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Fasciclin 2, the *Drosophila* orthologue of neural cell-adhesion molecule, inhibits EGF receptor signalling

Yanlan Mao and Matthew Freeman*

Adhesion proteins not only control the degree to which cells adhere to each other but are increasingly recognised as regulators of intercellular signalling. Using genetic screening in *Drosophila*, we have identified Fasciclin 2 (Fas2), the *Drosophila* orthologue of neural cell adhesion molecule (NCAM), as a physiologically significant and specific inhibitor of epidermal growth factor receptor (EGFR) signalling in development. We find that loss of *fas2* genetically interacts with multiple genetic conditions that perturb EGFR signalling. Fas2 is expressed in dynamic patterns during imaginal disc development, and in the eye we have shown that this depends on EGFR activity, implying participation in a negative-feedback loop. Loss of *fas2* causes characteristic EGFR hyperactivity phenotypes in the eye, notum and wing, and also leads to downregulation of Yan, a transcriptional repressor targeted for degradation by EGFR activity. No significant genetic interactions were detected with the Notch, Wingless, Hedgehog or Dpp pathways, nor did Fas2 inhibit the FGF receptor or Torso, indicating specificity in the inhibitory role of Fas2 in EGFR signalling. Our results introduce a new regulatory interaction between an adhesion protein and a *Drosophila* signalling pathway and highlight the extent to which the EGFR pathway must be regulated at multiple levels.

KEY WORDS: EGFR, Fasciclin 2, Adhesion, Signalling, *Drosophila*

INTRODUCTION

Animal cell behaviour is largely governed by intercellular signals that move between cells, bind to receptors and stimulate responses in the receiving cells. Much is known about the logic and molecular mechanisms of the signal transduction pathways downstream of receptors, but the picture of how the transmission and reception of the signal is regulated is much less clear. Unsurprisingly, cell-adhesion proteins can modulate signalling (reviewed by Comoglio et al., 2003; Christofori, 2003), but the relationship is complex and variable. From first principles, it is obvious that the degree to which cells stick to each other is likely to influence the transmission of signals between them. More specifically, there are numerous examples of specific adhesion complexes at the cell surface that appear to act as specialised sites of signal transmission (reviewed by McLachlan and Yap, 2007; Parsons, 2003). There are also well-documented examples of specific interactions between growth factor receptors and adhesion proteins that modulate receptor activity (e.g. reviewed in Comoglio et al., 2003). In these latter cases, this may be independent of the adhesion function of the protein. Beyond the spectrum of different types of relationships, the interaction between adhesion proteins and signalling can be cooperative or antagonistic: in some cases adhesion promotes signalling, in others it inhibits (e.g. Williams et al., 1994; Francavilla et al., 2007). Understanding the variety of relationships between signalling and adhesion is made more difficult by the fact that much of the information available has relied on cell culture and other in vitro methods. There are rather few cases where the in vivo significance of interactions between signalling and adhesion proteins is clear.

We have used *Drosophila* genetics to search for physiologically significant regulators of the epidermal growth factor receptor (EGFR) signalling pathway. The *Drosophila* EGFR is the orthologue of the four ErbB receptors in mammals, and has multiple functions in development (Shilo, 2003; Domínguez et al., 1998). In different contexts, EGFR signalling can trigger cellular responses as varied as differentiation, division, survival and migration. A consequence of this pleiotropy is that regulation of signalling strength and location must be precise. Moreover, signalling pathways appear to incorporate mechanisms of robustness against environmental perturbation. Our long-term goal is to understand the molecular machinery that provides these stringent control properties. In addition to revealing the logic of normal development, deregulation of ErbB activity is implicated in many human diseases, especially cancer (Uberall et al., 2008), further emphasising the importance of understanding the control of this pathway. An advantage of the forward genetic approach that we have used is that it makes no assumptions about the types of proteins needed for regulation; instead, it relies on random mutagenesis to reveal significant players – however novel or unexpected. It therefore complements more focused biochemical approaches. We have used genetic modifier screens in the *Drosophila* eye as a tool to identify novel regulators of EGFR signalling (Casci et al., 1999; Charroux et al., 2006). The eye develops in a well-characterised and stereotypical way (Wolff and Ready, 1993), and, because it is dispensable for viability, at least in the laboratory, it provides an excellent platform for genetic screening.

Many intracellular transducers and regulators that act downstream of the EGFR have been identified, but much less is known about the control of earlier events of signalling: the generation, transmission and reception of the ligand. Here, we report the identification of Fasciclin 2 in a genetic screen that was designed to focus on these earlier stages. The Fasciclin 2 (Fas2) protein is the *Drosophila* orthologue of the mammalian neural cell adhesion molecule (NCAM) family (Grenningloh et al., 1991; Cunningham et al., 1987), and our results show that it acts specifically to inhibit EGFR signalling during the normal development of the *Drosophila* eye, notum and wing.

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK.

*Author for correspondence (e-mail: mf1@mrc-lmb.cam.ac.uk)

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MATERIALS AND METHODS

Drosophila strains and genetics

All crosses were performed at 25°C unless otherwise stated. The following fly strains, described in FlyBase (http://flybase.bio.indiana.edu/), were used: *yw, GMR-argos, sev-rho, GMR-Gal4, sevEP-Gal4, C765-Gal4, UAS-EGFR, UAS-sprouty, UAS-tor*^{4021}*-EGFR, argos*$^{l\Delta 7}$*, rhomboid-1*$^{\Delta 5}$*, fas2*^{eb112} *sn FRT19A/FM7c* (Grenningloh et al., 1991) (gift from C. Fabre); *fas2*^{e76} (Grenningloh et al., 1991) (gift from L. Garcia Alonso); *fas2*^{G0293}*/FM7c, fas2*^{G0336}*/FM7c* (from Bloomington Drosophila Stock Centre); *w ubiGFP M(1)*^{osp}*FRT19A; A59/TM6b* (this study); *XA12 (R7-lacZ), P(w+, X81) rho-lacZ, spi*^{A14}*FRT40A/Cyo, Dl*^{rev10}*, GMRSu(H)DN, Hairless*^{P141} (gifts from S. Bray); *F76e, sev-wg, UAS-cadi,en-Gal4* (gifts from M. Bienz); *UAS-Smo5A,C765-Gal4* (a gift from S. Cohen); and *GMR>λ-btl, dof, trk*^{5}*, dlg*^{G0276}*, dlg*^{G0342}*, dlg*^{G0456}*, Nrg*^{l7}*, Nrg*^{l4}*, Nrg*^{G0099}*, Nrg*^{G0413}*, lgl*^{4}*, scrib*^{j7B3}.

Mitotic clones in the eye and wing discs were induced by the FLP/FRT technique (Xu and Rubin, 1993) in *Minute* and non-*Minute* background (Morata and Ripoll, 1975). Recombination was induced 48-72 hours after egg laying by a 60 minute heat shock at 37°C or by *eyeless*-induced FLP activity. Mutant clones were marked as appropriate by the absence of GFP or β-galactosidase (β-gal) antibody staining. The following genotypes of larvae were used for generating mutant clones: *fas2*^{eb112} *sn FRT19A/arm-lacZ FRT19A; eyflp/+* and *fas2*^{eb112} *sn FRT19A/ubi-GFP M(1)*^{osp}*FRT19A; MKRShsflp/+* (*Minute* background)

Immunostaining

Imaginal discs and pupal retinas (aged 40 hours at 25°C after pupation) were stained as described previously (Gaul et al., 1992). Primary antibodies used were: mouse anti-Fas2 1D4 (1:50), rat anti-Elav (1:200), mouse anti-Cut (1:100), mouse anti-Prospero (1:50), mouse anti-Yan (1:100), mouse anti-Achaete (1:10) (all from Developmental Studies Hybridoma Bank), rabbit anti-Armadillo (1:100) (a gift from M. de la Roche), guinea pig anti-Senseless (1:1000) (gift from H. Bellen), rabbit anti-pMad (1:200) (gift from E. Laufer and C. Heldin), rabbit anti-β-galactosidase (1:1000) (Cappel), rabbit anti-GFP (1:200) (Sigma). The appropriate fluorescently conjugated secondary antibodies from Molecular Probes and Jackson ImmunoResearch were used.

Confocal imaging and three-dimensional reconstruction

For three-dimensional reconstruction, discs were mounted under a coverslip supported by two strips of double-sided adhesive tape using Fluoromount-G (Southern Biotech). Discs were analysed with a BioRad Radiance 2100 laser scanning confocal microscope. Z-series were projected for three dimensional reconstruction using Volocity 2.5.1 software. All images were analysed using Adobe Photoshop.

Fluorescence intensity quantification

Fluorescence (pixel) intensities of confocal images were quantified using MetaMorph.

In situ hybridisation

The first 1.5 kb of the *fas2*-coding sequence was amplified from FasIIPA cDNA (a kind gift from Michael Hortsch) (primers were F, 5′-ATGGGTGAATTGCCGCCAAATTC-3′ and R, 5′-AGAGTAATA-CTGCCTCGTTACGGG-3′) and cloned into pCRBlunt IITOPO vector using standard techniques. DIG-labelled RNA antisense and sense probes were transcribed using Sp6 and T7 promoters, respectively, using manufacturer's instructions from Roche. In situ hybridisation on imaginal discs were carried out using standard procedures (Cubas et al., 1991).

Embryonic cuticle preparations

Overnight embryo collections were allowed to age for 24 hours. Embryos were dechorionated in 50% bleach, removed from their vitelline membranes, and mounted in 1:1 Hoyer's mountant:lactic acid. Cuticles were viewed under dark-field optics.

Scanning electron microscopy of adult eyes

Flies were frozen for at least 1 hour at –80°C, mounted onto aluminium electron microscope specimen stubs, and coated with 20 nm of a gold-palladium mixture. Samples were viewed on a Philips XL30 scanning electron microscope.

Photographs of adult flies

Flies were positioned appropriately and examined under a dissecting microscope. Images were taken at roughly 35 different focal planes with a Nikon D3 digital camera. The planes were then stacked using Helicon focus software.

RESULTS

Fasciclin 2 inhibits EGFR signalling in the developing eye

To identify regulators of the EGF receptor pathway that act close to the production, transmission and reception of the ligand, we performed a two-stage genetic modifier screen in the *Drosophila* eye. EGFR signalling was perturbed in opposing directions: upregulated by overexpression of Rhomboid-1 (Rhomboid – FlyBase), the protease that releases and activates membrane tethered EGF family ligands (Lee et al., 2001); and downregulated by the overexpression of Argos, an inhibitor that binds soluble ligand (Klein et al., 2004; Klein et al., 2008), preventing it from activating the receptor. The logic of using these two conditions is based on our experience that modifier screens are most efficient at identifying pathway components close to the site of perturbation. Rhomboid 1 and Argos act in ligand production and transmission, therefore we hoped to identify other components at this level of the pathway. For rigour, candidates were selected only if they interacted with both conditions and in opposite directions: that is, a suppressor of one needed to enhance the other. In one iteration of this strategy, a collection of approximately 350 P-elements on the X-chromosome was screened. Of these, 50 interacted with either *GMR-argos* or *sev-rhomboid-1*, of which 20 were identified as interacting with both in opposite directions. These 20 candidates were then narrowed down as follows: 10 were revertable by precise excision of the P-element (confirming that the P-element identifies the relevant gene), of which three showed consistent interactions with other components of the EGFR pathway; only one of these three also had a loss-of-function phenotype consistent with a role in EGFR signalling. This single gene, selected by this hierarchical triage, was an allele of the *fasciclin 2* (*fas2*^{G0293}) gene. It was identified as a suppressor of *GMR-argos* and as an enhancer of *sev-rhomboid-1* (Fig. 1A-D). These interactions were confirmed with other alleles of *fas2*, including *fas2*^{eb112} (Fig. 1E), an independently derived null allele (Grenningloh et al., 1991), and suggested that Fas2 might inhibit EGFR signalling. Fas2 is a member of the immunoglobulin superfamily and is the *Drosophila* orthologue of the conserved NCAM family of neural cell-adhesion molecules (Grenningloh et al., 1991). To investigate further the possibility that Fas2 inhibits EGFR signalling in the *Drosophila* eye, we examined genetic interactions with other components of the pathway. Consistent with the screen results, halving the dose of *fas2* enhanced the rough eye caused by overexpression of *Egfr* itself (Fig. 1F,G) and suppressed the rough eye caused by the overexpression of *sprouty*, another inhibitor of the pathway (Fig. 1H,I).

The *fas2* gene is expressed in a subset of neuronal cells in embryonic and post-embryonic nervous system. We examined the distribution of transcripts in the third instar imaginal eye disc by in situ hybridisation (Fig. 2A). *fas2* was not expressed in the undifferentiated cells anterior to the morphogenetic furrow but appeared strongly at the furrow; it then decreased for a few rows of ommatidia before strengthening again towards the posterior of the disc. Antibody staining confirmed this dynamic developmental pattern for the Fas2 protein (Fig. 2B): it was strongly upregulated just posterior to the furrow, prior to the expression of Senseless, the earliest marker of the R8 photoreceptor that seeds each ommatidium

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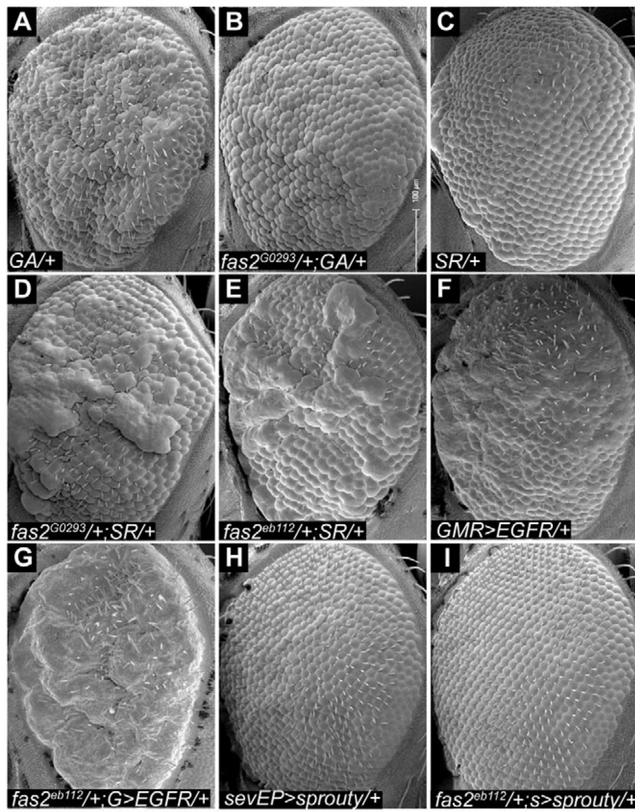

Fig. 1. *fas2* genetically interacts with EGFR pathway. (**A**) Control *GMR-argos (GA)*/+ eye is rough. (**B**) *GA*/+ rough eye was suppressed when heterozygous for the P-element allele *fas2*^{G0293}. (**C**) Control *sev-rhomboid-1 (SR)*/+ rough eye (grown at 18°C). (**D**,**E**) *SR*/+ rough eye was enhanced when heterozygous for *fas2*^{G0293} (D) and *fas2*^{eb112} (E) (grown at 18°C). (**F**,**G**) *GMR-Gal4*/+; *UAS-EGFR*/+ rough eye (F) was enhanced when one copy of *fas2* was removed (G). (**H**,**I**) *sevEP-Gal4*/+; *UAS-sprouty*/+ rough eye (H) was suppressed when one copy of *fas2* was removed (I).

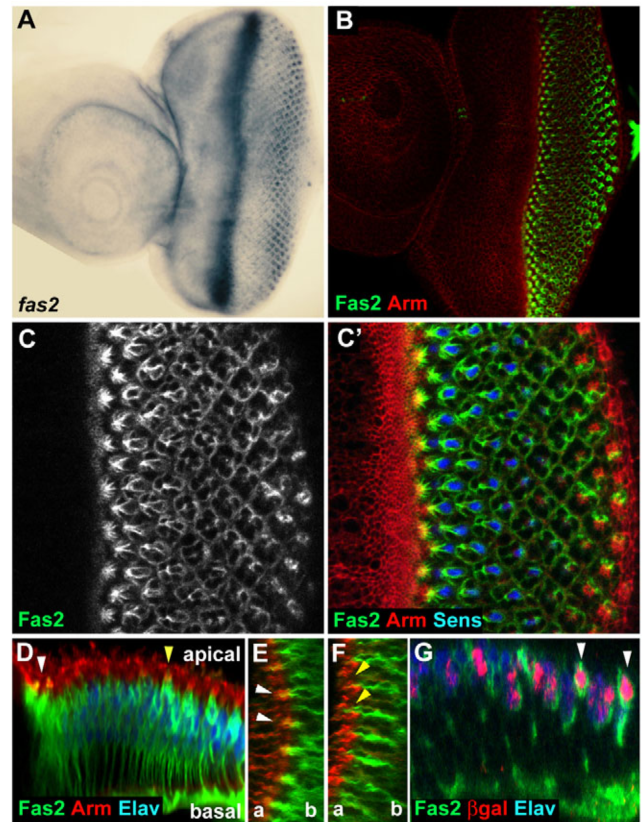

Fig. 2. Fas2 expression in the eye. In all images, anterior is towards the left, unless otherwise stated. (**A**) RNA in situ hybridisation of a third instar eye disc with a probe against *fas2*. (**B**) Third instar eye disc stained with antibodies against Fas2 (green) and Armadillo (Arm, red). (**C**,**C′**) A higher magnification view of Fas2 expression in a third instar eye disc co-stained with anti-Arm and anti-Senseless (Sens, blue). (**D**) A projection of *x/z* sections of a third instar eye disc stained with anti-Fas2, anti-Elav (photoreceptor marker, blue) and anti-Arm (marks apical compartment). Fas2 is mostly expressed basolaterally. White and yellow arrowheads indicate where Fas2 is also expressed apically. (**E**) *y/z* section in the plane of the white arrowhead in D with apical (a) towards left, stained with Fas2 (green) and Arm (red) showing some Fas2 in the apical compartment, white arrowheads. (**F**) Y/Z section in the plane of the yellow arrowhead in D with apical towards the left, stained with Fas2 (green) and Arm (red) showing some Fas2 apically, yellow arrowheads. (**G**) Third instar eye disc of XA12, a β-gal reporter line strongly expressed in R7s, stained with anti-β-gal (red), Fas2 (green) and Elav (blue). *x/z* section shows Fas2 is elevated in R7 cells, arrowheads.

(Fig. 2C). After the initial strong expression in the preclusters, Fas2 remained expressed at a low level in distinct ommatidial cluster patterns in the cell membranes of most photoreceptor cells. Eventually, one photoreceptor accumulated elevated levels of Fas2, and co-staining with an R7-expressing β-gal reporter line *XA12* (Hart et al., 1990), showed this to be the R7 photoreceptor (arrows, Fig. 2G). Significantly, although the bulk of Fas2 protein was detected basolaterally (Fig. 2D-F), in those cells with elevated levels (i.e. just posterior to the furrow and, later, R7s), we observed Fas2 staining in the apical compartment, where EGFR signalling occurs (arrows Fig. 2D-F).

Our data indicated that Fas2 is expressed in a specific pattern as the eye develops and that halving its dose perturbs EGFR signalling in the eye. If it is indeed a physiologically important regulator of the EGFR, loss-of-function mutations should have phenotypes characteristic of EGFR hyperactivity. The EGFR has multiple functions in eye development but one of its key roles is the recruitment of cells into the developing ommatidia. *fas2*-null mutants are lethal but it has previously been noticed that the escapers of the semi-viable transheterozygotes between a null (*fas2*^{eb112}) and a hypomorphic allele (*fas2*^{e76}) have rough eyes when grown at 18°C (Garcia-Alonso et al., 1995). We examined the pupal retinas from these rough eyes (Fig. 3B) and found that photoreceptor number was disrupted: 5% of ommatidia (n=158) had at least one extra photoreceptor [0% in wild type (WT), Fig. 3A]; 4% had fewer photoreceptors (0% in wild type); and about 4% of clusters appeared by location and photoreceptor number to be satellite 'mini-clusters' (0% in wild type, see below for discussion of these, and Fig. 3F′ for an example). Cone cell numbers were also disrupted: 3% of ommatidia (n=158) had extra cone cells (0% in wild type); 3% had fewer cone cells (0% in wild type); and 4% contained fewer cone cells in 'mini-clusters' (0% in wild type).

To determine whether the rough eye phenotype of these *fas2*^{eb112}/*fas2*^{e76} transheterozygotes could be modified by changing levels of EGFR signalling, we examined the phenotype of additionally halving the dose of *argos*, an EGFR-specific inhibitor. At 18°C, there were very few survivors, so the test was

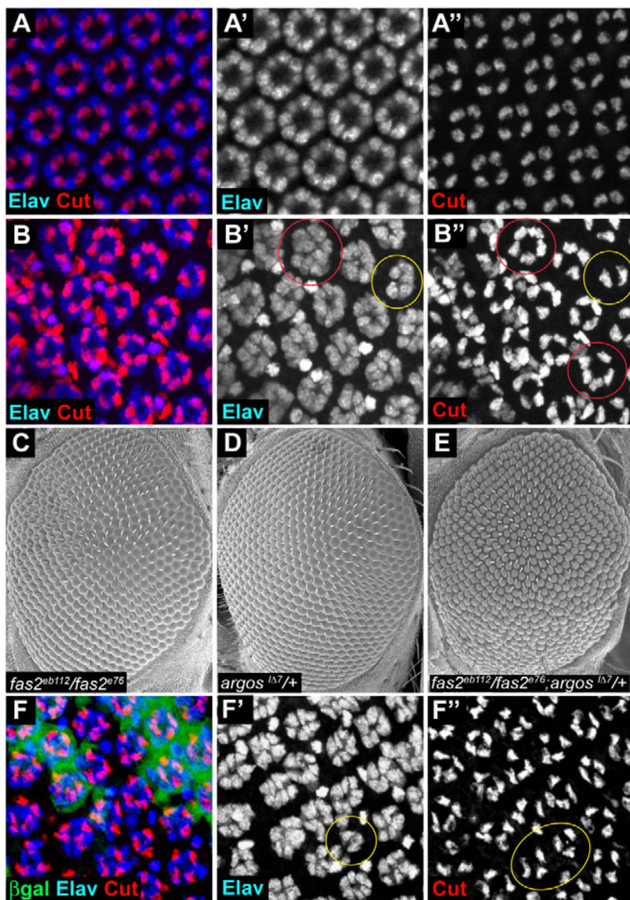

Fig. 3. *fas2* loss-of-function phenotypes in pupal retinas and adult eyes. (**A-A″**) Wild-type pupal retina stained with anti-Elav (blue; eight photoreceptors per ommatidium) and anti-Cut (red; four cone cells per ommatidium). (**B-B″**) Pupal retina of *fas2*^{eb112}/*fas2*^{e76} grown at 18°C. Circles (red, excess cells; yellow, missing cells) indicate ommatidia with abnormal numbers of photoreceptors (B′) and cone cells (B″). The strongly staining isolated Elav-positive cells in B′ are interommatidial bristles not photoreceptors. (**C**) *fas2*^{eb112}/*fas2*^{e76} eye is only mildly rough when grown at 20°C. (**D**) *argos*$^{l\Delta7}$/+ eye is wild type at 20°C. (**E**) Roughness of the *fas2*^{eb112}/*fas2*^{e76} eye is enhanced by heterozygosity for *argos*$^{l\Delta7}$ (grown at 20°C). (**F-F″**) *fas2*^{eb112} clones marked by lack of β-gal (green). 'Mini' ommatidia can be seen with Elav (circle, F′) and Cut reveals cone cell defects (circle, F″).

carried out at 20°C where viability was improved slightly. The *fas2*^{eb112}/*fas2*^{e76} phenotype was only very mildly rough at this temperature (Fig. 3C), but this was significantly enhanced by halving the dose of *argos* (Fig. 3E). This is consistent with the interactions described above, and suggests that reduction of Fas2 leads to upregulated EGFR signalling.

To obtain a clearer picture of Fas2 function, we examined clones of the null allele *fas2*^{eb112}. These clones have a similar but stronger phenotype to the transheterozygous hypomorphs (Fig. 3F). Nine percent of mutant ommatidia (n=376) had extra photoreceptors (0% in wild type), 12% had fewer (0% in wild type) and 4% appeared to be satellite mini-clusters (0% in wild type), whereas 3% had extra cone cells, 6% had fewer and 4% were in mini-clusters (0% in wild type for all cases). In principle, the phenotype caused by loss of *fas2* could be caused by abnormal cell recruitment or by later defects. We therefore examined *fas2*-null clones in third instar discs, when recruitment occurs. The earliest phenotype we saw was the presence of ectopic small clusters of Elav-positive cells between ommatidia, the same satellite 'mini-clusters' seen in pupal retinas (Fig. 4A). Staining for Senseless expression showed that these abnormal clusters had no R8 cell associated with them (Fig. 4B). Ectopic clusters of this kind are also seen in eye discs mutant for the EGFR inhibitors *argos* and *sprouty* (Freeman et al., 1992; Casci et al., 1999); moreover, hyperactive EGFR activity has been shown directly to trigger the differentiation of photoreceptors in the absence of a founding R8 cell (Domínguez et al., 1998). The presence of these ectopic photoreceptor clusters is therefore a hallmark of EGFR hyperactivity. In further correspondence with the pupal phenotype, ommatidia with extra photoreceptors were also observed, another phenotype of excess EGFR signalling. By contrast, unlike in pupal retinas, we were unable to observe ommatidia at this stage with too few photoreceptors, suggesting that the observed loss of photoreceptors may occur later in development.

We used cell-type specific markers to investigate the identity of the extra photoreceptors. In *argos* mutants, there is a frequent transformation of non-neuronal 'mystery cells' into extra R3/4 type photoreceptors, some of which break away to form ectopic mini-clusters; more rarely extra R7 cells are recruited to existing ommatidia (Freeman et al., 1992). Conversely, the extra cells in *sprouty* mutants are predominantly R7 type, although extra outer photoreceptors, forming ectopic mini-clusters, also occur (Casci et al., 1999). Photoreceptors in the ectopic mini-clusters in *fas2* mutants did not express markers that identified them as having a specific photoreceptor subtype, but the location of their initiation close behind the furrow and their positioning with respect to the ommatidia (Fig. 4A), is very similar to the ectopic clusters seen in *argos* and *sprouty* mutants. We therefore infer that they most closely resemble outer photoreceptors. In those *fas2*$^{-}$ ommatidia that show excess recruitment, the additional photoreceptors (Elav positive) were stained by anti-Prospero [an R7 and cone cell marker (Kauffmann et al., 1996)] and were therefore R7-like (Fig. 4C-D′). Interestingly, genotypically wild-type ectopic R7 cells were found at the borders of clones (Fig. 4D-D′); that is, a wild-type cell could adopt an abnormal fate if adjacent to *fas2* mutant cells, an indication of a non-autonomous effect. In summary, we detect two types of extra photoreceptors in *fas2*$^{-}$ clones: those that resemble outer photoreceptors, forming the mini-clusters; and R7-like cells, recruited into otherwise normal ommatidia.

The recruitment of ectopic clusters and excess photoreceptors associated with loss of *fas2* are specific characteristics of EGFR hyperactivity and therefore strongly support the case that Fas2 acts to inhibit EGFR signalling in the eye. An even more direct readout is the stability of the Yan protein, a transcriptional repressor of EGFR targets that is targeted for degradation by EGFR signalling (O'Neill et al., 1994; Rebay and Rubin, 1995). *fas2*-null clones showed a clear and reproducible, albeit modest, reduction in Yan protein levels, implying abnormally high levels of EGFR activity (Fig. 4E-G). Consistent with the Fas2 expression pattern, this phenotype was apparent only close to the furrow and towards the posterior of the eye disc: no effect was seen in the intervening zone of low Fas2 expression. The most prominent manifestation of this phenotype was the enlarged 'holes' in the Yan expression pattern, where Yan was clearly degraded more extensively than in neighbouring wild-type tissue (Fig. 4E′-G). This pattern of excess degradation presumably relates to the normal high level of Fas2

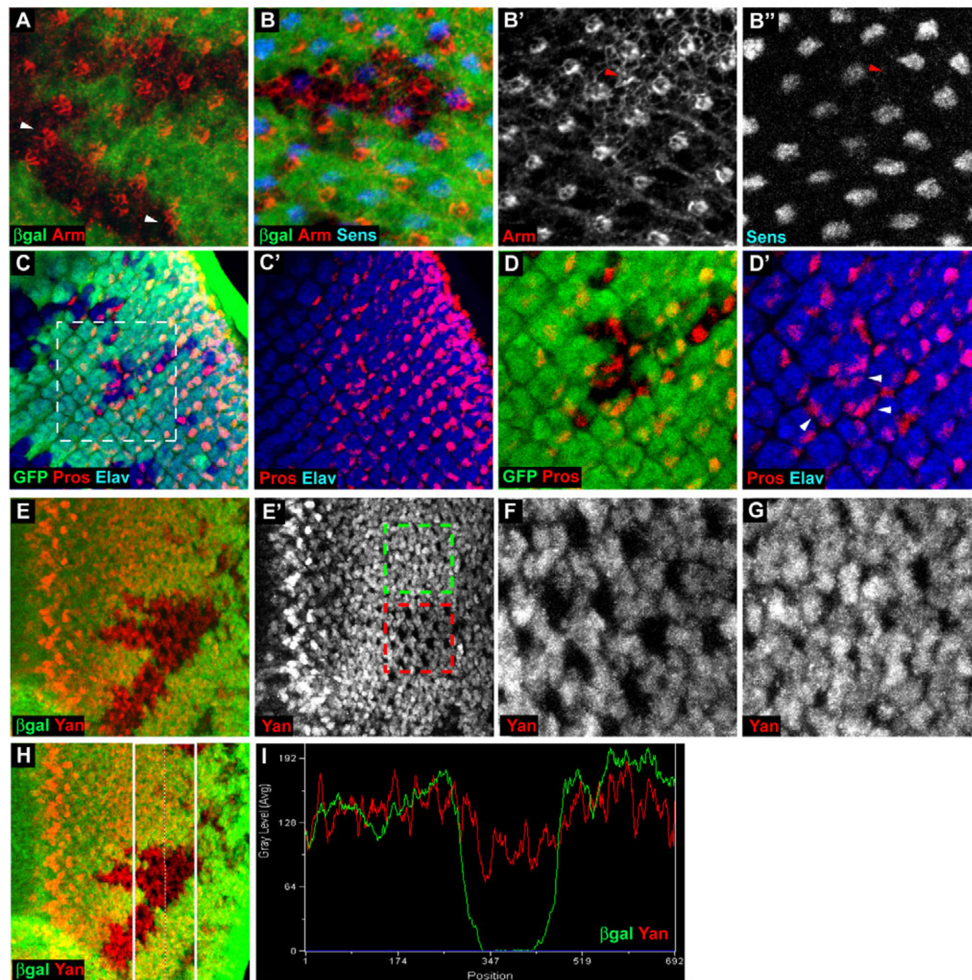

Fig. 4. *fas2* loss-of-function phenotypes in third instar eye discs. All images in this figure are of *fas2*^{eb112} clones, marked by lack of β-gal (green) or GFP (green). (**A**) Anti-Arm (red) reveals ectopic clusters in mutant tissue (white arrows). (**B-B″**) Co-staining with antibodies against Arm (red) and Sens (blue) revealed that the ectopic clusters (red arrowhead, B′) do not have R8 cells (missing Sens-positive cell, red arrowhead, B″). (**C-D′**) Co-staining with anti-Elav (blue) and anti-Prospero (red) revealed frequent cases where two cells per ommatidium stained positive for both Elav and Pros (pink cells) in clones. (D,D′) An enlargement of the area marked in C. Extra R7s can be seen within the clone (indicated by arrowheads); these were sometimes genotypically wild type (indicated by expression of GFP, lower two arrowheads in D′). (**E**,**E′**) Co-staining with anti-Yan (red) showed Yan downregulation in *fas2*$^{–}$ clones. (**F**) An enlargement of the region outlined in red in E′. (**G**) An enlargement of the region outlined in green in (E′). (**H**) White rectangle marks the area used in the fluorescence intensity quantification in I. (**I**) Graph showing the averaged relative fluorescence (pixel) intensities of Yan staining (red) and β-gal staining (green). The top of the rectangle in H is 0 on the position axis. Intensities are averaged across the anterior-posterior axis of the marked rectangle. Cells within the clone, marked by the absence of β-gal, have reduced levels of Yan.

expression being limited, at this stage in eye development, to the R7 cells. Therefore, loss of Fas2 would be expected primarily to affect R7 precursors and their neighbours (recall the local non-autonomous effects we observed in *fas2*$^{–}$ clones). To measure the reduction in Yan staining, we quantified the average fluorescent intensities of GFP and Yan staining across an area of wild-type and mutant tissue at the same developmental stages of nine different *fas2*$^{–}$ clones (example marked in Fig. 4H). This revealed a significant reduction in both overall Yan levels and the troughs of staining corresponding to the ‘holes’ in the staining pattern (Fig. 4I).

The evidence presented so far shows that *fas2* mutations genetically interact with components of the EGFR pathway in a direction that suggests that Fas2 inhibits EGFR signalling in the eye. The developmentally regulated expression pattern, the intracellular protein localisation and the phenotype of *fas2* mutants all support this functional relationship between the adhesion protein and the growth factor receptor. We next wanted to learn more about the physiological significance of this interaction and we started by asking whether it is confined to the developing eye.

Fasciclin 2 inhibits EGFR signalling in the developing notum and wing

The wing imaginal disc gives rise to the adult wing and the notum, and in both these structures the EGFR has well defined roles (Guichard et al., 1999; Culi et al., 2001). We therefore analysed Fas2 expression in the developing wing disc, both by RNA in situ hybridisation (Fig. 5A) and by antibody expression. Fas2 is expressed in the proneural clusters in the notum region of the wing disc (Fig. 5B), as well as in the wing vein and wing margin primordia (Fig. 5C); we also detected expression in proximal regions of the wing blade and the hinge region (Fig. 5B,C). The overlap in some cells between the expression of Fas2 and

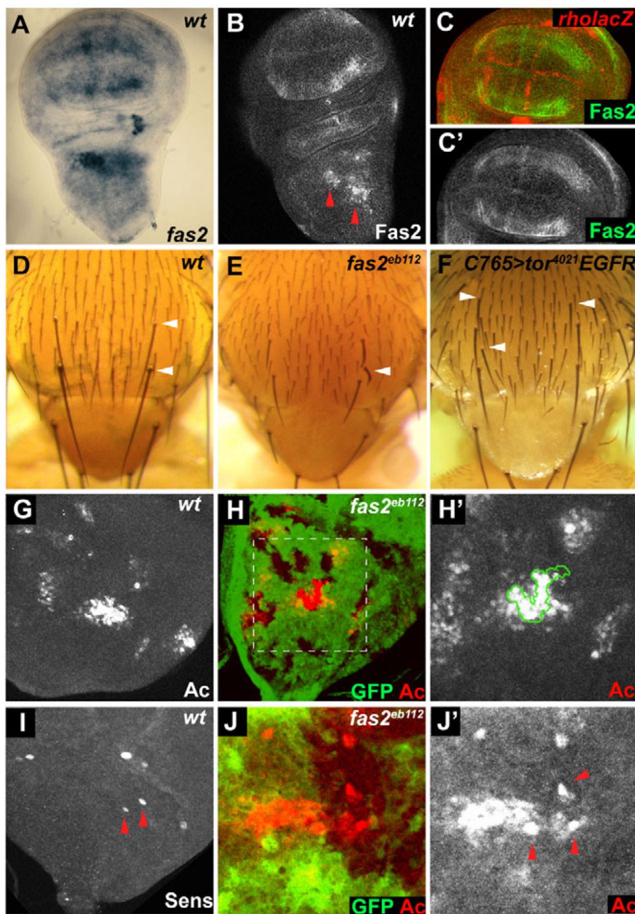

Fig. 5. Fas2 in the notum and wing. In all images of wing discs, dorsal is towards the bottom. (**A**) In situ hybridisation of a third instar wing disc with a probe against *fas2*. (**B**) Optical section through a third instar wing disc stained with anti-Fas2 showing expression in the proneural cluster regions of the prospective notum (arrowheads). (**C**,**C′**) *rho-lacZ* in third instar wing disc (red) marks wing veins and the wing margin; this colocalises with anti-Fas2 (green). (**D**) Wild-type adult notum with two dorsocentral (DC) bristles per heminotum (arrowheads). (**E**) *fas2^{eb112}* clones in the notum, marked by *singed,* resulted in extra DC bristles (arrowhead). (**F**) Hyperactivation of EGFR signalling by overexpressing a constitutively activated form of the EGFR in the notum also resulted in extra DC bristles (arrowheads). (**G**) Anti-Achaete (Ac) reveals the proneural cluster pattern in a wild-type late third instar wing disc. (**H**) Wing disc with *fas2^{eb112}* clones, marked by lack of GFP (green), and co-stained with anti-Ac (red). (**H′**) Enlargement of the area highlighted in H. The DC proneural cluster, marked by Ac, expands to fill the *fas2^{eb112}* clones (outlined in green). (**I**) Wild-type late third instar wing disc stained with anti-Sens to mark the sensory organ precursors (SOP). A maximum of two SOPs will form in the DC region of a wild-type disc (arrowheads). (**J**,**J′**) A *fas2^{eb112}* clone in the DC region of the notum of a late third instar wing disc. Three SOPs (arrowheads) have formed in the mutant DC region, as marked by the high accumulation of Ac (red in J, white in J′).

Rhomboid-1 is consistent with a functional relationship between EGFR signalling and Fas2. It should be emphasised, however, that the Fas2 expression is much wider than the domain of Rhomboid-1 expression, implying that other signals also participate in the determination of the Fas2 domain.

Loss-of-function clones of *fas2* in the adult notum resulted in the frequent presence of one or more ectopic dorsocentral (DC) bristles; other macro- and microchaetae were largely unaffected (Fig. 5E). On average there was an increase of 0.65 DC bristles (wild type=2) per heminotum with clones in the DC region (n=37). As not all clones span the whole DC region, we also calculated the number of extra bristles per mutant bristle, an average increase of 0.77 DC bristles. Excess EGFR activity also causes the formation of extra DC bristles by promoting proneural gene expression (Culi et al., 2001) (Fig. 5F). Furthermore, expression of a constitutively active form of the EGFR produces expanded proneural clusters (Y.M. and M.F., unpublished). Consistent with the idea that Fas2 is an inhibitor of EGFR signalling, null *fas2* clones had expanded proneural clusters and ectopic SOPs (Fig. 5G-J′). We note that it has previously been reported that Fas2 is required for the formation of specific proneural clusters in the developing head (Garcia-Alonso et al., 1995); this apparent opposite function is presumably an example of the context dependence of signalling. Another characteristic phenotype of EGFR hyperactivity is the formation of extra wing vein cells (Sturtevant et al., 1993); again, these are observed in *fas2* clones (see Fig. S1 in the supplementary material). Overall, these results in the notum and wing all support the conclusion that Fas2 acts to inhibit EGFR signalling in normal development, and they imply that it is not an eye-specific function.

We also noted that the wings of flies with the viable *fas2* hypomorphic genotype appeared abnormally elongated when grown at 18°C. By measuring the ratio of their length to breadth, it was confirmed that *fas2*$^{-}$ wings had a length to breadth ratio of 2.24±0.05 (mean±s.d.; n=19), significantly different (P<0.0001) from 2.13±0.03 in wild-type wings (also grown at 18°C, n=19; see Fig. S2 in the supplementary material). Although EGFR activity is implicated in the control of cell proliferation in imaginal discs, it has not previously been reported to control wing shape. We examined this directly and found that overexpression of wild-type EGFR, a condition that only moderately increases signalling, also leads to significant (P<0.0001) wing elongation, albeit less pronounced (2.18±0.02, n=20). Higher levels of EGFR signalling caused widespread transformation of intervein cells to ectopic veins (not shown), making the wings too abnormal to determine change in shape.

Feedback regulation by Fas2

Regulatory precision of the *Drosophila* EGFR pathway depends on multiple inhibitors. The expression of at least three of these, Argos, Sprouty and Kekkon-1, depends on EGFR signalling, meaning that they participate in a negative-feedback control strategy that limits the extent and/or strength of signalling (Shilo, 2003). Here, we report that Fas2 is a new inhibitor of EGFR signalling: is it also part of a negative-feedback loop? Overexpression of the EGFR activator Rhomboid-1 in the eye or the wing, under the respective control of the *sevenless* enhancer or Dpp-Gal4, did not induce detectable upregulation of Fas2, implying that EGFR signalling is not a rate-limiting determinant of Fas2 expression in these cells. However, loss of EGFR activity, in *spitz* mutant clones, caused a failure of Fas2 upregulation in the developing eye disc (31/31 clones) (Fig. 6), demonstrating a formal dependence on EGFR activity, and consequently the existence of a potential feedback loop. This conclusion needs to be qualified, however, because, in the absence of EGFR signalling, disc cells are not recruited to become photoreceptors, so the dependence may be indirect: the cells within the clones do not become photoreceptors. Nevertheless, the dependence on EGFR activity implies that Fas2 expression may participate in a self-limiting mechanism for EGFR signalling.

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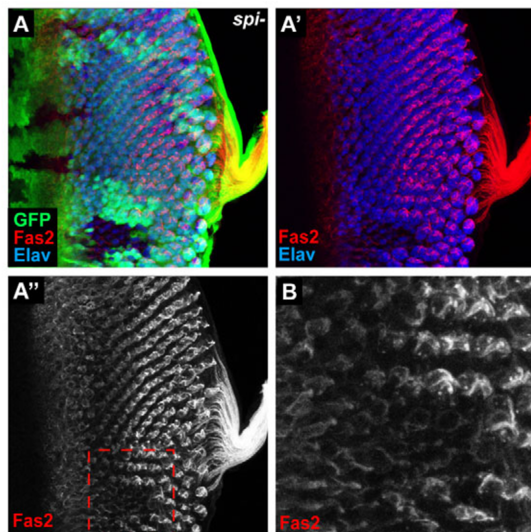

Fig. 6. Elevated Fas2 expression in the eye requires EGFR signalling. (**A-A″**) *spitz* null clones in third instar eye discs, marked by lack of GFP (green). Co-staining with anti-Elav (blue) and anti-Fas2 (red) revealed that Fas2 remains low in the absence of Spitz; note that non-R8 photoreceptors fail to differentiate in the clone. (**B**) An enlargement of the area marked in A″.

Specificity of interaction between EGFR and Fas2

Fas2 is a homophilic adhesion molecule, and disrupting the degree of contact between cells may have quite broad and non-specific effects. We therefore tested whether mutations in *fas2* were also able to disrupt signalling by other major pathways. To analyse the Notch, Wingless and Hedgehog pathways, we used several genetically sensitised conditions, analogous to our original screen for modifiers of EGFR signalling, to ask whether halving the dose of *fas2* modulated pathway activity. In no case was any phenotypic modification observed (see Fig. S3 in the supplementary material). We also used a similar logic to test whether signalling by two other receptor tyrosine kinases, Torso and FGFR, was affected by reduction of Fas2. No modification of Torso activity was observed (see Fig. S3 in the supplementary material). By contrast, halving the dose of *fas2* suppressed the phenotype caused by misexpression in the eye of an activated form of the FGF receptor Breathless in conjunction with Downstream-of-FGF-receptor (Dof) (Zhu et al., 2005) (see Fig. S3 in the supplementary material). Note, however, that this genetic interaction is the opposite of the effect we see with the EGFR: it implies that Fas2 is a positive regulator of the FGFR pathway, which is consistent with other contexts where it has been reported that Fas2 induces FGFR activity to stimulate neurite outgrowth (Forni et al., 2004). In the absence of a suitable genetically sensitised condition for assaying Dpp modulation, we assayed the levels of phosphorylation of the Dpp transducer Mad in *fas2*-null clones in the wing disc. We were unable to see any changes (see Fig. S3 in the supplementary material). These experiments do not rule out subtle effects of Fas2 on signalling by these pathways, but they do imply that a robust and consistent inhibitory effect is specific to EGFR signalling.

Another specificity issue is whether EGFR signalling is broadly sensitive to changes in adhesion between cells, or specifically to changes in Fas2. A degree of specificity was already indicated by the fact that no other adhesion molecules were isolated in our extensive genetic screens for EGFR modifiers. To test the specificity of Fas2 more thoroughly, other components of the basolateral junction, which are known to function in a complex with Fas2 (Knust and Bossinger, 2002), were also tested in the screen. Halving the dose of Discs-large, Neuroglian, Lethal-giant-larvae and Scribble did not modify either of the interaction conditions (overexpressed *EGFR* or *argos*, data not shown), implying that the effect of Fas2 on EGFR signalling is distinct from its adhesion function in basolateral junctions. We also found that Capricious and Tartan (Milan et al., 2001), two LRR repeat proteins related to Kekkon-1, a known inhibitor of the EGFR (Ghiglione et al., 1999), failed to modulate EGFR signalling (Mao et al., 2008). On the basis of these results, we conclude that the relationship between Fas2 and the EGFR is specific: Fas2 does not substantially inhibit a number of other common signalling pathways, including those controlled by other receptor tyrosine kinases; and several other adhesion components do not substantially modulate EGFR signalling.

DISCUSSION

Our results demonstrate that the NCAM orthologue Fasciclin 2 specifically inhibits EGFR signalling activity during the normal development of the *Drosophila* eye, notum and wing. Interestingly, like other *Drosophila* EGFR inhibitors, Fas2 participates in a potential negative-feedback loop to regulate signalling, although the developmental significance of this remains to be established. The evidence for the interaction between Fas2 and EGFR relies on genetic interactions, diagnostic phenotypes of loss of function *fas2* mutants, and a direct readout in *fas2* clones of reduction of Yan, a transcriptional repressor targeted for degradation by EGFR activity. Furthermore, the results in the eye are supported by similar genetic logic in the developing notum and wing. Despite this, *fas2* phenotypes are not identical to those of other known EGFR inhibitors. This is less surprising than it first appears, as the phenotypes of none of the known EGFR inhibitors in *Drosophila* (which currently include Argos, Kekkon-1, Echinoid, Sprouty, as well as some less specific proteins such as Gap-1) are as strong as constitutive activation of the receptor, and all are distinct (Freeman et al., 1992; Casci et al., 1999; Ghiglione et al., 1999; Bai et al., 2001; Gaul et al., 1992). The explanation for the variation in strength and detail of phenotype is that each of the inhibitors has a different molecular mechanism and site of action in the pathway, as well as different sites of expression. For example, Argos is specific to the EGFR and is a diffusible molecule that sequesters ligand. By contrast, Sprouty, a cytoplasmic protein, inhibits a range of receptor tyrosine kinases, whereas Echinoid and Kekkon-1 are cell surface proteins that bind directly to the EGFR. It is evident that EGFR regulation depends on a patchwork of overlapping effects of multiple different types of modulators, each of which has greater or less importance in different developmental contexts. Presumably, this network of regulators underlies the observed precision and robustness of signalling.

Loss of Fas2 in the eye triggers at least two distinct types of extra photoreceptor recruitment. The ectopic mini-clusters appear at the same time that the normal outer photoreceptors are recruited and, by analogy with *argos* mutations (Freeman et al., 1992), we believe them to be caused by transformation of the 'mystery cells'. In normal development these form part of the precluster, but are ejected prior to the onset of photoreceptor differentiation. It is also possible that some of the mini-clusters are derived from de novo photoreceptor determination occurring in undifferentiated interommatidial cells, which is known to be triggered by excess EGFR activity (Domínguez et al., 1998). The second recognisable

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type of extra photoreceptors are the R7-like, Prospero-positive cells. These are presumably the product of abnormal recruitment of cone cell precursors as R7s, a switch of fates within the R7 equivalence group, which is sensitive to altered levels of receptor tyrosine kinase signalling (Tomlinson and Ready, 1986; Van Vactor et al., 1991; Freeman, 1996).

Our genetic data do not reveal a molecular mechanism for the inhibition of EGFR by Fas2 – that will require future biochemical analysis – but its location at the plasma membrane and the non-autonomy we detected at the border of mutant clones point to three classes of model:

(1) Fas2 reduces EGFR ligand production, presumably the TGFα homologues Spitz or Keren, for example by direct sequestration of the mature ligand.

(2) Fas2 inhibits EGFR signalling, either by direct interaction with the receptor, or by indirectly downregulating its level or activity; in this case the observed non-autonomy would be indirect and caused by the well established positive feedback loop, whereby EGFR signalling activates expression of Rhomboid 1, which itself generates processed ligands.

(3) Perhaps slightly less plausibly, the extracellular domain of Fas2 might be able to span the intercellular gap, thereby interacting with and inhibiting EGFR molecules on adjacent cells.

Precedence leads us to favour the second model. Two other adhesion proteins, Kekkon-1 (Ghiglione et al., 1999) and Echinoid (Spencer and Cagan, 2003), interact directly with the EGFR. Similarly, mammalian E-cadherin can inhibit the EGFR by direct binding (Qian et al., 2004). Of particular relevance to this work, it has recently been reported that mammalian EGFR can be inhibited by NCAM, the Fas2 orthologue (Povlsen et al., 2008). In these experiments using explanted mouse neurons combined with transfected mammalian cell lines, NCAM stimulates neurite outgrowth by blocking EGFR function. Preliminary results lead the authors to favour a mechanism of NCAM-induced downregulation of EGFR levels, although direct parallels with our work are difficult to draw because the cytoplasmic domains of NCAM and Fas2 are not similar.

Beyond the evidence for inhibition of the EGFR described here and in the recent paper discussed above, Fas2/NCAM has now been implicated in several other signalling systems. The best characterised of these is an interaction with FGFR signalling, where, both in *Drosophila* and mammals, FGFR activity is required for Fas2/NCAM induced neurite outgrowth (Williams et al., 1994; Forni et al., 2004) and direct binding of NCAM activates FGFR (Kiselyov et al., 2003; Christensen et al., 2006). By contrast, and an illustration of the context dependence of such interactions, it has also recently been reported that NCAM can inhibit FGFR activation by its ligand FGF (Francavilla et al., 2007). Less well studied links between NCAM and growth factors include the observation that NCAM can act as a signalling receptor for GDNF (Paratcha et al., 2003), and that it participates in the response of oligodendrocyte precursors to PDGF (Zhang et al., 2004). The work we report here is the first genetic evidence to imply a role for Fas2 in the physiological inhibition of EGFR activity. It is important to set this discussion in the context of the well established role of Fas2/NCAM as a neural cell-adhesion molecule, with roles in axonal growth and pathfinding, as well as in synaptic maturation (Grenningloh et al., 1991); Schuster et al., 1996).

Overall, it is becoming clear that the EGFR pathway is regulated by multiple partially overlapping mechanisms, presumably because of the importance of regulatory precision and robustness of such a central and pleiotropic pathway. Notably, negative-feedback control is a recurring theme. Much less is known about physiologically significant regulators of EGFR signalling in mammals, and it will be interesting to determine whether feedback control is a conserved strategy. As there are many other signalling pathways and adhesion proteins that contribute to normal development, the total potential number of regulatory interactions between these key cell surface proteins is enormous and, indeed, many have been observed in vivo and in vitro. Of course, some of these might not occur in normal biological contexts, emphasising the value of a genetic approach to revealing which relationships between adhesion proteins and signalling pathways are physiologically relevant.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/3/473/DC1

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