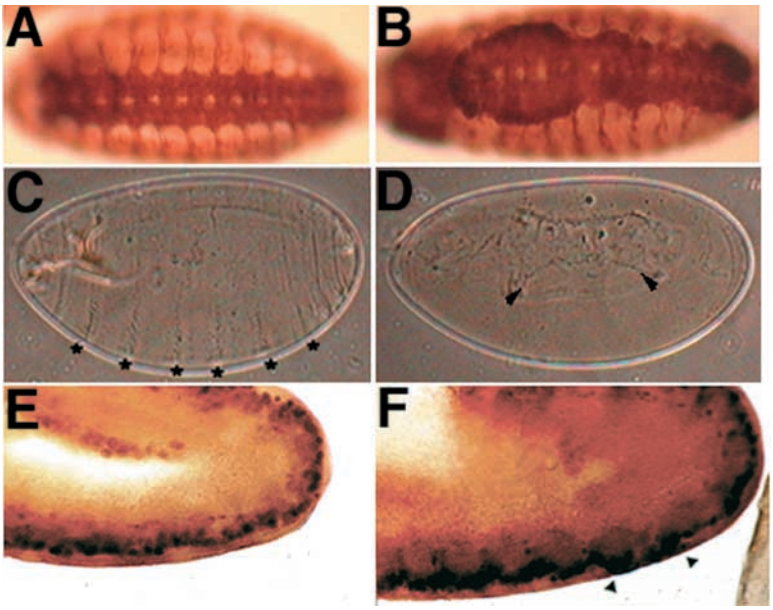

Fig. 2. Embryonic phenotypes associated with *ed* mutations. Ventral views of wild-type (WT) (A) and ed^{2B8}/ed^{2B8} (B) embryos stained with anti-HRP antibody. ed^{2B8} homozygous embryos exhibit a hyperplasia of the CNS. This hyperplasia is accompanied by loss of epidermal structures. (C-F) Lateral views of the embryos. Cuticle preparations of WT (C) and ed^{2B8}/ed^{2B8} (D) embryos. Asterisks in C mark denticle belts, characteristic structures of the ventral cuticle. ed^{2B8} homozygous embryos show an extensive loss of ventral and procephalic cuticle. (E,F) Lateral views of WT (E) and ed^{2B8} homozygous (F) embryos labeled with anti-Deadpan antibody. Arrowheads in F mark the ectopic neuroblasts present in *ed* mutant embryos.

allele. Additional lethal P-element insertion lines mapping to the 24D region were obtained from a collection of recessive lethal P-element insertion lines generated by the Berkeley Drosophila Genome Project (BDGP). The pupal lethal P-element insertion line *l(2)k01102* carries a P-element in the first intron of the *ed* transcription unit and was identified as an *ed* allele based on non-complementation of ed^{2B8} (Bai et al., 2001) (this study). The mutation associated with ed^{ts} was mapped to a single base change, G^{1834} to A (data not shown). This results in the replacement of the conserved Asp^{610} residue in the IgC2 domain V with an Asn residue. ed^{ts} is viable in homozygosity or in transheterozygosity with other alleles of *ed* at temperatures up to 25°C, but is embryonic lethal in transheterozygosity over ed^{2B8} at 29°C. The molecular basis of ed^{m1} has not yet been determined. Sequencing of the ed^{m1} allele ORF did not reveal a mutation. Thus, the molecular change associated with the ed^{m1} allele may be located in *ed* introns or *ed* cis-regulatory regions.

Immunostaining of homozygous (ed^{2B8}, ed^{3C2} and ed^{m1}) or transheterozygous *ed* mutant embryos [$Df(2L)ed1/ed^{2B8}$, ed^{2B8}/ed^{m1}, ed^{2B8}/ed^{ts}, and ed^{2B8}/ed^{3C2}] with anti-HRP antibody, which recognizes all neuronal cells of the CNS and PNS (Jan and Jan, 1982), reveals hyperplasia of the CNS. The observed hyperplasia ranges from mild bulges in the CNS to an increase in the size of the entire CNS. Fig. 2 shows an intermediate phenotype (Fig. 2A,B). Analysis of *ed* mutant embryos with antibodies against a neural precursor marker, Deadpan (Bier et al., 1992), shows that the increase in the number of neurons is preceded by an increase in the number of neural precursors (Fig. 2E,F). The enlargement of the CNS is accompanied by a parallel loss of epithelium, manifested as loss of cuticle, from the ventral and procephalic regions of the embryo. The cuticular defects range from a fusion of denticle belts to a complete absence of ventral and ventrolateral cuticle, as well as procephalic cuticle (Fig. 2C,D and data not shown). The *ed* mutant phenotype is reminiscent of the phenotypes displayed by loss of function mutations of neurogenic genes that are part of the Notch signaling pathway (Lehmann et al., 1983).

Ed has multiple functions during *Drosophila* development

ed has previously been shown to be essential for proper eye and wing formation (Bai et al., 2001; Islam et al., 2003). To further examine *ed* function during postembryonic development, we analyzed the phenotype of several transheterozygous viable combinations of *ed* alleles. Flies transheterozygous for ed^{k01102} and ed^{m1} (ed^{k01102}/ed^{m1}) generally die as pupae, but escaper flies can be isolated from low-density cultures and their adult phenotype examined. Flies transheterozygous for ed^{ts}/ed^{m1} or ed^{ts}/ed^{2B8}, shifted to the nonpermissive temperature of 29°C for three days at the end of larval stage 2, and then shifted back to 18°C, can also survive to adulthood. These flies show defects in the wings, legs, sensory bristles and eye. Frequently, transheterozygous *ed* mutant flies show extra wing vein material, irregular thickening of wing vein II and notching at the distal tip (Fig. 3A,B and data not shown). The leg phenotypes visible in transheterozygous flies were generally mild. Thus, the legs of ed^{k01102}/ed^{m1} males had proper number of segments, but the sex combs were misarranged (Fig. 3C,D). The sensory bristles, macrochaetae and microchaetae, are arranged in a stereotypical pattern on the adult dorsal thorax (Jan and Jan, 1993). ed^{ts}/ed^{m1} or ed^{ts}/ed^{2B8} flies that were shifted to the nonpermissive temperature as third instar larvae (see above) show extra macrochaetae. These extra macrochaetae were generally found next to macrochaeta normally present in the wild type (WT) (Fig. 3E,F). The microchaetae were also increased in density. In addition, ed^{ts}/ed^{m1} or ed^{ts}/ed^{2B8} flies and the ed^{k01102}/ed^{m1} showed a rough eye phenotype (not shown), similar to the eye phenotype described previously (Bai et al., 2001).

Overexpression of the extracellular region of Ed results in a dominant-negative phenotype

The extracellular region of Ed has seven IgC2-type domains and two fibronectin-type III repeats. Transgenic fly lines expressing a truncated Ed protein (EdExt) containing the entire extracellular and TM region, but lacking most of the

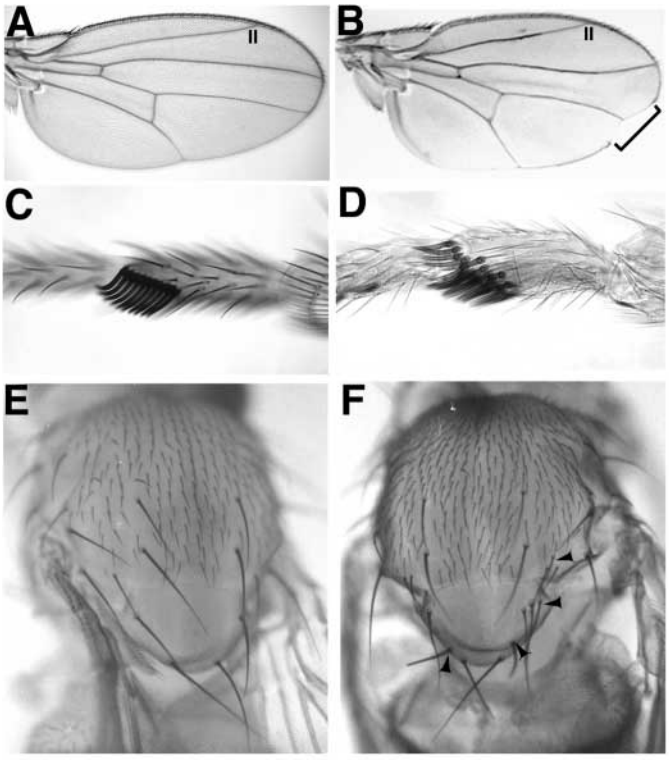

Fig. 3. Adult phenotypes associated with *ed* mutations. (A) Wing of wild-type (WT) and (B) ed^{2B8}/ed^{ts} flies. ed^{2B8}/ed^{ts} flies shifted to 29°C for three days at end of larval stage 2 show wings with irregular thickening of wing vein II and/or slight notching (indicated by the bracket in B) of the distal margin. In addition, extra macrochaetae and an increased density of microchaetae are evident on the thorax of ed^{2B8}/ed^{ts} flies (F), compared with the WT (E). Arrowheads point to ectopic bristles. Foreleg of WT (C) and ed^{k01102}/ed^{m1} (D) males. The sex combs are malformed.

intracellular region (except for the first 11 amino acids of the intracellular region) were generated. EdExt was expressed in wing discs of otherwise WT flies using various *GAL4* drivers. Ectopic expression of EdExt in the wing disc using either the *T80 GAL4* or the *69B GAL4* driver lines resulted in extra wing vein material at the distal end of wing vein II and rare (10%, *n*=30 wings) notching of the distal wing tip (Fig. 4B,C and data not shown). These phenotypes are similar to those seen in the adult hypomorphic *ed* mutant flies. In addition, and similar to adult flies carrying hypomorphic *ed* alleles, these animals also show extra sensory bristles (macrochaetae and microchaetae) (Fig. 4E and data not shown). Extra macrochaetae were always found in close proximity to a normally positioned bristle. The extra sensory bristles phenotype was more prominent when a stronger wing disc driver, *pnr-GAL4*, was used. *pnr-GAL4* mediates expression in the mesothorax region (Heitzler et al., 1996b). Expression of EdExt in this region resulted in an increase in the number of macrochaetae. This was especially evident in the scutellar region. Extra bristle specification was accompanied by an apparent loss of epidermal tissue, resulting in a smaller scutellum (Fig. 4F). The loss of epidermal tissue appears to be more severe than might be expected from inappropriate SOP specification alone. This might indicate additional roles of Ed in epidermogenesis. In addition, microchaetae were duplicated or quadrupled and missing their respective sockets, or missing completely (Fig. 4G). The phenotypes observed upon overexpression of the EdExt are qualitatively similar to those observed with various transheterozygous combinations of *ed* mutant alleles. These observations suggest that the EdExt construct behaves as a dominant-negative protein.

ed interacts with genes of the Notch signaling pathway

The neurogenic phenotype of homozygous *ed* mutant embryos as well as the extra sensory bristle phenotype of *ed* transheterozygous adults suggests that *ed* gene function, similar to the function of Notch signaling pathway genes, may be required to suppress neural fate. We therefore performed genetic interaction tests to investigate the possibility of functional interactions and/or epistatic relationship between *ed* and genes of the Notch signaling pathway.

Activation of the Notch signaling pathway results in suppression of neuronal cell fate (Bray, 1998; Greenwald, 1998). Overexpression of the intracellular region of N (N^{act}) results in a dominant activated phenotype (Fortini et al., 1993; Rebay et al., 1993). Accordingly, when N^{act} is expressed in embryos using the *Kr-GAL4* driver, an almost complete loss of neurons in parasegments 4-6 (Kr expression domain) is detected (Lieber et al., 1993) (Fig. 5C,G). In contrast, ed^{ts}/ed^{2B8} transheterozygous embryos, grown at the restrictive temperature of 29°C, show a phenotype opposite to that caused by ectopic N^{act} expression. Here, hyperplasia of the CNS with local bulging of the ventral nerve cord is evident (Fig. 5B,F). Reduced *ed* activity results in a strong suppression of the N^{act} overexpression phenotype, as is evident in the CNS of *UAS-*N^{act}*:* ed^{ts}/ed^{2B8}*; Kr-GAL4/+* embryos (Fig. 5D,H). Hence, reduction of *ed* activity can compensate for ectopic Notch signaling pathway activity.

To test for genetic interactions between *ed* and other genes of the Notch signaling pathway we performed dosage-sensitive interaction assays. For this purpose, a homozygous viable *Dl* mutation, Dl^{via1} (Vaessin and Campos-Ortega, 1987) and $E(spl)^{8D06}$, a deletion of the *E(spl)* complex (Knust et al., 1987), were used. Flies heterozygous for Dl^{via1} (+/+; Dl^{via1} /+) have wings indistinguishable from the WT. ed^{ts}/ed^{m1}; +/+ flies, grown at 25°C, have wings with irregular thickenings in wing vein II (Fig. 5J). In contrast, the wings of ed^{ts}/ed^{m1}*;* Dl^{via1} /+ flies, grown at 25°C, display significantly increased irregular thickenings of wing vein II. In addition, extra wing vein material is evident in the posterior cell of the wing (Fig. 5K). Wings of +/+; $E(spl)^{8D06}$/+ flies, raised at 29°C, are generally WT, but may display with low penetrance (<20%) extra vein material in the posterior cell (Fig. 5M). Flies heterozygous for ed^{ts} (ed^{ts}/+; +/+), raised at 29°C, have morphologically normal wings (Fig. 5L). The wings of ed^{ts}/+; $E(spl)^{8D06}$/+ flies, raised at 29°C, showed an enhanced phenotype compared with +/+; $E(spl)^{8D06}$/+ flies: the extra vein material is expanded in ed^{ts}/+; $E(spl)^{8D06}$/+ flies and is visible with full penetrance (Fig. 5N). Thus, synergistic interactions can be observed between *ed* and *Dl*, as well as *ed* and the *E(spl)C*.

We also analyzed the ability of the dominant-negative EdExt protein to functionally interact with Notch signaling pathway genes. In these experiments EdExt was ectopically expressed using the *Eq-GAL4* driver. *Eq-GAL4* mediates expression in the

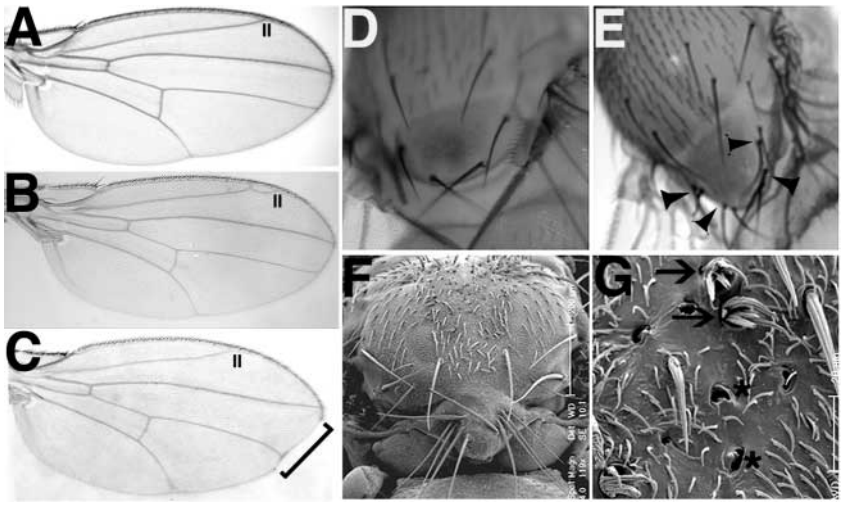

Fig. 4. EdExt has dominant-negative activity. (A) Control wing of *UAS-EdExt/+* fly. (B) *UAS-EdExt/69B GAL4* fly wing have irregular wing vein II. (C) *T80 GAL4/+; UAS-EdExt/+* fly wing exhibits irregular wing vein II and rare notches (indicated by the bracket) in the distal wing margin. (D) Partial dorsal thorax of control (*UAS-EdExt/+*) fly showing the normal number of sensory bristles on the scutellum. (E) Scutellums of *T80 GAL4/+; UAS-EdExt/+* flies show extra sensory bristles (indicated by arrowheads). The extra sensory bristle phenotype is more evident when *pnr GAL4* is used to mediate EdExt expression. A loss of epithelium is also noticeable (F). (G) Magnification of a part of the mesothoracic region of the thorax in F. The microchaetae are missing (asterisk) or quadrupled (arrows).

anterior region of the presumptive notum in the wing disc, with a stronger expression in the anterior midline region (Pi et al., 2001). *UAS-EdExt/+; Eq-GAL4/+* flies exhibit a slight increase in the number of microchaetae (Fig. 6B). Overexpression of full-length Dl has been shown to cause an increase in the number of sensory organs (Doherty et al., 1997) (Fig. 6C). It has been argued that this is because of the ability of Dl to autonomously inhibit N signal reception in Dl-expressing cells. Hence, when all cells of the proneural clusters express high levels of Dl, N signal reception would be inhibited, causing an increase in the number of sensory organs (Doherty et al., 1996; Doherty et al., 1997). Simultaneous overexpression of Dl and EdExt using the *Eq-GAL4* driver resulted in a dramatic increase in the number of microchaetae that exceeds the additive combination of the respective phenotypes (Fig. 6D). Hence, dominant-negative EdExt causes increased reduction in Notch signaling pathway-mediated lateral inhibition caused by ectopic expression of Dl. Accordingly, an increase in the activity of the Notch signaling pathway should suppresses the phenotype caused by EdExt overexpression. Flies overexpressing EdExt in the *pnr* expression domain (*UAS-EdExt/+; pnr-GAL4/+*) show extra sensory organs (Fig. 4F,

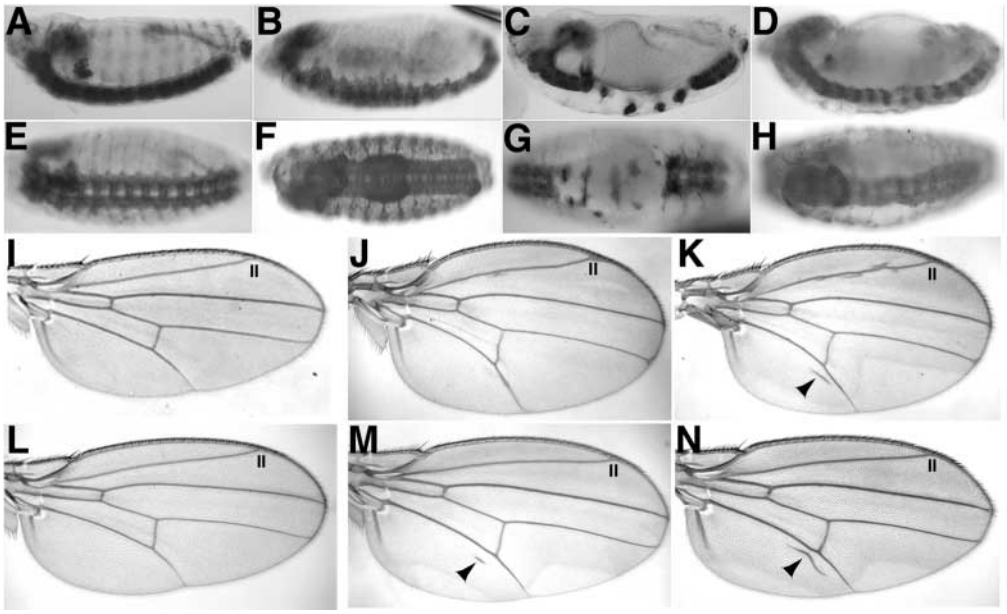

Fig. 5. Genetic interactions between *ed* and genes of the Notch signaling pathway. Anti-HRP labeling of neurons in the embryos (A-H). (A-D) Lateral views; (E-H) ventral views. Embryos in A-H were grown at 29°C. (A,E) Wild-type (WT) embryos. (B,F) Examples of the extent of hyperplasia of the CNS evident in ed^{2B8}/ed^{ts} embryos. Ectopic expression of N^{act} in the parasegments 4-6 mediated by the *Kr-GAL4* driver, results in the suppression of neuronal cell fate in this region (C,G). ed^{ts}*:UAS-*N^{act}/ed^{2B8}*; Kr-GAL4/+* embryos show strong suppression of the N^{act} overexpression phenotype, resulting in a near WT morphology (D,H). (I) Control wing of $Dl^{via1}/+$ fly. (J) ed^{ts}/ed^{m1} flies grown at 25°C show a mild thickening of wing vein II. (K) ed^{ts}/ed^{m1}*;* $Dl^{via1}/+$ flies grown at 25°C show an enhancement of the thick wing vein II phenotype and show additional wing vein material (indicated by the arrowhead) in the posterior cell. (L) $ed^{ts}/+$heterozygous flies raised at 29°C show a WT wing morphology. (M) $E(spl)^{8D06}/+$ flies raised at 29°C show extra wing vein material (arrowhead) in the posterior cell with low penetrance. (N) $ed^{ts}/+$*;* $E(spl)^{8D06}/+$ flies at 29°C display more ectopic vein material (arrowhead) with full penetrance.

Fig. 6F). Ectopic expression of E(spl)m7, a downstream target of an activated Notch signaling pathway, suppresses sensory organ specification resulting in the absence of bristles (Ligoxygakis et al., 1999). Flies expressing ectopic EdExt and E(spl)m7 simultaneously are indistinguishable from those expressing only E(spl)m7 ectopically (Fig. 6H), implying that increasing the activity of Notch signaling pathway can compensate for reduced Ed activity.

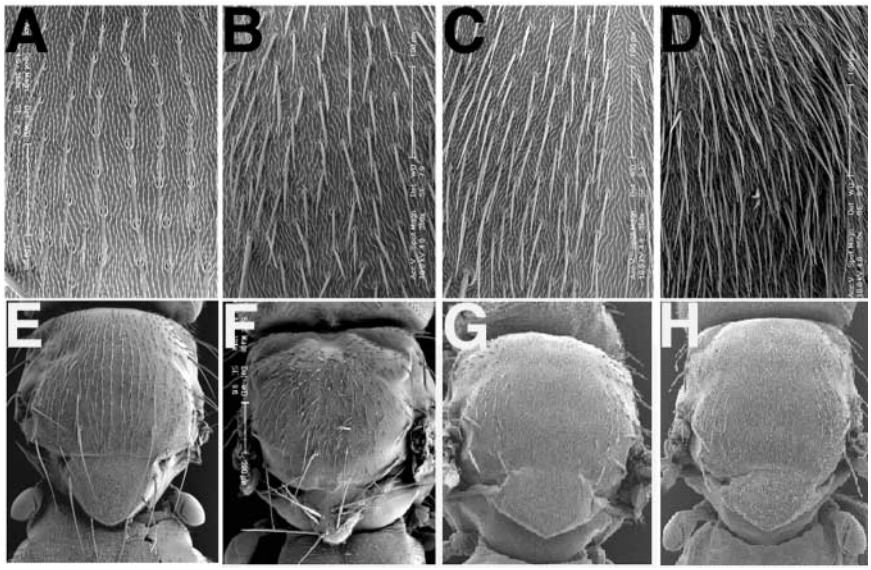

Fig. 6. The dominant-negative activity of EdExt is modulated by the Notch signaling pathway. (A-D) SEM of anterior midline region of adult thorax. (A) Normal microchaetae distribution in wild type (WT) flies. Ectopic expression of EdExt mediated by *Eq-GAL4* driver results in a mild increase in the microchaetae density (B). Ectopic expression of Dl also results in an increased microchaetae density (C). (D) *UAS-EdExt/+; UAS-Dl/Eq-GAL4* flies show significantly increased microchaetae density. (E) Arrangement of sensory bristles on the notum of an *UAS-EdExt/+* fly. An extra bristle can be seen rarely. *pnr-GAL4*-mediated ectopic expression of EdExt results in formation of extra bristles, which is accompanied by loss of epidermis (F). Overexpression of E(spl)m7 results in suppression of sensory bristle formation, resulting in a bald mesothorax region (G). Overexpression of E(spl)m7 completely suppresses the phenotype of EdExt overexpression (H). Thus *UAS-EdExt/+; UAS-E(spl)m7/pnr-GAL4* flies (H) are indistinguishable from *UAS-E(spl)m7/pnr-GAL4* flies (G).

Discussion

Echinoid is required for the restriction of neuronal development

Using the embryonic lethal alleles of *ed*, we have shown that *ed* is essential for normal embryonic neurogenesis. The phenotypes associated with loss of function mutations of *ed* have characteristic hallmarks of mutant phenotypes shown by neurogenic genes such as *N*, *Dl* or the *E(spl)* gene complex, namely hyperplasia of the nervous system and corresponding loss of epidermal structures. Similarly, reduction of *ed* function during sensory organ formation in the wing disc results in specification of extra sensory organs. This phenotype implies that *ed* is also required for the process of lateral inhibition in the proneural clusters during adult sensory organ development. A main difference between the mutant phenotypes of neurogenic genes and of *ed* alleles lies in the severity of the observed hyperplasia of the embryonic CNS. Embryos homozygous for apparently amorphic *ed* alleles show a less extensive neural hyperplasia than that caused by loss of genes such as *N* or *Dl* and resemble embryos homozygous/hemizygous for hypomorphic alleles of other neurogenic genes. Strong maternal effects contribute to weak phenotypes of various amorphic mutant alleles of neurogenic genes such as *N*, *mam* or *groucho*. Indeed, maternal *ed* transcripts can be readily detected in Northernblot analysis of 0-2 hour-old embryos (data not shown). However, it remains to be determined if a maternal contribution can account for the relatively weak neural hyperplasia exhibited by *ed* mutant embryos.

ed RNA and protein expression during early neurogenesis indicates that *ed* gene products become restricted to the neuroectodermal cell layer, whereas no *ed* products are detectable in the delaminated neuroblasts. The dynamics of *ed* RNA and protein distribution during neuroblast delamination implies that *ed* function might be required in cells that remain in the ectodermal cell layer. In such a scenario, similar to N, *ed* function would be required in the cells receiving the lateral inhibitory signal. However, it should be noted that at the time when the differential distribution of *ed* RNA and protein becomes detectable, the neuroblast segregation has already been initiated.

Ed expression during embryogenesis is dynamic and seen in many developing organ systems. The widespread expression of *ed* indicates that *ed* might be required for the development of multiple organs. Indeed, analysis of the trachea and muscles in *ed* mutant embryos reveals defects in the proper formation of these organ systems (data not shown). The requirement of *ed* for normal development of multiple tissues is not limited to embryogenesis. Adult flies with reduced *ed* activity show defects in leg, wing and eye development (Bai et al., 2001; Islam et al., 2003). A similar widespread expression and requirement in multiple organs has also been observed for the Notch signaling pathway during *Drosophila* development (Artavanis-Tsakonas et al., 1999).

The ectopically expressed extracellular domain of Ed has dominant-negative activity

Ed protein missing its intracellular region interferes with the process of lateral inhibition, as overexpression of EdExt in the developing wing disc results in an increase in the number of macrochaetae and microchaetae. Additional phenotypes include the irregular thickening of wing vein II and infrequent notching of the wing margin. These phenotypes are similar to those seen upon reduced *ed* function. Thus, ectopic expression of EdExt interferes with the function of endogenous Ed. A dominant-negative activity of the extracellular portion is not unusual for receptors that bind to ligands and then transduce a signal intracellularly (Rebay et al., 1993). Thus, it is possible that the EdExt competes with the WT Ed for a limited amount of ligand. Because EdExt is missing its intracellular region, its

binding to the ligand may have no functional consequence other than limiting the amount of available ligand. The ability of the extracellular domain to act as a dominant-negative molecule and the observation that the temperature-sensitive allele of *ed* has a mutation associated with Ig C2 domain V implies that the interaction of the extracellular domain with a putative ligand is an essential component of Ed function. Two isoforms of Neuroglian (Nrg) have recently been identified as activating ligands for the antagonistic effect of Ed on the EGFR pathway in the eye disc (Islam et al., 2003). Both isoforms (Nrg^{180} and Nrg^{167}) are expressed in the wing disc (Hortsch et al., 1990) and thus overlap in their expression with Ed. It has yet to be determined whether Nrg also functions as a ligand for Ed during sensory organ development. Ed has been shown to act as a homophilic cell adhesion molecule (Islam et al., 2003) (A.A., S.C. and H.V., unpublished). In the eye disc, it has been shown that the Nrg-mediated heterophilic activity of Ed in repressing the EGFR signaling pathway is redundant with the homophilic activity of Ed (Islam et al., 2003). Thus, it is possible that the dominant-negative construct interferes with Ed activity by competing for homophilic binding.

Ed interacts synergistically with the Notch signaling pathway

Ectopic expression of an activated form of N results in suppression of neuronal specification (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). In contrast, reduced *ed* gene activity results in increased specification of neurons. Ectopic expression of N^{act} in ed^{2B8}/ed^{ts} embryos results in a near WT nervous system. The observation of compensating, as opposed to an epistatic phenotype, does not support the formulation of a straightforward epistatic relationship between *ed* and *N* gene function. Rather, although both WT *N* and *ed* have a similar antineurogenic function during neurogenesis, they might be acting in functionally synergistic, yet possibly parallel regulatory pathways.

The observation of dosage-sensitive interactions between mutations in two genes can also be indicative of closely related roles. We have observed dosage-sensitive interactions between *ed* and *Dl* and *ed* and *E(spl)*. The mild wing phenotype exhibited by ed^{m1}/ed^{ts} flies raised at 25°C is enhanced by loss of one copy of *Dl*. Similarly, the wing phenotype of $E(spl)^{8D06}/+$ flies is enhanced by reduction of Ed activity. These observations imply that, in the wing disc also, the Notch signaling pathway and *ed* are acting synergistically.

Genetic interaction between *ed* and the Notch signaling pathway is also observed during the development of the adult PNS. Ectopic expression of EdExt results in specification of extra macrochaetae and microchaetae. Overexpression of Dl results in an increase in the number of sensory bristles (Doherty et al., 1997). Simultaneous ectopic expression of EdExt and Dl has a phenotype much stronger than what would be the result of additive combination of the individual phenotypes. The EdExt protein interferes with the activity of endogenous Ed and the decrease in Ed activity increases the neurogenic phenotype caused by Dl overexpression. These observations imply that Ed acts in concert with Dl. An epistatic interaction was also observed with E(spl)m7. Ectopic expression of E(spl)m7 completely suppressed the extra bristles phenotype obtained upon EdExt expression. The complete suppression of the dominant-negative phenotype would imply that E(spl)m7 functions downstream to the *ed*. However, it is possible that this suppression is a result of the strong antineurogenic activity of E(spl)m7. Although it is presently not clear whether *ed* and the genes of the Notch signaling pathway function in the same or parallel pathway, our observations establish that *ed* and the Notch signaling pathway genes act synergistically in both embryonic and postembryonic development.

Ed, Fred and the N and EGFR signaling pathway

Ed has previously been shown to be a negative regulator of the EGFR pathway. *ed* mutations enhance the rough eye phenotype of Elp^{B1}, a gain-of-function *EGFR* allele, and *ed* genetically interacts with several components of the EGFR pathway during eye development. As a consequence, *ed* mutant phenotypes include the generation of extra photoreceptor and cone cells (Bai et al., 2001; Rawlins et al., 2003; Spencer and Cagan, 2003). We have shown that *ed* mutations result in neural hyperplasia in the embryo and the formation of extra sensory organs on the notum of adult flies, and that *ed*, in these processes, interacts synergistically with the Notch signaling pathway. We have recently described an Ed paralog, Fred, and have shown that Fred is required to suppress SOP specification and is required for proper eye development (Chandra et al., 2003). Similar to *ed*, *fred* interacts synergistically with the Notch signaling pathway during SOP specification and also during eye development. *fred* also interacts with the EGFR pathway. Furthermore, *ed* and *fred* show a dosage-sensitive interaction during eye development, indicating that the two genes function in close concert (Chandra et al., 2003). Hence, *ed* and *fred* define a subgroup of two closely related IgC2-type TM proteins that interact synergistically with the Notch signaling pathway and antagonistically with the EGFR pathway.

Putative roles of a cell adhesion protein in neuronal precursor specification

Our data point to a role of Ed in the cell-cell communication processes that lead to the selection of the future neural precursor from the proneural cluster. In this process, Ed functions synergistically with the Notch signaling pathway. In this role Ed might be part of the cell-cell communication process itself. However, keeping in mind its cell-cell adhesion function, it could be argued that Ed may also be involved in the execution of the developmental decisions that result from cell-cell communication. In this scenario downregulation of *ed* expression in the future neuroblast may contribute to neuroblast delamination, whereas continued *ed* expression in the future epidermal precursors maintains cell adhesion and stabilizes their fate. Thus, Ed might be functioning at the level of cell-cell communication and at the level of coordinating cell-cell signaling with morphogenesis.

Note added in proof

A paper by Escudero et al. (Escudero et al., 2003) with similar conclusions appears in this issue.

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