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The cell adhesion molecule Echinoid defines a new pathway that antagonizes the *Drosophila* EGF receptor signaling pathway

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SUMMARY

Photoreceptor and cone cells in the *Drosophila* eye are recruited following activation of the epidermal growth factor receptor (EGFR) pathway. We have identified *echinoid* (*ed*) as a novel putative cell adhesion molecule that negatively regulates EGFR signaling. The *ed* mutant phenotype is associated with extra photoreceptor and cone cells. Conversely, ectopic expression of *ed* in the eye leads to a reduction in the number of photoreceptor cells. *ed* expression is independent of EGFR signaling and ED is localized to the plasma membrane of every cells throughout the eye disc. We present evidence that *ed* acts nonautonomously to generate extra R7 cells by a mechanism that is *sina*-independent but upstream of Tramtrack (TTK88). Together, our results support a model whereby ED defines an independent pathway that antagonizes EGFR signaling by regulating the activity, but not the level, of the TTK88 transcriptional repressor.

Key words: Echinoid, EGF receptor, *Drosophila*, Signaling

INTRODUCTION

The *Drosophila* compound eye is composed of approximately 800 ommatidia, each of which contains eight photoreceptor cells (R1-R8), four non-neuronal cone cells, and eight accessory cells arranged in a highly ordered pattern. In third instar larvae, patterning of the ommatidial field begins at the posterior margin of the eye imaginal disc, with the morphogenetic furrow sweeping across the disc epithelium in a posterior to anterior direction. Behind the furrow, different cell types are recruited sequentially. The R8 photoreceptor is the first cell to differentiate, followed by R2/R5, R3/R4, R1/R6 and R7. Addition of cone and accessory cells to the photoreceptor cluster produces the final ommatidial unit (reviewed in Wolff and Ready, 1993). The *Drosophila* epidermal growth factor receptor (EGFR) is required for differentiation of all the cell types, with the exception of R8 (Freeman, 1996; Dominguez et al., 1998). A model describing the reiterative activation of the EGFR by the opposing action of the EGFR ligand, Spitz (SPI) and an antagonist, Argos (AOS), has been proposed to explain the successive recruitment of each cell type in the developing eye (Freeman, 1997).

The *Drosophila* EGFR signaling pathway is subject to modulation at multiple levels by various positive and negative mechanisms (reviewed in Perrimon and Perkins, 1997; Wasserman and Freeman, 1997; Schweitzer and Shilo, 1997). There are multiple EGFR ligands, SPI, Vein and Gurken that activate the receptor tyrosine kinase (RTK) at different stages of development. In addition, Rhomboid (RHO), a multiple transmembrane domain protein, can potentiate EGFR signaling by regulating SPI processing and presentation (reviewed in Wasserman and Freeman, 1997; Bang and Kintner, 2000). The activated EGFR triggers a conserved signal transduction cascade that includes DRK, SOS, RAS1, KSR, RAF, MAPK (reviewed by Perrimon and Perkins, 1997). While SOS activates RAS1 by promoting the exchange of GDP for GTP, the GTP-activating protein, GAP1, inactivates RAS1 by stimulating its intrinsic GTPase activity (Gaul et al., 1992). Furthermore, activated MAPK is thought to propagate the RAS cascade signal into the nucleus by phosphorylating two members of the ETS family of transcription factors, YAN (AOP – FlyBase) and Pointed (PNT) (Brunner et al., 1994a; Brunner et al., 1994b; O'Neill et al., 1994). PTP-ER, a cytoplasmic tyrosine phosphatase, binds to and downregulates activated MAPK (Karim and Rubin, 1999). Therefore, both GAP1 and PTP-ER act in the cytoplasm to negatively regulate EGFR signaling. YAN is a transcriptional repressor that inhibits the production of both photoreceptor and cone cells (Lai and Rubin, 1992). Upon phosphorylation, YAN moves to the cytoplasm for degradation, leading to the differentiation of both cell types (Rebay and Rubin, 1995). In addition, *tramtrack (ttk)*, which encodes two alternatively spliced, zinc finger proteins, TTK69 and TTK88 (Read and Manley, 1992; Xiong and Montell, 1993), plays a central role in photoreceptor cell differentiation. TTK88 functions as a transcriptional repressor to inhibit photoreceptor but not cone cell differentiation (Lai et al., 1996; Yamamoto et al., 1996). Upon EGFR activation, two other nuclear proteins, Phyllopod (PHYL), which is

transcriptionally regulated by the RAS/MAPK pathway, and Seven In Abstentia (SINA), form a complex that binds to TTK88. This association leads to the targeting of TTK88 for degradation which results in the differentiation of photoreceptor cells (Tang et al., 1997; Li et al., 1997).

In addition to the cytoplasmic (GAP1, PTP-ER) and nuclear (YAN and TTK88) repressors, EGFR signaling can also be down-regulated by the production of AOS, Kekkon 1 (KEK1), and Sprouty (STY), via negative feedback loops following EGFR activation. AOS, a secreted molecule functions nonautonomously to repress EGFR signaling by blocking both the receptor dimerization and the ability of SPI to bind the EGFR (Freeman et al., 1992; Schweitzer et al., 1995; Jin et al., 2000). KEK1, an adhesion molecule protein, might execute its inhibitory effect through direct association of its extracellular and transmembrane domain with the EGFR (Ghiglione et al., 1999). Finally, the intracellular but inner membrane-associated STY has been proposed to act through its direct binding to DRK and GAP1, thereby blocking the activation of RAS1 (Casci et al., 1999).

Studies of the EGFR have also revealed that heterologous pathways can also modulate the activity of this RTK (reviewed in Moghal and Sternberg, 1999; Tan and Kim, 1999). For example, when growth hormone binds to its receptor, it activates JAK2, which promotes tyrosine phosphorylation of a GRB2 binding site on the EGFR, thereby leading to the activation of RAS and MAPK (Yamauchi et al., 1997). In addition, occupied and aggregated integrins can collaborate with growth factors by triggering tyrosine phosphorylation of EGFR (Miyamoto et al., 1996; Moro et al., 1998). In contrast, extracellular matrix proteins such as collagen, can promote retinoid-induced differentiation of normal human bronchial epithelial cells, by reducing the level of EGFR-dependent MAPK activation (Moghal and Neel, 1998). Thus, molecules involved in cell adhesion can act either positively or negatively to regulate EGFR signaling.

In this report, we describe the identification of the gene *echinoid* (*ed)* as a novel negative regulator of EGFR signaling. Ommatidia of *ed* mutant flies contain extra photoreceptor and cone cells. In contrast, ectopic expression of *ed* in the eye leads to a reduction in the number of photoreceptors. We show that *ed* is not transcriptionally regulated by EGFR signaling and that it encodes a putative cell adhesion protein which is primarily localized to the plasma membrane of every cells throughout the eye disc. Our genetic analyses demonstrate that *ed* acts nonautonomously to generate extra R7 cells by a mechanism that is *sina*-independent but upstream of TTK (TTK88). Together, our results support a model whereby ED defines an independent pathway that antagonizes EGFR signaling by regulating the activity of the TTK88 transcriptional repressor.

MATERIALS AND METHODS

Genetics

The stocks used were ed^{slH8}, ed^{slA12}, ed^{lF20} (de Belle et al., 1993); *l(2)k01102* (Torok et al., 1993); Elp^{B1} (Baker and Rubin, 1989); $Gap1^{B2}$ (Chou et al., 1993); sos^{e4G} (Simon et al., 1991); *hs-aos* (Freeman, 1994); rl^{SEM} (Brunner et al., 1994); sev^{d2} (Simon et al., 1991); *sev-*Ras^{V12} (Fortini et al., 1992); *sev-*Ras^{N17} (Karim et al., 1996); *sev-*tor^{4021}*Egfr* (Reichman-Fried et al., 1994); *sev-*tor^{4021}*Raf* (Dickson et al., 1992); pnt^{1277}, $pnt^{\Delta 88}$ (Brunner et al., 1994); *sev-*yan^{ACT} (Rebay and Rubin, 1995); $sina^{2}$, $sina^{3}$ (Carthew and Rubin, 1990); ttk^{0219} (Lai et al., 1996); *GMR-ttk88* (Tang et al., 1997); $sprouty^{S88}$ (Casci et al., 1999); *UAS-kek1* (Ghiglione et al., 1999); *hs-rho* (Dominguez et al., 1998); *GMR-GAL4* (Freeman, 1996); and *MS1096-GAL4* (Capdevila and Guerrero, 1994).

Production of mosaic clones

Mitotic clones in the eye were induced by using *ey-FLP* (kindly provided by B. Dickson). The genotype of these flies was *w,*sev^{d2}*/Y;* ed^{slA12}*,*FRT^{40A}*/ P[w+]*^{30C}*,* FRT^{40A}*; ey-FLP/+*.

Molecular biology

A 8 kb *Eco*RI genomic DNA flanking the *l(2)k01102* P-element insertion was isolated by plasmid rescue and used to screen an eye disc cDNA library (provided by Dr A. Cowman). Two classes of cDNAs were isolated and sequenced. One is 5.1 kb and encodes an open reading frame (ORF) of 1332 amino acids. We refer to it as *ed* cDNA. The other cDNA is 4.5 kb and encodes a noncoding RNA: the longest open reading frame (ORF) would encode a polypeptide of 102 amino acids but its AUG codon is in a poor position for translation initiation (Cavener, 1987). The ORF region of the *ed* cDNA was generated by PCR and inserted into the *pCaSpeR-hs* and *pUAST* transformation vectors (Brand and Perrimon, 1993) to create *hs-ed* and *UAS-ed. UAS-*$ed^{\Delta intra}$ was made by inserting by PCR a stop codon 31 amino acids after the transmembrane domain of ED. All the constructs were sequenced. Transgenic lines were generated by P-element-mediated transformation (Spradling and Rubin 1982). *hs-ed* was tested for its ability to rescue the lethality of ed^{slH8} by heat shock in a 37° water bath for 20 minutes every 12 hours throughout development.

ed-specific RT-PCR was performed as follows. Five late third instar larval eye discs of different genotypes were dissected and subjected to cDNA synthesis with a Cells-to-cDNA system (Ambion). 20 cycles of PCR amplifications were carried out with primer pairs from the seventh (CGATGCCCGGAAATGAATGG) and ninth exon (GCGTATGACGCGACGGTTT) of *ed* genomic DNA. 18S rRNA primers (Ambion) were used as internal controls.

Histology

Fixation, embedding and sectioning of adult retina were performed as described by Wolff and Ready (Wolff and Ready, 1991). Scanning electron micrographs were prepared as described by Kimmel et al. (Kimmel et al., 1990). Cobalt sulphide staining of pupal retinas was performed as described by Wolff and Ready (Wolff and Ready, 1991). Immunohistochemical staining of imaginal discs was performed as described in Xu and Rubin (Xu and Rubin, 1993). Polyclonal rabbit α-ED antibodies were generated against a synthetic peptide, corresponding to the N-terminal region of ED (MRRKTVTKGTAIVNSRSARRAATTI) and were used at a dilution of 1: 200. α-ELAV (rat, 1: 250, Developmental Studies Hybridoma Bank); αi-Cut (mouse, 1: 5, Developmental Studies Hybridoma Bank); α-β-galactosidase (rabbit, 1: 1000, Cappel); α-TTK88 (mouse, 1:100); and α-Boss (mouse, 1:1000); Cy3-, Cy5-, FITC-conjugated secondary IgGs are from Jackson Immunological Laboratories. Confocal microscopy was performed using a Zeiss Model 310.

GenBank Accession Number

The accession number for the *ed* sequence reported in this paper is AF275903.

RESULTS

Identification of a negative regulator of EGFR signaling pathway

Elp^{B1} is a gain-of-function allele of the *Egfr* (Baker and Rubin,

1989). We carried out a genetic modifier screen for components of the EGFR pathway that dominantly enhance or suppress the rough eye phenotype caused by Elp^{B1} (Fig. 1B). *1X5* was isolated as an EMS induced mutation which strongly enhances the rough eye phenotype associated with Elp^{B1} (Fig. 1C). The dominant enhancer activity of *1X5* is similar to the effect of *Gap1* (Fig. 1D) or *yan* (data not shown) mutations, two known negative regulators of the EGFR signaling pathway.

Consistent with the genetic interaction with Elp^{B1}, *1X5* also enhances the eye phenotype caused by $sev\text{-}tor^{4021}Egfr$ (Fig. 1E,F), another constitutively active form of the EGFR (Reichman-Fried et al., 1994). To define further the role of *1X5* in the EGFR signaling pathway, we examined the genetic interactions between *1X5* and *rho*, a specific activator of EGFR pathway, and *aos*, a specific EGFR inhibitor. Interestingly, we found that *1X5* enhances the rough eye phenotype caused by ectopic expression of *rho* (Fig. 1G,H), and suppresses the rough eye phenotype caused by misexpression of *aos* (Fig. 1I,J).

Further genetic interactions between the EGFR pathway and *1X5* were also detected in the wing. *1X5* enhances the extra wing-vein phenotype caused by the overactive Elp^{B1} mutation, as well as rl^{SEM}, a constitutively active MAPK (Brunner et al., 1994; data not shown). In addition, flies heterozygous for both *1X5* and *Gap1*, or both *1X5* and sty^{S88} (data not shown), exhibit extra vein materials, although heterozygosity for either mutation alone causes no phenotype. Therefore, the genetic interactions observed between *1X5* and several components of the EGFR pathway suggest that *1X5* is a negative regulator of the EGFR signaling pathway during eye and wing vein development.

1X5 is allelic to *echinoid* and is required for the formation of photoreceptor and cone cells

1X5 was mapped to 24D3-4 using three overlapping deficiencies: *Df(2L)ed1 (24A3-4;24D3-4)*, *Df(2L)ed-dp (24C3-5;25A2-3)* and *Df(2L)M24F11 (24D3-4;25A2-3)*. This region contains the *ed* gene and we found that ed^{lF20} (de Belle et al., 1993) fails to complement *1X5* and enhances the Elp^{B1} rough eye phenotype, as well as the extra wing vein phenotype of rl^{SEM} (data not shown). Thus *1X5* is allelic to *ed*, and we refer to it as such below. All *ed* mutations are pupal lethal in homozygotes with the exception of ed^{slH8}, which is a weaker allele. Homozygous ed^{slH8}, as well as ed^{slH8} in combination with all other *ed* alleles, including *Df(2L)ed-dp*, are semi-lethal. Emerging adults have rough eyes (Fig. 2B) and extra wing veins (see Fig. 5J). When sectioned, 33% of ommatidia contain extra R7-like cells with small and centrally positioned rhabdomeres (Fig. 2D). To exclude that these extra cells with small rhabdomeres are R8, third instar larval imaginal discs of ed^{1X5}/ed^{slH8} transheterozygotes were stained with anti-Boss, an R8-specific antibody. Single R8 cell was seen in each mature ommatidium (Fig. 2E), confirming that the extra photoreceptor cells are indeed R7. In addition, 26% of ommatidia exhibit extra outer-photoreceptor cells while 6% of the ommatidia show reduced outer-photoreceptor cells. Further, ed^{slH8} hemizygotes animals have more R7 cells than ed^{1X5}/ed^{slH8} transheterozygote animals indicating that the *ed* alleles are loss of function. ed^{slH8} hemizygotes have 1.68 R7 cells in average (n=64), compared with 1.34 (n=164) in ed^{1X5}/ed^{slH8}. To determine the origins of the extra photoreceptor cells, ed^{1X5}/ed^{slH8} transheterozygote discs were stained with the

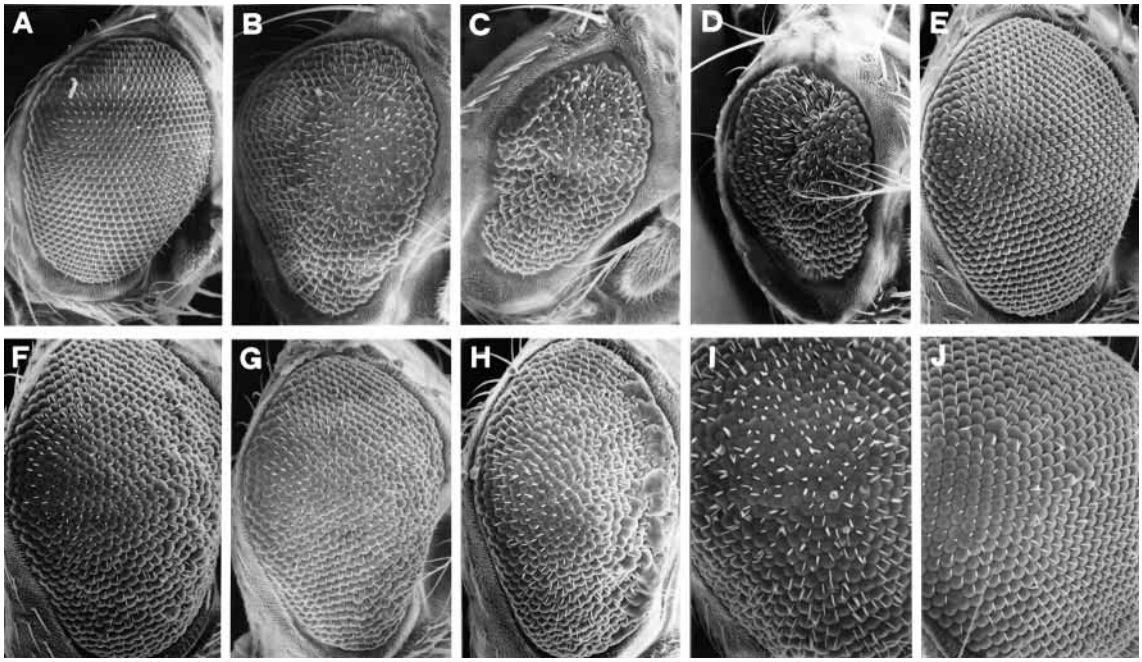

Fig. 1. *1X5* genetically interacts with mutations in the EGFR pathway during eye development. A wild-type eye possesses around 750 ommatidia arranged in a highly ordered pattern (A). $Elp^{B1}/+$ eyes are rough (B) and this phenotype is enhanced when heterozygous for *1X5* (C), and $Gap1^{B2}$ (D). The eye phenotype of $sev\text{-}tor^{4021}Egfr/+$ (E) is enhanced when heterozygous for *1X5* (F). The rough eye phenotype associated with *hs-rho*/+ (G) is enhanced when heterozygous for *1X5* (H). Overexpression of *aos* under the heat shock promoter (*hs-aos*/+) causes a weak rough eye (I), and this phenotype is suppressed when heterozygous for *1X5* (J).

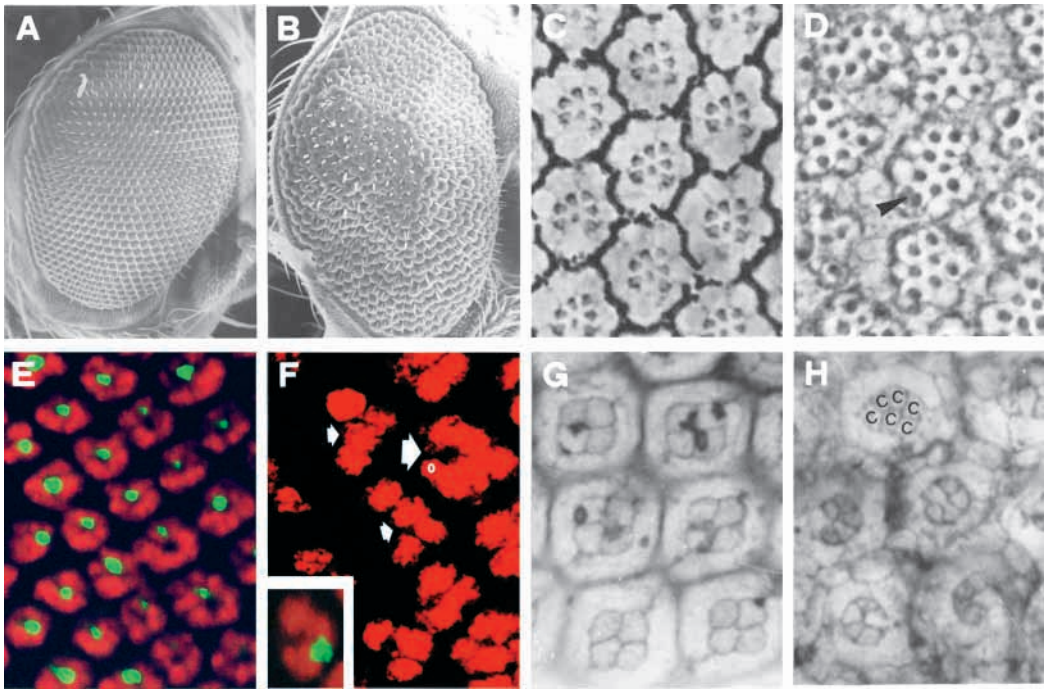

Fig. 2. *ed* mutant eyes contain extra cone and photoreceptor cells. Scanning electron micrographs (A,B) of adult eyes; apical sections through adult retinas (C,D); third instar eye imaginal discs stained for the R8 specific marker, Boss (green), and neuronal marker, Elav (red) (E,F); and pupal retina stained with cobalt sulphide (G,H). (A,C,G) Wild type; (B,D-F,H) are from ed^{1X5}/ed^{slH8} flies. ed^{1X5}/ed^{slH8} transheterozygote animals have large, rough eyes (B). Thin section analysis of these eyes reveals that 26% of the ommatidia contain extra R1-R6 photoreceptor cells, 33% contain extra R7 cells (D) and 6% have a decreased number of photoreceptor cells. The arrowhead indicates an ommatidium that contains two R7 and either six or seven (right) outer photoreceptors. There is only a single R8 cell in each mature ommatidium (E). In some ommatidia, one extra Elav-positive cell (small arrow in F) was first detected in row 2-3 where R8/R2/R5 are located. Inset in (F) shows the four-cell cluster only contains a single R8 cell. In addition, one Elav-positive mystery cell (circle) was found in row 4 (large arrow in F). Together, these may contribute to the formation of the supernumerary photoreceptors that are seen in ed^{1X5}/ed^{slH8} animals. The morphogenetic furrow is to the left. Cobalt-sulfide staining of the ed^{1X5}/ed^{slH8} pupal eye imaginal discs reveals that 69% of the ommatidia have five to six (H), instead of four cone cells (G).

anti-Elav neural marker (Fig. 2F). Extra Elav-positive cells were first detected in rows 2 and 3, where R8/R2/R5 are located. However, these four-cell clusters contain only single R8. In addition, one or two extra Elav-positive mystery cells were detected adjacent to R3 and R4 cells four row of cells behind the furrow. Mystery cells will normally leave the five-cell precluster and disappear; however, as in *sty* or *yan* mutants (Casci et al., 1999; Lai et al., 1992), they are transformed into neuronal photoreceptor cells in the *ed* mutant discs.

We also examined the *ed* mutant phenotype during pupariation. At this stage there are four cone cells and two primary pigment cells in wild-type discs (Fig. 2G). However, 69% of ommatidia in ed^{1X5}/ed^{slH8} transheterozygotes exhibit five or six cone cells (Fig. 2H) and 10% contain three primary pigment cells (data not shown). Together, the over-recruitment of photoreceptor, cone and pigment cells in *ed* mutants is consistent with ED acting as a negative regulator of EGFR because previous analyses have shown that EGFR is required for differentiation of these three cell types (Freeman, 1996).

ed encodes an adhesion molecule-like protein with six immunoglobulin domains

We identified a P-element insertion, *l(2)k01102* (Torok et al., 1993) located at 24D3-4, that failed to complement either ed^{1X5} or ed^{lF20}. To characterize *ed* molecularly, we recovered the DNA region flanking *l(2)k01102* by plasmid rescue (see Materials and Methods). A 8 kb genomic DNA was isolated and used to screen an eye disc cDNA library. A 5.1 kb cDNA was isolated and sequence analysis revealed that it encodes an open reading frame of 3996 bp, which predicts a protein of 1332 amino acids (Fig. 3B). The translated protein contains six immunoglobulin (Ig) C2 type domains (Williams and Barclay, 1988) (Fig. 3C), a fibronectin type III domain (Hynes, 1986) (Fig. 3D) and a transmembrane domain, followed by a 315 amino acid C-terminal tail with no identifiable functional motif. A comparison of the genomic and cDNA sequence indicates that *l(2)k1102* is inserted in the first intron, which is upstream of the coding region (Fig. 3A). To establish that the 5.1 kb cDNA identifies *ed*, we expressed the cDNA under the control of a heat shock promoter. Following heat shock treatments, we found that the hs-*ed* transgene rescues the lethality associated with the weak ed^{slH8} allele (data not shown).

To detect the expression pattern of ED, we stained embryos with an antibody generated against the N-terminal ED peptide. The ED protein is widely expressed in the epidermis and is localized to the plasma membrane (Fig. 4A). Further, we find that ED is uniformly detected in all cells throughout the third instar larval eye and wing disc (data not shown).

The expression of *aos* and *kek1*, two other negative regulators of the EGFR pathway, is regulated by the EGFR pathway. To determine whether *ed* is regulated by the EGFR pathway, we examined the expression of *ed* in *GMR-aos* (Fig. 4B-D) and *sev-*Ras^{V12} (data not shown) eye discs. In each case, the level of *ed* mRNA is not affected, as revealed by either the X-gal staining of the P insertion *l(2)k1102*. (Fig. 4B,C) or the *ed*-specific relative RT-PCR (Fig. 4D). These results indicate that *ed*, unlike *aos* and *kek1*, is not transcriptionally regulated by the activation of EGFR pathway.

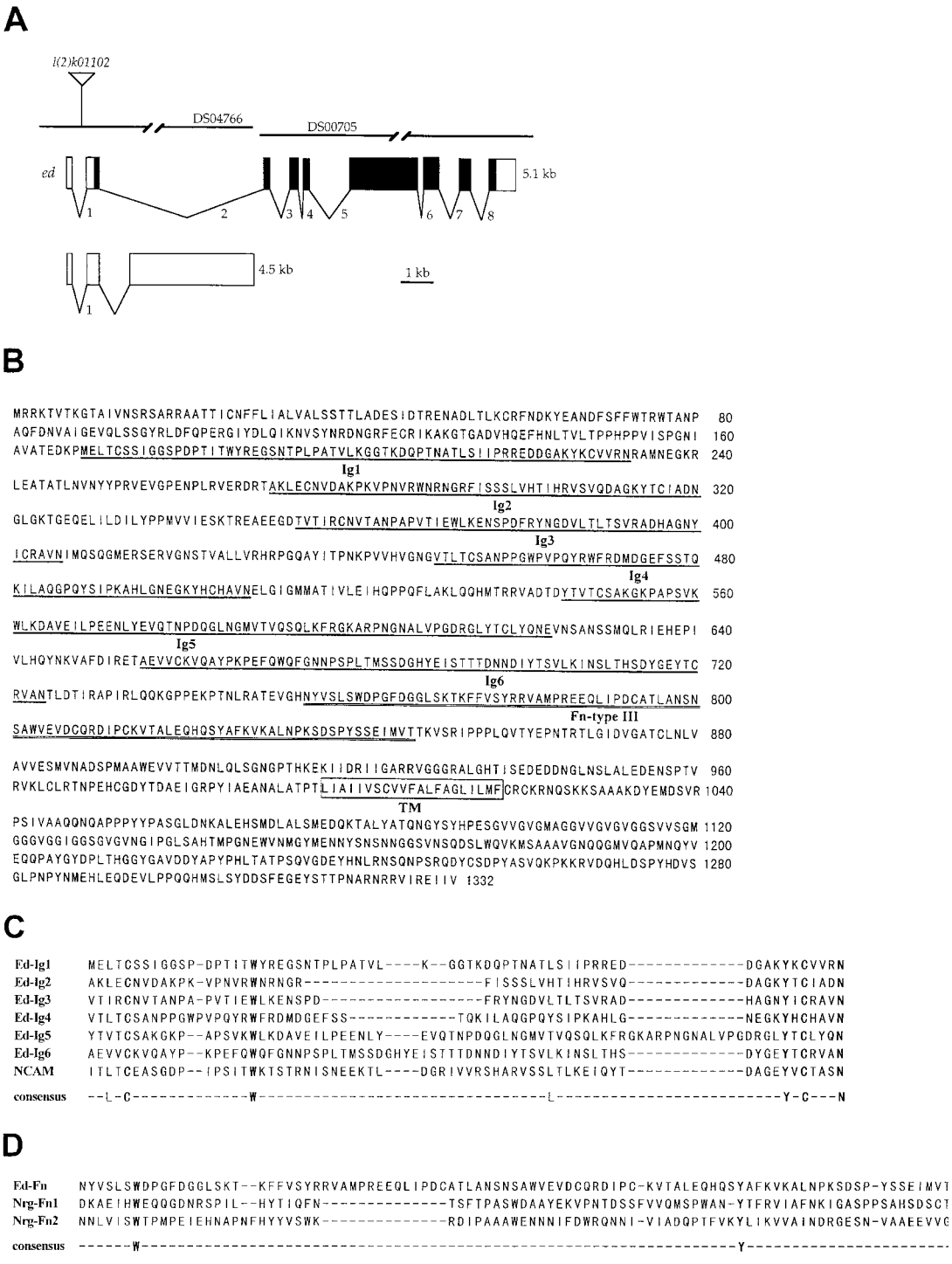

Fig. 3. Molecular characterization of the *ed* locus. (A) Genomic organization of the *ed* gene. The *ed* locus is encompassed by two P1 phage, DS04766 and DS00705. The *ed* cDNA and the direction of transcription is indicated. The black boxes represent the coding region of *ed*, whereas the white boxes represent the untranslated regions. *ed* is composed of nine exons and eight introns. The size of introns from 1 to 8 are 866 bp, 35 kb, 4.79 kb, 62 bp, 17.7 kb, 583 bp, 6.3 kb and 5.8 kb, respectively. The position of the P element *l(2)k01102* was mapped to the first intron. The other cDNA shares the first two exons but encodes a noncoding RNA, as the longest open reading frame (ORF) would encode a polypeptide of 102 amino acids but its AUG codon is in an poor context for translation initiation (Cavener, 1987). (B) Amino acid sequence of ED. *ed* encodes a putative transmembrane protein of 1332 amino acids. The extracellular domain contains six immunoglobulin (Ig) C2 type domains (unbroken lines) and one fibronectin type III domain (double lines). The transmembrane (TM) domain is boxed. (C) Alignment of Ig domains. The consensus sequence of Ig C2 type domain (Williams and Barclay, 1988) is shown. (D) Alignment of the fibronectin type III domain. The consensus sequence of fibronectin type III domain (Hynes, 1986) is shown.

Overexpression of *ed* antagonizes EGFR activity in the eye and wing

As shown above, loss of *ed* function is required for the formation of photoreceptor, cone and primary pigment cells. To determine the effect of overexpression of *ed* in the eye, we expressed *UAS-ed* using the GMR-Gal4 driver. *GMR-Gal4; UAS-ed* flies exhibit a small rough eye (Fig. 5A) and a reduced number of photoreceptors (Fig. 5B); this effect correlates with the reduced number of Elav-positive cells (Fig. 5C) in the eye disc. There are only four or five Elav-positive cells per cluster. In contrast, no obvious defects in the formation of cone cells were observed in response to *ed* overexpression, as most ommatidia still contain four Cut-positive cells (data not shown). Flies carrying two copies of GMR-GAL4-driven UAS-*ed* exhibit complete absence of the eye (data not shown).

To further document the interaction between ED and the EGFR pathway, we examined the effect of ectopic expression of *ed* in flies where other regulators were overexpressed. Overexpression of *UAS-sty* alone by *GMR-GAL4* produces small rough eye (Fig. 5D). This phenotype can be partially

suppressed by halving the dose of *ed* (Fig. 5E), and enhanced by GMR-GAL4-driven *UAS-ed* (Fig. 5F). Similar genetic interactions can also be observed between *ed* and *kek1*. The rough eye phenotype caused by GMR-GAL4-driven *UAS-kek1* (Fig. 5G) is enhanced by GMR-GAL4-driven *UAS-ed* (Fig. 5H). Therefore *ed*, like *sty* and *kek1*, is a repressor of EGFR signaling during eye development.

Similarly, during wing vein development, *ed* genetically interacts with several components in the EGFR pathway. Flies of ed^{1X5}/ed^{slH8} have increased size of wing and extra wing vein (Fig. 5J). However, ectopic expression of *ed* using *MS1096 GAL4* results in severe reduction in the size of wing, ranging from one quarter to one fifth of normal wing size. In addition, there is no vein material present (Fig. 5K).

The intracellular domain of ED is required for the repression

ED contains six Ig domains and a 315 amino acid intracellular domain. To determine whether the intracellular domain of ED is required for the repression of the EGFR signaling, we generated $UAS\text{-}ed^{\Delta intra}$ flies. Overexpression of $UAS\text{-}ed^{\Delta intra}$ using *GMR-GAL4* had no phenotypes in the eye, indicating that the cytoplasmic domain of ED is required for the repression of the EGFR signaling pathway.

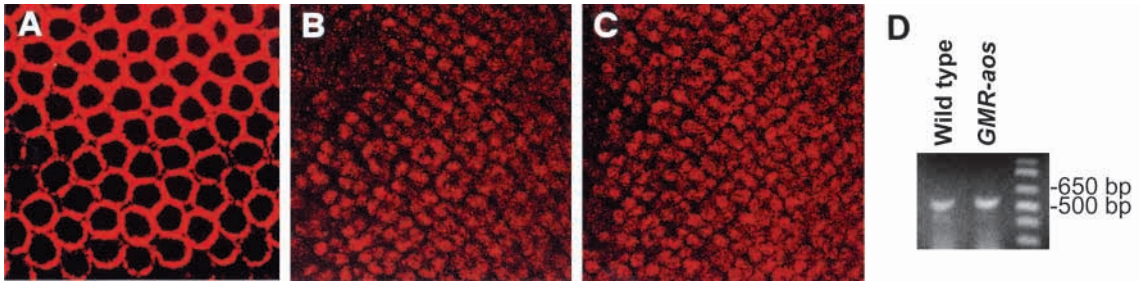

Fig. 4. *ed* is not transcriptionally regulated by EGFR signaling. Embryos of cellular blastoderm stage were labeled with antibodies against ED. The ED immunostaining is uniformly distributed at the membrane of each cell (A). Expression of the enhancer trap reporter gene in $ed^{l(2)k01102}/+$ is detected in the photoreceptor cells of wild type (B) and *GMR-aos* (C) discs. The *lacZ* expression of *l(2)k1102* mimics the ED expression pattern. The relative levels of *ed* mRNA from eye discs were measured by RT-PCR and the predicted 554 bp products were visualized on a 1.2% agarose gel (D).

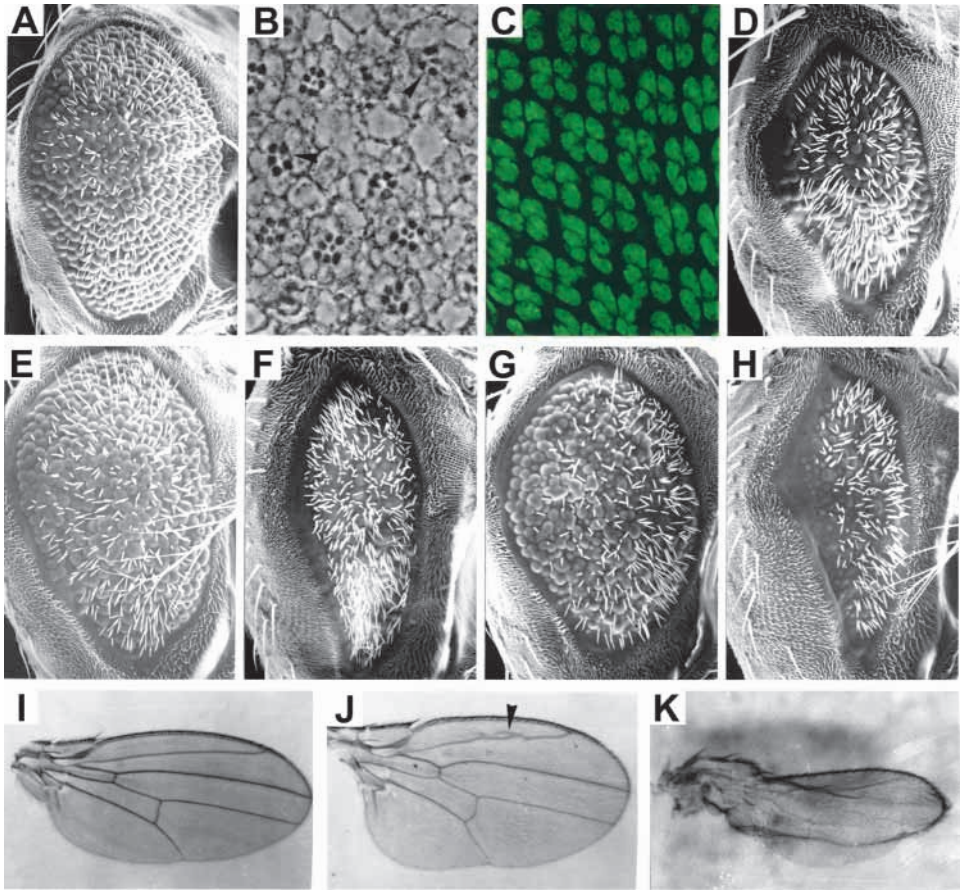

Fig. 5. Overexpression of *ed* antagonizes the activity of the EGFR signaling pathway. *GMR-GAL4/+; UAS-ed/+*. eyes are rough and reduced in size (A), and the number of photoreceptors is reduced (B). Note that some ommatidia still contain seven photoreceptors while others only contain four or five photoreceptors (arrowhead). In *GMR-GAL4/+; UAS-ed/UAS-ed* third instar eye discs (C), there are only four or five Elav-positive cells per cluster. The morphogenetic furrow in this section is to the left. The eyes of *GMR-GAL4; UAS-sty* flies are rough (D). However, this phenotype is suppressed when heterozygous for ed^{lF20} (E), but enhanced in the presence of a *UAS-ed* transgene (the genotype of the eye shown in F is *GMR-GAL4; UAS-sty; UAS-ed*). Similarly, the eye of *GMR-GAL4; UAS-kek1* are rough (G), and this phenotype is enhanced in *GMR-GAL4; UAS-kek1; UAS-ed* flies (H). *ed* also antagonizes the activity of the EGFR signaling pathway during wing vein formation. In wild type, the veins are arranged in a stereotyped pattern (I), while ed^{1X5}/ed^{slH8} transheterozygote animals show an increased size of the wing and extra wing vein (arrowhead in J). Overexpression of *UAS-ed* by *MS1096-GAL4* results in severe reduction in the vein material and size of wing, ranging from one quarter to one fifth of normal wing size (K).

ed acts parallel or downstream of *sina* but upstream of *ttk* to specify R7 cells

To determine where in the RAS/RAF/MAPK signaling pathway *ed* acts, we conducted a number of genetic epistasis experiments. sev^{d2} is a loss-of-function *sevenless (sev)* allele (Simon et al., 1991) and sev^{d2} mutant flies lack R7 cells (Fig. 6A). Although ommatidia within a ed^{1X5}/ed^{slH8} mutants contain an average of 1.34 R7 cell (Fig. 2F), we found that ommatidia within a sev^{d2}; ed^{1X5}/ed^{slH8} double mutant contain an average of 1.37 R7 cells (*n*=61) (Fig. 6B). This demonstrates that in *ed* mutants, the formation of supernumary R7 cells is independent of *sev* function. In addition, ed^{1X5} enhances the rough eye phenotype caused by overexpressing constitutive active forms of either the EGFR (Fig. 1C,F), RAS1 (Fig. 6C,D), or RAF (Fig. 6G,H). Conversely, ed^{1X5} suppresses the rough eye phenotype caused by overexpressing dominant negative RAS1 (Fig. 6E,F). While 61% of ommatidia in a sev-Ras^{N17}/+ mutant lack R7 cells (*n*=164), only 10% of ommatidia in ed^{1X5}/ed^{slH8}; sev-Ras^{N17}/+ double mutants lack R7 photoreceptors. In addition, at 25°, ed^{1X5} also rescues the lethality of Raf^{HM7}, a temperature-sensitive *Raf* allele. Therefore, *ed* acts either downstream of the *Ras/Raf* pathway or in parallel.

To determine whether ED acts in the nucleus, we generated flies double mutant for *ed;pnt*, *ed;yan* or *ed;sina*. We found that $pnt^{\Delta 88}/pnt^{1277}$ (Fig. 6I) and sev-yan^{ACT}/+ (Fig. 6K) ommatidia contain an average of 0.69 (*n*=264) and 0.05 (*n*=250) R7 cells, respectively. However, ed^{1X5}/ed^{slH8}; $pnt^{\Delta 88}/pnt^{1277}$ (Fig. 6J) and ed^{1X5}/ed^{slH8}; sev-yan^{ACT}/+ ommatidia (Fig. 6L) contain an average of 1.44 (*n*=102) and 1.01 (*n*=125) R7 cells, respectively. Strikingly, ed^{1X5}/ed^{slH8}; $sina^{2}/sina^{3}$ ommatidia (Fig. 6N) contain an average of 1.29 (*n*=180) R7 cells, as compared with 0.01 (*n*=173) R7 cells in $sina^{2}/sina^{3}$ mutant (Fig. 6M). Therefore, in *ed* mutants, the formation of supernumary R7 cells is independent of *sina* function. Finally, loss of *ttk* activity has been shown to produce ectopic R7 cells in a *sina*-independent manner (Lai et al., 1996). To determine whether *ed* acts downstream of *ttk*, we overexpressed *ttk* in *ed* mutants. Overexpression of TTK88 under the control of either the *GMR* enhancer that completely inhibits photoreceptor cell development (Fig. 6L) or the *sev* enhancer that only deletes R3, R4 and R7 photoreceptors (data not shown). However, this TTK88-mediated neuronal repression cannot be suppressed by removing *ed* activity (Fig. 6P), indicating that *ed* acts upstream of *ttk* to specify R7 development. Together, our genetic epistatic analysis suggests that *ed* acts either parallel or downstream of *Ras*, *Raf*, *pnt*, *yan* and *sina*, but upstream of *ttk* to specify R7 cell fates.

ED does not regulate *ttk88* expression or protein stability

Our genetic epistatic analyses suggest that *ed* acts upstream of

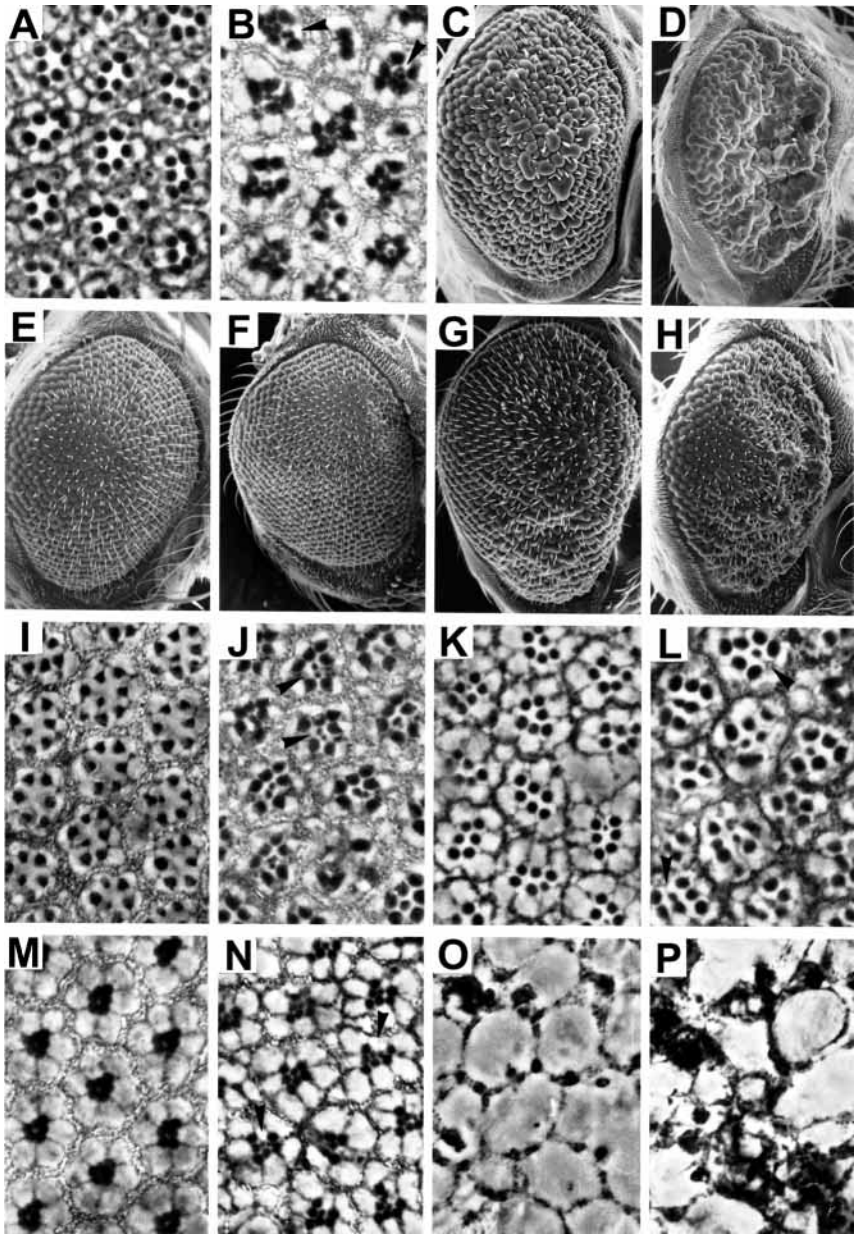

Fig. 6. Genetic epistatic analysis of *ed*. sev^{d2} ommatidia have no R7 cells (A) but sev^{d2}; ed^{1X5}/ed^{slH8} ommatidia contain ectopic R7 cells (B, arrowheads). The rough eye phenotype caused by sev-$Ras1^{V12}$/+ (C) is enhanced when heterozygous for ed^{1X5} (D). Overexpression of Ras^{N17} under the control of the *sevenless* enhancer (sev-Ras^{N17}/+) causes a rough eye (E), and this phenotype is suppressed when heterozygous for ed^{1X5} (F). The rough eye phenotype associated with sev-$tor^{4021}Raf$/+ (G) is enhanced when heterozygous for ed^{1X5} (H). Ommatidia within a $pnt^{\Delta 88}/pnt^{1277}$ (I) and sev-yan^{ACT}/+ (K) mutants contain 0.69 and 0.05 R7 cell, respectively. However, ommatidia within ed^{1X5}/ed^{slH8}; $pnt^{\Delta 88}/pnt^{1277}$ (J) and ed^{1X5}/ed^{slH8}; sev-yan^{ACT}/+ (L) double mutants contain 1.44 and 1.01 R7 cells (arrowheads), respectively. $sina^{2}/sina^{3}$ (M) ommatidia contain 0.01 R7 cells, however, ed^{1X5}/ed^{slH8}; $sina^{2}/sina^{3}$ (N) double mutants contain 1.29 R7 cells (arrowhead). Overexpression of *ttk88* under the control of the GMR enhancer *(GMR-ttk88/+)* blocks photoreceptor determination (O), and this phenotype can not be suppressed in ed^{1X5}/ed^{slH8}; *GMR-ttk88*/+ (P).

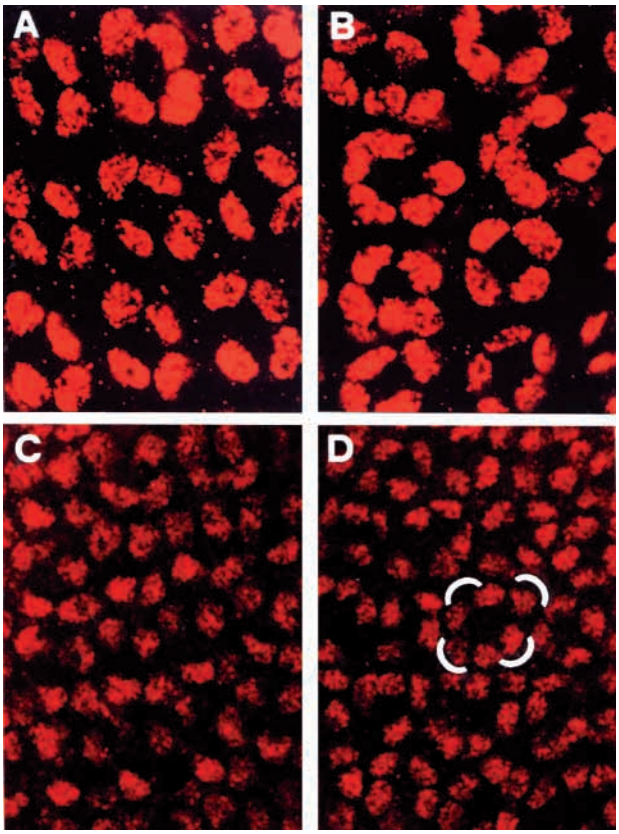

Fig. 7. *ed* does not regulate *ttk88* expression or protein stability. ttk^{0219} enhancer trap reporter gene is expressed in the cone cells (A) using anti-β-galactosidase. The expression levels of ttk^{0219} are unaffected in ed^{1X5}/ed^{slH8} mutant disc (B), which contain extra cone cells. In wild type, TTK88 is shown in the cone cell nuclei (C) using anti-TTK88. The levels of TTK88 do not change in ed^{1X5}/ed^{slH8} mutant disc (D). A six-cell cluster is marked.

ttk88 to specify R7. *ed* might regulate *ttk88* mRNA expression or TTK88 protein levels. Alternatively, *ed* might regulate the activity of TTK88 through protein modification, like phosphorylation. To determine whether *ed* regulates *ttk* expression, we examined the expression of *ttk* in *ed* mutant disc using the X-gal staining of the P-element insertion ttk^{0219} (Li et al., 1997) and detected no obvious changes (Fig. 7B). Furthermore, TTK88 is expressed at high levels in the cone cells but is not expressed in developing photoreceptor cells (Li et al., 1997; Dong et al., 1999). To determine whether *ed* regulates TTK88 protein levels, we examined TTK88 levels in *ed* (Fig. 7D) and *GMR-Gal4; UAS-ed* eye discs (data not shown). In each case, the level of TTK88 was unaffected. Together, our results suggest that ED does not regulate *ttk88* mRNA expression or TTK88 protein stability.

The *ed* mutation acts nonautonomously to generate extra R7 cells

To determine in which cells *ed* is required, we used *ey-FLP* (Newsome et al., 2000) to generate clones of homozygous ed^{slA12} mutant cells in a sev^{d2} background. As shown in Fig. 6A, no R7 cells develop in the sev^{d2} background. We scored 54 mosaic ommatidia that contain R7-like cells. Among them, 57% of the R7-like cells were ed^{-} (Fig. 8A), while 43% were

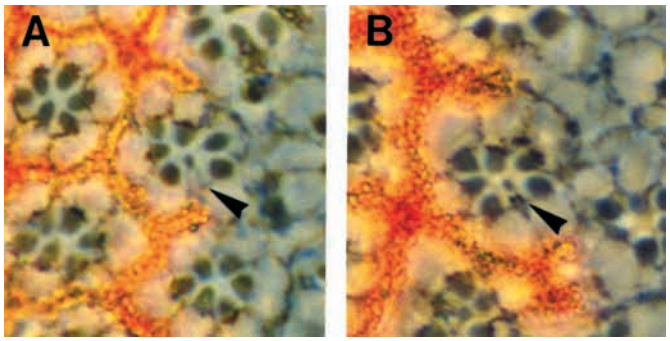

Fig. 8. *ed* functions in a non cell-autonomous manner. Phase-contrast images of section through an ed^{slA12} homozygous mutant clone induced in a sev^{d2}/Y; $ed^{slA12}/+$ animals. The clones are marked by the lack of pigmentation. sev^{d2} ommatidia have no R7 cells. A total of 54 phenotypically normal mosaic ommatidia were scored for the presence of pigment in the R7 cells. The ectopic R7 cells (arrowheads) can be derived from either *ed* mutant (A) or wild-type cells (B), indicating that the *ed* mutation acts cell non-autonomously in the generation of supernumerary R7 cells.

ed^{+} (Fig. 8B). Similar results were obtained when we generated *ed* mutant clones in *sina* and $sev\text{-}yan^{ACT}$ mutant backgrounds (data not shown). The observation that R7 cells can be derived from either wild-type or *ed* mutant cells, leads us to propose that the *ed* mutation acts cell non-autonomously in the generation of supernumerary R7 cells.

ED is not a universal repressor

ED is uniformly expressed in the follicle cells during stage 1-10 oogenesis (data not shown). To determine whether *ed* acts during oogenesis in the establishment of EGFR-dependent dorsal/ventral polarity, we examined the eggs derived from $ed^{slH8}/Df(2L)ed\text{-}dp$ females. These females are fertile and do not exhibit any overt morphological defects (data not shown). As loss-of-function mutations in many cell adhesion molecule have subtle mutant phenotypes (Ghiglione et al., 1999), we overexpressed *UAS-ed* in the follicle cells using the GAL4 drivers T155 or CY2 (Queenan et al., 1997; Ghiglione et al., 1999). The eggs derived from such females have completely normal dorsal appendages (data not shown) suggesting that ED does not interfere with EGFR signaling in follicle cells.

DISCUSSION

The EGFR plays important roles at various stages of *Drosophila* development and is subject to modulation by multiple positive and negative regulators. We have identified ED as a novel adhesion molecule-like protein that negatively regulates the EGFR signaling pathway. *ed* genetically interacts with several components in the EGFR pathway. Flies of *ed* mutant produce extra photoreceptor and cone cells. Conversely, ectopic overexpression of *ed* in the eye leads to reduction of photoreceptor number. We demonstrate that ED acts by converging on TTK88, the most downstream component known in EGF receptor signaling. Our results not only demonstrate the active role of an adhesion molecule in the EGFR signal transduction pathway but also identify a previously unknown regulatory mechanism.

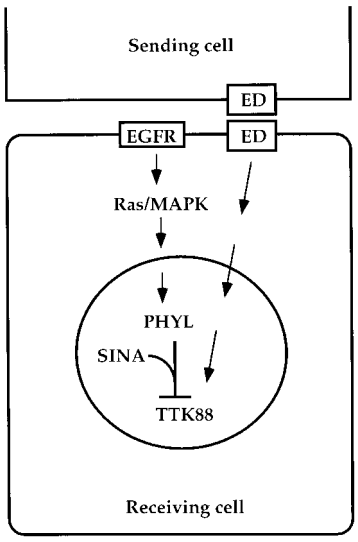

Fig. 9. Model for ED function. The membrane-spanning ED protein transmits the negative signal, via homotypic interactions, into the receiving cell where it antagonizes the EGFR signaling by converging on TTK88.

ed acts nonautonomously

ED contains six Ig domain with extensive homology to vertebrate neural adhesion molecule L1. The L1-family of cell adhesion molecules exert their functions through homophilic or heterophilic interactions with other Ig domain-containing adhesion molecule (Hortsch, 1996). We found that ED is expressed in every cell of the eye disc. In addition, our genetic analysis demonstrate that *ed* acts in a cell nonautonomous manner to generate extra R7 cells. If ED transmits the negative signal from the sending cell via homophilic interaction to the receiving cell, loss of *ed* in either sending or receiving cells would result in the same phenotype, owing to the failure to receive the inhibitory signal. Therefore the extra R7 cells found in the receiving cells could be either wild type or mutant for *ed*. However, if ED transmits the negative signaling via heterophilic interaction, *ed* is only required in the sending cells but not the receiving cells. Therefore, the extra R7 cells found in the receiving cells could be either wild type or mutant for *ed*. Alternatively, ED might act as a ligand that activates an unidentified receptor on receiving cells. All three models are consistent with our results showing that *ed* functions cell nonautonomously. However, only the homophilic interaction model would require the cytoplasmic domain of ED to be required in both the sending and receiving cells. Since we found that the cytoplasmic domain of ED is required for the repression of the EGFR pathway, we favor the homophilic interaction model between ED molecules to specify photoreceptor cell formation (Fig. 9).

ed define an independent pathway to repress EGFR signaling

Studies on RTK signaling in both vertebrates and invertebrates have converged on an evolutionarily conserved DRK/RAS/RAF/MAPK signaling cassette that is required to transmit the signal from the receptor to the nucleus. Previous studies on TORSO (TOR) signaling, however, indicated that TOR RTK transduces its signals through both a RAS-dependent and an unidentified RAS-independent pathways that converge on RAF (Hou et al., 1995). In addition, genetic analysis of *daughter of sevenless (Dos)* has revealed that it functions upstream of RAS1 and defines a signaling pathway that is independent of direct binding of DRK/GRB2 to the SEV RTK (Raabe et al., 1996; Herbst et al., 1996). Nevertheless, either a RAS- or KSR-independent pathway still acts underneath a RTK.

In contrast to the previous examples, our genetic data indicates that ED functions either downstream of RAS1/MAPK/PNT/YAN or in a parallel pathway. However, based on the following data we argue that ED is unlikely to act downstream of PNT/YAN/SINA in the nucleus, but instead defines an independent pathway that antagonizes EGFR signaling (Fig. 9). First, antiserum against ED N-terminal peptide localizes ED to the plasma membrane, but not the nucleus, of every cell in the eye disc. Second, ED functions non-autonomously in the signal-sending cells. Third, *ed* is not transcriptionally regulated by the activation of the EGFR pathway, a situation that is different from other negative regulators of EGFR such as *aos*, *sty* and *kek1*. Fourth, SINA has been shown to form a complex with PHYL to target TTK for degradation. The production of ectopic R7 cells in mutations of most negative regulators, like *Gap1* and *yan*, all require *sina* (Gaul et al., 1992; Lai and Rubin, 1992). However, the formation of extra R7 cells in *ttk* mutant is only partially *sina* dependent. This observation led Lai et al., to suggest that the production of extra R7 cells in *ttk* mutant is partly influenced by both the normal R7 developmental signals, which are *sina* dependent, and another *sin*-independent signaling (Lai et al., 1996). Our genetic analysis demonstrates that the production of ectopic R7 cells in *ed* mutant is completely *sina* independent. The observation that *ed* functions upstream of *ttk88*, implies that the independent inhibitory pathway, although *sina* independent, converges on TTK.

Mechanism of inhibition of the EGFR by ED

ED, a putative cell adhesion protein, is constitutively expressed on every cells on the eye disc. There are several ways in which *ed* expression can influence signaling. For example, it could induce polarization and adherens junction formation of undifferentiated cells. The EGFR is localized to the apical microvillar border where it binds its inductive ligand (Zak and Shilo, 1992). The apical restriction of EGFR may concentrate these receptors at a high density and allows efficient capture of the SPI ligand, thus restricting SPI diffusion over a long distance. In the absence of ED, the EGFR might diffuse to the basolateral membrane and the density of EGFR may be too low to capture SPI efficiently. According to this model, SPI may diffuse to distant cells. These cells which normally do not encounter the ligand would then differentiate extra photoreceptor or cone cells. In this case, ED would function as a mechanical force to affect the binding efficiency of EGFR and the diffusion distance of SPI. However, we do not favor this mechanism because we observe that halving the dose of *ed* can enhance the rough eye phenotype caused by constitutively active EGFR (*sev-tor*^{4021}*Egfr*), which does not require SPI.

The other possibility is that ED, via homophilic interactions, may directly transmit a negative signal. This signal would

counteract the basal ligand-independent activity of RTK, caused by nonspecific RTK oligomerization, and establish an inherent inhibitory network to prevent cells from differentiating as photoreceptor or cone cells. According to this model, only when a cell receives its ligand can it activate its RTK and antagonize this negative effect and differentiates. Thus, loss of *ed* activity behind furrow produces ectopic photoreceptor and cone cells. In this case, a photoreceptor differentiation response would be elicited in region where only the RTK signaling pathway was activated. Thus, RTK activation functions in a permissive manner.

Loss of *ed* gene activity results in ectopic photoreceptor and cone cells formation. Thus ED, like GAP1 and YAN, functions as a general repressor of differentiation in the developing eye. The negative signal that ED transmits might be mediated through TTK to repress photoreceptor cell formation and another negative regulator to repress cone cells formation. How does *ed* transmit an inhibitory signal into the receiving cells? The neurite outgrowth and axonal fasciculation mediated by L1-family neural adhesion molecule require both the homotypic or heterotypic interactions of extracellular Ig domains and the conserved ankyrin binding site (FIGQY) in the cytoplasmic domain (Dubreuil et al., 1996; Hortsch et al., 1998). Unlike members of L1-family, ED contains a 315 amino acid cytoplasmic domain with no apparent sequence homology to the conserved FIGQY ankyrin-binding site. However, deletion analysis indicates that the intracellular domain is required for the repression of the EGFR pathway. Finally, the repressing effect of ED signaling in photoreceptors is mediated through TTK. There are several ways that ED signaling might affect TTK. It might elevate the repressing activity of TTK through posttranslational modification, like phosphorylation, to increase its DNA binding affinity to its target genes. Alternatively, it might directly upregulate *ttk* mRNA or its protein levels. The activation of EGFR signaling has been shown to downregulate TTK88 protein stability (Tang et al., 1997; Li et al., 1997). We favor the former possibility, as we found that the levels of both *ttk* mRNA and ED protein are unaffected in *ed* mutant background.

ED is a tissue specific repressor

ED is widely expressed at various stages of *Drosophila* development (J.-C. H., unpublished observations). Our results demonstrate that ED is a negative regulator of EGFR and Sev signaling pathways during eye and wing development. However, ED does not appear to be involved in EGFR signaling during oogenesis. Therefore, ED differentially functions as an inhibitor of RTK in a tissue-specific manner.

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