Egfr signalling defines a protective function for ommatidial orientation in the *Drosophila* eye

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Summary

Ommatidial rotation in the *Drosophila* eye provides a striking example of the precision with which tissue patterning can be achieved. Ommatidia in the adult eye are aligned at right angles to the equator, with dorsal and ventral ommatidia pointing in opposite directions. This pattern is established during disc development, when clusters rotate through 90°, a process dependent on planar cell polarity and rotation-specific factors such as Nemo and Scabrous. Here, we demonstrate a requirement for epidermal growth factor receptor (Egfr) signalling in rotation, further adding to the manifold actions of this pathway in eye development. Egfr is distinct from other rotation factors in that the initial process is unaffected, but orientation in the adult is greatly disrupted when signalling is abnormal. We propose that Egfr signalling acts in the third instar imaginal disc to 'lock' ommatidia in their final position, and that in its absence, ommatidial orientation becomes disrupted during the remodelling of the larval disc into an adult eye. This lock may be achieved by a change in the adhesive properties of the cells: cadherin-based adhesion is important for ommatidia to remain in their appropriate positions. In addition, we have evidence that there is an error-correction mechanism operating during pupal stages to reposition inappropriately orientated ommatidia. Our results suggest that initial patterning events are not sufficient to achieve the precise architecture of the fly eye, and highlight a novel requirement for error-correction, and for an Egfr-dependent protection function to prevent morphological disruption during tissue remodelling.

Key words: Eye, Patterning, Ommatidial rotation, Adhesion, Epidermal growth factor receptor, *spitz*, *keren*, *scabrous*, *nemo*, *roulette*, *argos*

Introduction

Development of complex organisms requires tissues to undergo significant morphogenesis and remodelling. One example of such a process occurs in the *Drosophila* eye, where the developing ommatidia undergo a precise 90° rotation in order to reach their final orientation. Although ommatidial rotation is an eye-specific phenomenon, it shares characteristics with other examples of cell motility and tissue morphogenesis: in both cases, cells must remodel intercellular contacts and move with respect to their neighbours. Here we present evidence that this remarkably precise example of morphogenesis relies not only on an initial patterning mechanism but also requires error correction and active maintenance functions.

Eye differentiation begins in the third larval instar, when the morphogenetic furrow sweeps across the eye imaginal disc from posterior to anterior, leaving clusters of differentiating cells behind it (reviewed by Wolff and Ready, 1993; Ready et al., 1976). The R8 photoreceptor cell is the first to be specified, followed sequentially by the other photoreceptors in a defined order (Tomlinson, 1985; Tomlinson and Ready, 1987). Non-neuronal cone and pigment cells are subsequently recruited to make up the complete ommatidium. As well as this posterior to anterior organisation, the eye disc is also polarised in the dorsoventral axis. The rhabdomeres of the adult photoreceptors are arranged in a trapezoidal shape, and ommatidia in dorsal and ventral halves of the disc are of opposite chiral forms, being mirror-symmetric about the equator. This asymmetry arises in the third instar disc, when the R3 and R4 cell fates are specified from an equivalent pair of neuronal cells: the cell closest to the equator becomes R3, and the more polar cell differentiates as R4. Subsequently, the ommatidia initiate rotation in opposite directions on either side of the equator. Determination of chirality is under the control of planar cell polarity (PCP) (reviewed by Adler, 2002; Mlodzik, 1999; Strutt and Strutt, 1999). Mutations in PCP components, such as the Wnt receptor Frizzled, display defects in chirality, with R3 and R4 being incorrectly specified, and rotation being initiated in the wrong direction (Zheng et al., 1995). Consequently, no equator is visible in a *frizzled*$^{-}$ eye.

In addition to determination of chirality and direction of rotation, the developing ommatidial clusters must also rotate by the correct degree. Visual processing in flies involves a precise mapping of the pattern of photoreceptors onto neurons in the optic lobe (Clandinin and Zipursky, 2002; Meinertzhagen and Hanson, 1993). Therefore, if the ommatidia are not properly rotated, the photoreceptor array will be disorganised, leading to a loss of visual acuity. The precision of ommatidial rotation is remarkable and is easily seen in sections through adult eyes (see, for example, Fig. 1A). In the wild-type (WT) disc, the ommatidia first rotate through 45°, then pause before reinitiating rotation to complete the full 90° turn (shown schematically in Fig. 1E). As well as affecting

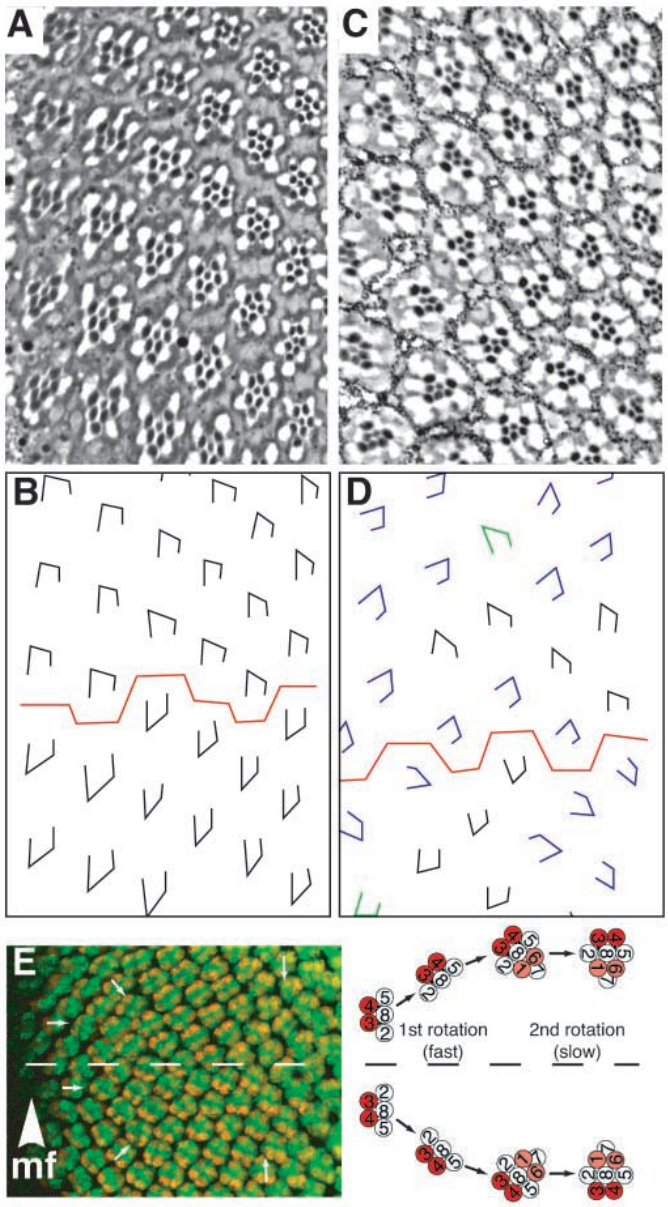


Fig. 1. Rotational defects in eyes overexpressing Keren. (A-D) Sections through adult eyes (A,C), with schematics shown below (B,D). (A,B) In wild-type eyes, all ommatidia are orientated at precisely 90° to the equator. Dorsal and ventral ommatidia are of opposite chiral forms. (C,D) Eyes in which full-length *keren* is misexpressed under the control of *sev-Gal4* (referred to as *UAS-keren*) show severe defects in rotational angle. All ommatidia, however, are of correct chiral form. In B and D, and all subsequent schematics, black trapezoids represent correctly orientated ommatidia, green trapezoids show underrotations, and blue trapezoids overrotations. The red line marks the position of the equator. In this and all images, anterior is to the left. (E) Confocal image and schematic of rotation in the third larval instar disc. (Left) Confocal projection of *svp-lacZ/+* disc stained with α-Elav (green) to mark photoreceptors and α-β-galactosidase (red) to highlight R1,3,4 and 6. The R3/R4 pair is initially parallel to the morphogenetic furrow (MF, arrowhead); ommatidia then undergo a fast 45° rotation, followed by a second, slower turn to 90°. Rotational positions are shown by white arrows. (Right) Schematic of the events occurring in left panel, showing rotation of, and recruitment of further photoreceptors to, the five-cell cluster.

chirality, *frizzled* mutants show disruptions in the degree of rotation (Zheng et al., 1995), although this does not seem to be true for all PCP components; mutations in the atypical cadherin *fat*, for example, show significant chiral defects but ommatidia still rotate through 90° (Rawls et al., 2002; Yang et al., 2002). Chirality and rotation, although intimately linked, are therefore separable processes, with the control of rotation being much less well understood than chirality.

Only a few mutations that specifically disrupt rotation have been identified. These include *nemo*, *roulette* and *scabrous*. Mutations in *nemo*, which encodes a protein kinase distantly related to the MAPK family, cause ommatidial clusters to arrest at 45°, and not to initiate the second phase of rotation (Choi and Benzer, 1994). The *roulette* mutation causes apparent randomisation of ommatidial rotation, with both over- and underrotated ommatidia (Choi and Benzer, 1994). Scabrous, a fibrinogen-related secreted protein (Baker et al., 1990; Mlodzik et al., 1990a), has been implicated in controlling the normal arrest of rotation at 90°, because mutations in *scabrous* show overrotation defects (Chou and Chien, 2002). It has been proposed that Nemo may act as a driving force for the second 45° rotation, and that the function of Scabrous is to counter this force, and thus cause ommatidia to stop at the appropriate point (Chou and Chien, 2002). In addition, it has recently been suggested that RhoA, previously considered as one of the core group of PCP components (Fanto et al., 2000; Strutt et al., 1997), may be specific for rotation (Strutt et al., 2002). *Drosophila* Rho kinase also seems to show rotational defects (Winter et al., 2001). How any of these factors act to control rotation at a mechanistic level, however, remains to be addressed, as does their relationship to each other.

Here, we identify a role for the *Drosophila* epidermal growth factor receptor (Egfr) signalling pathway in the control of rotation. Egfr signalling plays several important roles during eye development. Notably, it is responsible for the recruitment of all cell types except R8 in the developing ommatidium – in the absence of Egfr signalling, only R8 differentiates, and overactivating the pathway leads to excess photoreceptor and cone cell recruitment (Freeman, 1996; Freeman, 1997). As well as its role in recruitment, this pathway also controls ommatidial spacing, promotes cell proliferation behind the morphogenetic furrow, and protects cells against apoptosis (Baker and Yu, 2001; Baonza et al., 2001; Bergmann et al., 1998; Domínguez et al., 1998; Kumar et al., 1998; Kurada and White, 1998; Spencer et al., 1998).

Both over- and underactivation of the Egfr signalling pathway gave similar rotational defects in the adult eye. To our surprise, however, we found that the initial process of ommatidial rotation is not dependent on Egfr signalling. Instead, the rotational angle becomes disrupted at a later stage in development, suggesting that this pathway may be required to prevent ommatidia from reinitiating rotation during pupal stages, or to protect them against rotational distortion during the substantial morphogenetic movements that occur during the formation of the mature retina. These results demonstrate a previously unrecognised additional role for Egfr signalling in eye formation, further emphasising the reiterative functions of a single signalling pathway in development. Moreover, they provide us with the opportunity of using the well-characterised system of the fly eye to analyse mechanisms of regulating cell motility and tissue remodelling. As an initial step in this

direction, we have evidence that ommatidial rotation may depend at least partly on cadherin-based adhesion.

Materials and methods

Fly strains

The following *Drosophila* stocks were used. *argos*^{w11}, *HS-Gal4*, *mδ05-lacZ* (Cooper and Bray, 1999), *mys*^{1}, *nemo*^{P1}, *pnt*$^{\Delta 88}$, *pnt*^{1277}, *ru*^{1}, *rlt*^{1}, *S*^{5671}, *sev-argos*, *sevEPGal4*, *shg*^{2}, *shg*^{IG29}, *shg*^{K03401}, *spi*^{scp1}, *spi*^{scp2}, *P[lacW]svp*^{07842}, *UAS-mkeren* (Urban et al., 2002), *UAS-DN-Egfr*, *wb*^{09437}. Unless otherwise referenced, all stocks are as described in FlyBase (http://flybase.bio.indiana.edu/). All crosses were performed at 25°C. Clones of *argos*^{w11} were generated using standard techniques.

Histology

Adult heads were embedded as described in Freeman et al. (Freeman et al., 1992). Larval eye discs and pupal retinae were stained as described (Gaul et al., 1992). The following antibodies were used: mouse anti-β-galactosidase (1:100) (Promega), rabbit anti-β-galactosidase (1:100) (Cappel); mouse anti-Cut (1:100) (Blochlinger et al., 1990) and rat anti-Elav (1:200) (O'Neill et al., 1994) (both obtained from DSHB); and rabbit anti-BarH1 (1:50) (Higashijima et al., 1992). Alexa-568 and Alexa-647 (Molecular Probes) and FITC-conjugated secondary antibodies were used at 1:200 (Jackson ImmunoResearch). Fluorescent images were taken on a BioRad Radiance confocal microscope.

Analysis of rotational angles

In those cases in which rotational angles were measured accurately, this was done using the program XIMDISP (Smith, 1999). Adult eye sections were photographed and images imported into XIMDISP. The angle calculated was that between a vector drawn along the equator and a vector from rhabdomeres R1-R3. All correctly specified ommatidia in a section were analysed. In all other cases, ommatidia in adult eye sections or larval discs were scored as being misrecruited, correctly orientated or misrotated. Frequencies of each type were then calculated.

Results

Misexpressing Keren in the eye causes rotational defects

We misexpressed the Egfr ligand Keren in developing photoreceptors and cone cells under the control of *sev-Gal4*. Surprisingly, this caused a disruption in the orientation of ommatidia relative to WT (Fig. 1A-D) – a phenotype not previously associated with excess Egfr signalling. In the WT adult eye, all ommatidia are orientated at 90° relative to the equator (shown schematically in Fig. 1B). By contrast, when Keren was misexpressed, we found many ommatidia were abnormally orientated (Fig. 1C,D), with some ommatidia having rotated more than 90° (blue trapezoids) and some less than 90° (green trapezoids). In general, excess Egfr signalling leads to over-recruitment of cells in the eye, but photoreceptor recruitment was not affected when Keren was expressed at these levels. However, analysis of the pupal retina showed that Keren misexpression did cause over-recruitment of cone cells (data not shown), consistent with it acting through the Egfr (Freeman, 1996). Previous work has shown that recruitment of cone cells is more sensitive than photoreceptors to Egfr overactivation (Freeman, 1996); our observations support this, and also suggest that rotation is more sensitive than photoreceptor recruitment to perturbation of Egfr signalling.

Further examination of the adult phenotype indicated that it is rotation specifically that is disrupted on overexpressing Keren; the chirality (i.e. the correct specification of R3 and R4) of the ommatidia remains unaffected. This distinguished the *UAS-keren* phenotype from disruption of PCP components, which can cause both rotational and chiral defects (Theisen et al., 1994; Zheng et al., 1995).

Rotational defects are caused by disrupting Egfr signalling

Keren resembles the Egfr ligands Spitz and Gurken and can activate the Egfr (Reich and Shilo, 2002; Urban et al., 2002). The absence of a *keren* mutant, however, prevents us from being sure that it does not act in another pathway. The unexpected rotational disruption could be explained either by a previously unrecognised function of the Egfr, or by Keren acting through a different mechanism. Analysis of a role for Egfr signalling in rotation is made difficult by the fact that this pathway plays many roles in eye development. For example, disrupting signalling usually affects photoreceptor recruitment, making ommatidial rotation unscorable. We therefore examined another condition in which the Egfr pathway is only moderately hyper-activated: a hypomorphic mutation in the Egfr inhibitor *argos* (Schweitzer et al., 1995) (Fig. 2A,B). In *argos*^{w11} clones, although many of the ommatidia had too many photoreceptors (circles), a significant proportion had the correct number and we observed that many of these ommatidia were misrotated. This implies that the rotation phenotype caused by misexpressing Keren is a consequence of overactivating the Egfr pathway, rather than being a non-Egfr-related function of Keren. We note that these data do not address whether Keren normally functions in ommatidial rotation; instead they simply demonstrate that Egfr hyperactivity – including that triggered by Keren – leads to misrotation. Below we consider the possible ligands involved.

Is Egfr activity normally required for correct rotation? We examined several conditions that decrease Egfr signalling, including a haploinsufficient *Star* allele [which has previously been noted to have slight rotational defects (Heberlein and Rubin, 1991)], *rho3/ru* mutants (Wasserman et al., 2000), and expression of dominant-negative Egfr (Freeman, 1996) under the control of heatshock *HS-Gal4* (Fig. 2C-H). In all these cases, rotational defects were clearly seen in correctly specified ommatidia. In order to quantify and compare the rotational defects further, we measured the rotation angles of approximately 600 ommatidia each in WT, *UAS-keren* and *ru*^{1} eyes. (Fig. 2K) (see Materials and methods). Strikingly, defects caused by too little or too much Egfr activity were very similar – ommatidia were over- or underrotated, although in both cases there was a bias towards rotation angles of greater than 90°. The similarity of the rotational defects caused by increasing and decreasing pathway activity is reminiscent of some PCP mutations (Strutt et al., 1997; Tomlinson et al., 1997).

All known cases of Egfr signalling in *Drosophila* are transmitted through the canonical Ras/Raf/MAPK pathway, and through a transcriptional output. The transcription factor Pointed is involved in most circumstances: PointedP2 is directly phosphorylated and activated by MAPK, and upregulates the expression of PointedP1; both factors mediate the transcription of downstream genes (Brunner et al., 1994; Klämbt, 1993; O'Neill et al., 1994). In the case of rotation,

which we envisage as being a specialised case of cell motility or tissue remodelling, it seemed possible that Egfr signalling might influence the cytoskeleton directly, rather than exerting its effects by transcriptional control. We therefore tested whether a *pointed* hypomorph showed rotational defects (Fig. 2I,J). Although, as expected, many ommatidia showed under-recruitment of photoreceptors, rotational defects were frequent in those ommatidia that were correctly specified, indicating that this function of the Egfr pathway relies on Pointed-mediated transcription.

The rotational phenotypes caused by perturbation of Egfr signalling were very similar to the published phenotype of the *roulette* mutation, one of the few mutations previously reported to specifically disrupt rotation and not chirality (Choi and Benzer, 1994). Interestingly, *roulette* turns out to be allelic to *argos* (K. Choi, personal communication). We confirmed this by non-complementation of *roulette* by $argos^{w11}$, and by rescue of the *roulette* phenotype by a *sev-argos* transgene (Fig. 2L-Q). This result is therefore consistent with our discovery of a role for the Egfr pathway in controlling ommatidial rotation. We hereafter refer to the *roulette* mutations as $argos^{rlt}$.

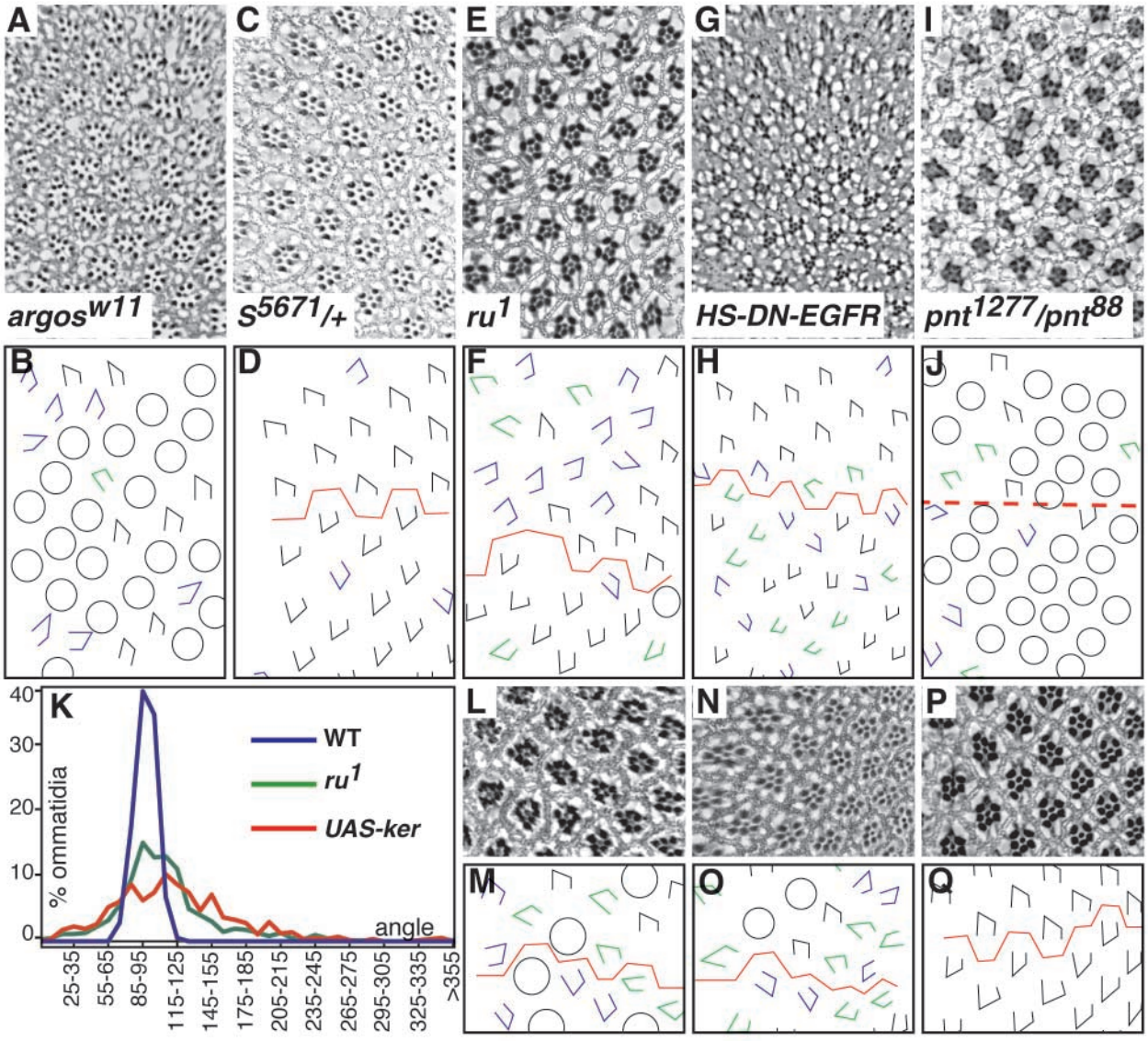
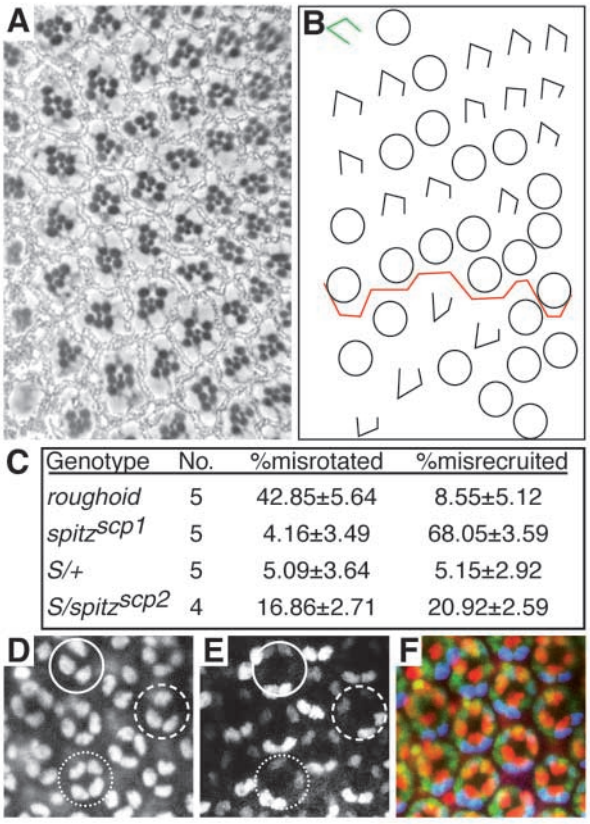


Fig. 2. Perturbing Egfr signalling disrupts ommatidial rotation. Upper panels show sections through adult eyes, lower panels are schematics of these images. (A,B) aos^{w11} clone (note, in this section, the equator can not be seen, but runs left to right as in all other images); (C,D) $S^{5671}/+$; (E,F) ru^1; (G,H) *DN-Egfr* expressed under the control of *HS-Gal4*; (I,J) $pnt^{1277}/pnt^{\Delta 88}$ (position of equator cannot be accurately determined because of the large proportion of mis-specified ommatidia). In all cases, misrotated ommatidia can be seen. (K) Graph showing rotational angles in wild type (blue), *sev-Gal4*, *UAS-keren* (red) and ru^1 (green) eyes. Data is plotted as percentage of ommatidia at each angle. In each case, 5-600 ommatidia were scored from 5-6 eyes. *UAS-keren* and ru^1 have qualitatively similar effects on rotation. (L-Q) *roulette* is allelic to *argos*. (L,M) rlt^1 mutants show similar phenotypes to Egfr pathway mutants (compare L with other images in Fig. 2). (N,O) The rlt^1 mutant fails to complement aos^{w11}. (P,Q) rlt^1 phenotype can be rescued by overexpression of one copy of the *sev-argos* transgene. Colour coding in schematics is as for Fig. 1 (black, correctly orientated; green, underrotated; blue, overrotated; red line, equator). Black circles indicate mis-specified ommatidia in this and all subsequent Figures.

More than one Egfr ligand controls rotation

There are four ligands that activate the *Drosophila* Egfr: Spitz, Gurken and Keren, which resemble mammalian TGFα, and Vein, a neuregulin-like molecule (Neuman-Silberberg and Schüpbach, 1993; Reich and Shilo, 2002; Rutledge et al., 1992; Schnepp et al., 1996; Urban et al., 2002). Spitz is thought to mediate most of the Egfr functions in eye development (Freeman, 1994; Freeman, 1997; Tio et al., 1994; Tio and Moses, 1997), although *spitz* clones do not phenocopy *Egfr* clones in all respects. Specifically, *spitz* clones do not show defects in cell survival or ommatidial spacing, which are seen in *Egfr* loss-of-function clones (Domínguez et al., 1998). We examined *spitz* hypomorphic eyes to determine whether these show rotational defects (Fig. 3A,B). Under-recruited ommatidia are very common in the spi^{scp1} hypomorph, indicating that Egfr activity is substantially impaired – to beneath the threshold for photoreceptor recruitment. Despite this, very few misrotated ommatidia are seen (see Fig. 3C). In comparison, ru^{1} eyes show only minor recruitment defects, indicating a less dramatic reduction of Egfr activity than spi^{scp1}. ru^{1} eyes, however, show severe rotational defects. These data suggest that Spitz is not essential for normal rotation. They do not, however, rule out the possibility that Spitz acts redundantly with another ligand. To test this, we looked for a genetic interaction between *Star* and a *spitz* hypomorph (see Fig. 3C). As expected, heterozygosity for *spitz* enhanced the recruitment defects in the *S/+* eye. We also observed a significant enhancement of rotational defects, implying that Spitz does function in ommatidial orientation. Together, these results suggest that Spitz acts redundantly with another Egfr ligand to control rotation. The fact that loss of Rho3/ru, a protease that activates Egfr ligands (Wasserman et al., 2000), results in rotational defects, whereas *spitz* mutants do not, implies the involvement of another cleaved ligand. Gurken is restricted to the germline. By elimination, we therefore tentatively conclude that Keren also acts in the Egfr-dependent regulation of ommatidial rotation. Note, however, that *keren* expression is too low to detect by in situ hybridisation in any tissue (Reich and Shilo, 2002) (K.E.B. and M.F., unpublished) so we cannot tell whether it is transcribed appropriately. Confirmation of our hypothesis awaits the identification of a *keren* mutant.



Genotype	No.	%misrotated	%misrecruited
roughoid	5	42.85±5.64	8.55±5.12
$spitz^{scp1}$	5	4.16±3.49	68.05±3.59
S/+	5	5.09±3.64	5.15±2.92
$S/spitz^{scp2}$	4	16.86±2.71	20.92±2.59

Fig. 3. The role of Spitz in rotation and evidence that the Egfr acts directly. (A,B) Section through adult eye of spi^{scp1} hypomorph. Many ommatidia show under-recruitment defects; misrotations, however, are very rare (green trapezoid). Colour coding in schematics is as for previous Figures (black, correctly orientated; green, underrotated; blue, overrotated; black circles, mis-specified ommatidia; red line: equator). (C) Quantification of rotational defects in spi^{scp1} versus ru^{1} and *S/+* versus spi^{scp2}. 'No.' indicates the number of eyes scored for each genotype. In spi^{scp1}, very few misrotations are seen relative to the proportion of misrecruitments; the converse is seen in ru^{1}. However, spi^{scp2} dominantly enhances rotational defects of *S/+*, suggesting Spitz plays some role in the control of rotation. (D-F) *S/+; svp-lacZ/+* 40 hour pupal retina stained with α-cut (D; red in F), α-*lacZ* (E; blue in F) and α-Elav (green in F). Ommatidial orientation and cone cell number are not correlated: ommatidia with too few cone cells may be either correctly (solid circle) or incorrectly (broken circle) orientated, and incorrectly orientated ommatidia may also have the correct number of cone cells (dotted circle).

The Egfr acts directly in ommatidial rotation

Perturbing cell recruitment could have an impact on the packing of the cells within the retina, and might therefore affect the orientation of ommatidia as a secondary function. If this were the case, however, rotational defects should also be observed in *spitz* hypomorphs, in which there are substantial recruitment defects of all ommatidial cell types. Because in all cases orientation was only scored in ommatidia with the correct number of photoreceptors, rotational defects cannot be secondary to photoreceptor recruitment. However, this does not address a possible role for other cells. We examined this question in several ways. First, we found that in eyes misexpressing Keren, almost all ommatidia had misrecruited cone cells; despite this, a proportion were rotationally normal. Second, we investigated whether there was any correlation between cone cell recruitment and rotation defects in *Star/+* pupal retinae (Fig. 3D-F). In these we found that 50% (n=98) of ommatidia (all with the appropriate number of photoreceptors) with reduced numbers of cone cells were rotationally normal and, conversely, that 26% (n=66) of misrotated ommatidia had a normal complement of cone cells. Finally, we can rule out rotation defects being secondary to pigment cell misrecruitment on several grounds: the lack of misrotations in *spitz* hypomorphic eyes, the fact that pigment cells are not recruited until long after the requirement for Egfr activity (see below), and the fact that pigment cells are recruited simultaneously across the eye, whereas the rotational function of the Egfr sweeps across the eye in the wake of the morphogenetic furrow (see below). Together, these data provide compelling evidence that the Egfr function in ommatidial rotation is direct and not secondary to its function in recruitment.

Initial rotation is unaffected by the Egfr

At what stage in ommatidial development does the Egfr control rotation? We used several markers to look at rotation in the eye disc: α-Bar, which stains R1 and R6 (Higashijima et al., 1992), *svp-lacZ*, which is strongly expressed in R3 and R4 and more weakly in R1 and R6 (Mlodzik et al., 1990b), and *mδ0.5-lacZ*, which highlights R4 only (Cooper and Bray, 1999). The first two markers enable visualisation of the rotational angle during disc development, and the third shows which cell of the R3/R4 pair develops R4 fate, thus providing a marker for chirality. *mδ0.5-lacZ* staining of discs misexpressing *keren* showed no defects in R3/R4 specification (compare Fig. 4A and Fig. 4C), which correlates well with the lack of chiral defects in the adults. Surprisingly, rotational defects were also very minor in the third instar disc (Fig. 4E-L). The vast majority of ommatidia reach 45° as expected, and by the back of the disc have turned to 90°. This is in stark contrast to the adult eye, in which approximately 28% have rotated less than 90°, and 6.2% less than 45°, as well as 65% being rotated greater than 90°. Occasional misrotations can be seen in the larval disc (arrowheads in Fig. 4E,G), but analysis showed that the frequency of these (4.9%; 1275 ommatidia in 10 discs) is not significantly different from WT (5.1%; 1354 ommatidia in 8 discs). This result demonstrates that the eye defects we see in the adult must arise at a stage later in development than the third instar imaginal disc.

When do rotational defects occur?

In order to try and determine when Egfr signalling affects rotation, we took two approaches. First, we examined eyes at stages intermediate between the third instar larva and the adult. Fig. 5A-D show discs taken from WT and flies misexpressing *keren* at 6 hours post-pupariation. In the WT disc, ommatidia have reached 90° and stopped rotating several rows before the back of the disc. If disrupting Egfr signalling leads to a failure to stop rotation, then defects should be obvious by this stage. However, discs misexpressing *keren* looked indistinguishable from WT, even at the posterior of the disc, implying that the effects of perturbing Egfr signalling are only apparent later than 6 hours post-pupariation. By 30 hours post-pupariation, rotational defects were clearly visible in the retina (Fig. 5E-H), indicating that rotation becomes disrupted between 6 and 30 hours post-pupariation.

The second approach we took was to ask when Egfr signalling was required in order to influence rotation. We used *HS-Gal4* driving a dominant-negative form of the Egfr to disrupt the pathway at specific times through development, and then looked at the effects of these heatshocks in the adult eye.

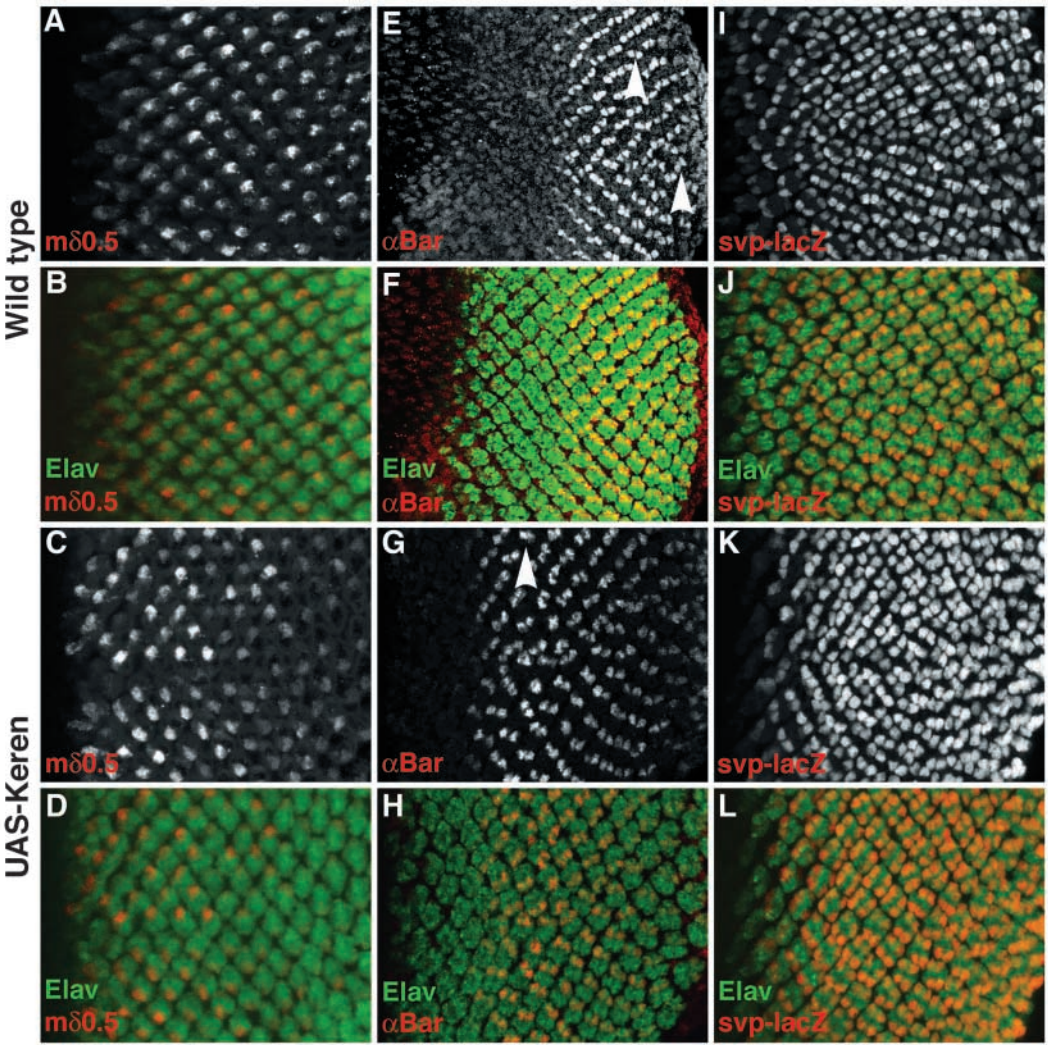


Fig. 4. Eye discs misexpressing Keren are indistinguishable from wild type (WT). All images show eye discs taken from crawling third instar larvae. In all cases, green is α-Elav, marking photoreceptors. Upper panels show the red channel alone; lower panels are merges. (A-D) *mδ0.5-lacZ* staining (red) in WT (A,B) and *UAS-keren* (C,D) discs. *mδ0.5-lacZ* highlights the R4 cell and acts as a marker for chirality; R4 determination is normal in *UAS-keren* discs. (E-H) α-Bar staining (red), highlighting R1 and R6 in WT (E,F) and *UAS-keren* (G,H) discs. Rare misrotations can be seen in both WT and mutant discs (arrowheads). (I,L) *svp-lacZ* (red) staining in WT (I,J) and *UAS-keren* (K,L) discs. *seven-up* is expressed strongly in R3 and R4 (outer pair in each ommatidium) and weakly in R1 and R6 (inner pair). Note the similarity between WT and *UAS-keren* with all three markers.

The results of these experiments are summarised in Table 1. In each case, flies were heatshocked 3 times at 35°C for 30 minutes, with 90 minutes of recovery between heatshocks. Misrotated ommatidia can be seen in a dorsal-ventral stripe of rows across the eye, although, under these mild conditions, only a small proportion of ommatidia are incorrectly orientated. Very few ommatidia are misrotated outside this band. Consistent with the posterior to anterior progression of eye development, the older the flies were at the time of heatshock, the more anterior the band of misrotation.

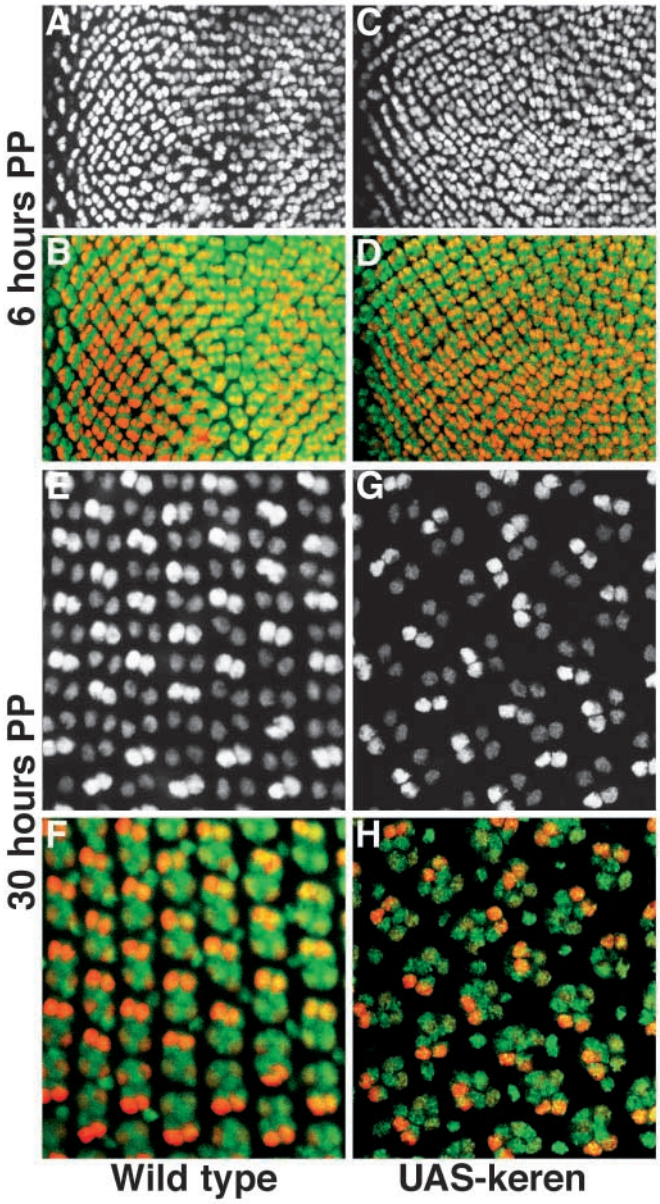


Fig. 5. Rotational defects arise during pupal eye development. All images are confocal projections of *svp-lacZ/+* retinae stained with α-Elav (green) and α-β-galactosidase (red). Upper panels show the red channel only; lower panels are merges. *svp-lacZ* highlights R1,3,4 and 6. (A-D) Discs taken from wild type (WT) (A,B) and *UAS-keren* (C,D) flies at 6 hours post-pupariation. At the back of the WT disc, ommatidia are arrested at 90°. *UAS-keren* discs show no disruption in rotation, even at the back of the disc where ommatidia are oldest. (E-H) Retinae taken from WT (E,F) and *UAS-keren* (G,H) flies at 30 hours post-pupariation. By this stage, rotational defects are clear in the *UAS-keren* retinae (compare E with G).

The wave of rotational defects shown by these heatshock experiments can be used to deduce the time of susceptibility to loss of Egfr signalling. In the case of white prepupae, the band of misrotation spreads from approximately 14 rows from the posterior margin, to approximately 21 rows. At this stage, the morphogenetic furrow (which moves approximately one row every two hours) has progressed approximately 28 rows from the posterior. Given the likelihood of a delay between time of heatshock and expression of the dominant-negative construct, this suggests that the period of sensitivity to loss of Egfr signalling corresponds to approximately 10-15 rows behind the furrow. This coincides with the second 45° rotation: ommatidia reach 45° at approximately row 6, and 90° at approximately row 15-16. Data from other time points are remarkably consistent with this analysis, both in regard to the initiation and duration of susceptibility (see Table 1). Furthermore, we calibrated the time of susceptibility of rotation to Egfr signalling by comparing the position of rotational defects with photoreceptor recruitment defects in *HS-Gal4/DN-Egfr* eyes. Consistent with the times deduced above, rotation defects occurred immediately posterior to recruitment defects (Table 1). This clearly shows that the Egfr function in rotation follows very soon after its role in photoreceptor recruitment. This corresponds to a period during or immediately after the second 45° rotation, even though defects resulting from disruption of the pathway are not apparent until significantly later than this.

Genetic interactions with other known rotation mutants

Apart from Argos and other members of the Egfr pathway, Nemo and Scabrous are the main factors known to cause rotation-specific defects (Choi and Benzer, 1994; Chou and Chien, 2002). We therefore tested potential genetic interactions between the Egfr pathway, *nemo* and *scabrous*. It is already known that *nemo*, $argos^{rlt}$ double mutants show a *nemo* phenotype (Choi and Benzer, 1994); this was also observed on misexpressing *keren* in a $nemo^{P1}$ mutant background (compare Fig. 6A,B with Fig. 6C,D). In addition, ru^{1} $nemo^{P1}$ double mutants were indistinguishable from the $nemo^{P1}$ single mutant (Fig. 6E,F), implying that there is no synergy between the Egfr pathway and *nemo*. In conjunction with the observations that Nemo is required for the onset of the second 45° rotation, whereas Egfr activity is not required until later, this suggests that they act in separate processes. Moreover, these data imply that Egfr activity is not required unless ommatidia rotate beyond the initial 45°.

In the case of *scabrous*, we only saw minor rotational defects in adult eyes. Many ommatidia were incorrectly specified, but of those ommatidia that had the correct number of photoreceptors, most had rotated accurately to 90° (Fig. 6G,H). Approximately 11% were misrotated, and of these, half were underrotated. This adult phenotype appears inconsistent with the overrotation defects observed in discs (Chou and Chien, 2002), and hints at the existence of an error-correction mechanism that acts during pupal development (see Discussion). In order to try and determine whether Egfr signalling and Scabrous might be closely linked in controlling rotation, we made double $scabrous^{1}$; $argos^{rlt}$ and $scabrous^{1}$; ru^{1} mutants and examined rotation defects in adult eyes. These

Table 1. Heatshocks reveal a window of susceptibility to Egfr disruption

Time of heat shock	Posterior edge of misrotation (rows)	Anterior edge of misrotation (rows)	Posterior edge of misrecruitment (rows)	Width of misrotated band (rows)
Third instar	10-12 (*n*=5)	17-18 (*n*=4)	18-21 (*n*=4)	6-8
Prepupae	13-15 (*n*=5)	21 (*n*=1)	22 (*n*=1)	7
2 hours PP	15-16 (*n*=5)	23 (*n*=1)	ND	7
4-5 hours PP	15-17 (*n*=7)	25 (*n*=2)	24-25 (*n*=2)	8
9 hours PP	19 (*n*=1)	ND	ND	ND
12 hours PP	21 (*n*=1)	ND	ND	ND
15 hours PP	23 (*n*=1)	ND	ND	ND
20 hours PP	23 (*n*=1)	31/32 (*n*=1)	32 (*n*=1)	8/9
24 hours PP	24-26 (*n*=4)	32 (*n*=1)	None	7

Table shows the effects of overexpressing DN-Egfr by heatshock at different times through larval and pupal (post-pupariation; PP) development. Misrotated ommatidia appear in a dorsalventral stripe – the posterior and anterior edges of which are indicated where possible; rows were counted from the posterior edge of the adult eye. Note that the anterior edge of misrotation closely abuts the posterior edge of photoreceptor misrecruitment, implying that the rotational function of the Egfr immediately follows its recruitment function.

n, the number of eyes scored for each time point (posterior edges were easier to identify because of the orientation at which eyes were sectioned).

ND indicates that the edge and therefore the width of the band of misrotation could not be determined.

phenotypes indicated that removing Scabrous in either *argos*^{rlt} or *ru*^{1} backgrounds does not significantly alter the Egfr pathway phenotypes (data not shown).

Cadherin-based adhesion is involved in rotation

Our results demonstrate that Egfr signalling is required for the maintenance through eye development of the correct orientation of ommatidia. We speculated that rotation may rely at least partly on the adhesive properties of the cells. In an initial attempt to examine this hypothesis, we looked for genetic interactions between components of the Egfr pathway and various adhesion molecules. We used a *Star* heterozygote, in which Egfr signalling is slightly reduced (Kolodkin et al., 1994), as a background in which to look for interactions, because this phenotype is very weak (see Fig. 2C,D), allowing any enhancement of rotational defects to be easily recognised. Halving the dose of α-laminin [*wing blister* (Martin et al., 1999)] and the integrin β subunit [*myospheroid* (MacKrell et al., 1988)] did not modify the *Star*/+ phenotype. In contrast, alleles of E-cadherin [*shotgun* (Tepass et al., 1996)] showed a significant interaction with *Star*, with many more misrotated ommatidia (Fig. 7). Under the strongest condition, there was also an enhancement of the rare misrecruitment defects seen in *Star*/+ eyes, but the enhancement of the rotational defect was independent of this by two criteria. First, the rotational defects were only measured in correctly specified ommatidia; and second, the weaker alleles of *shotgun* affected rotation without enhancing recruitment. On the basis of these results, we conclude that the control of rotation by Egfr signalling is linked to cadherin-based adhesion.

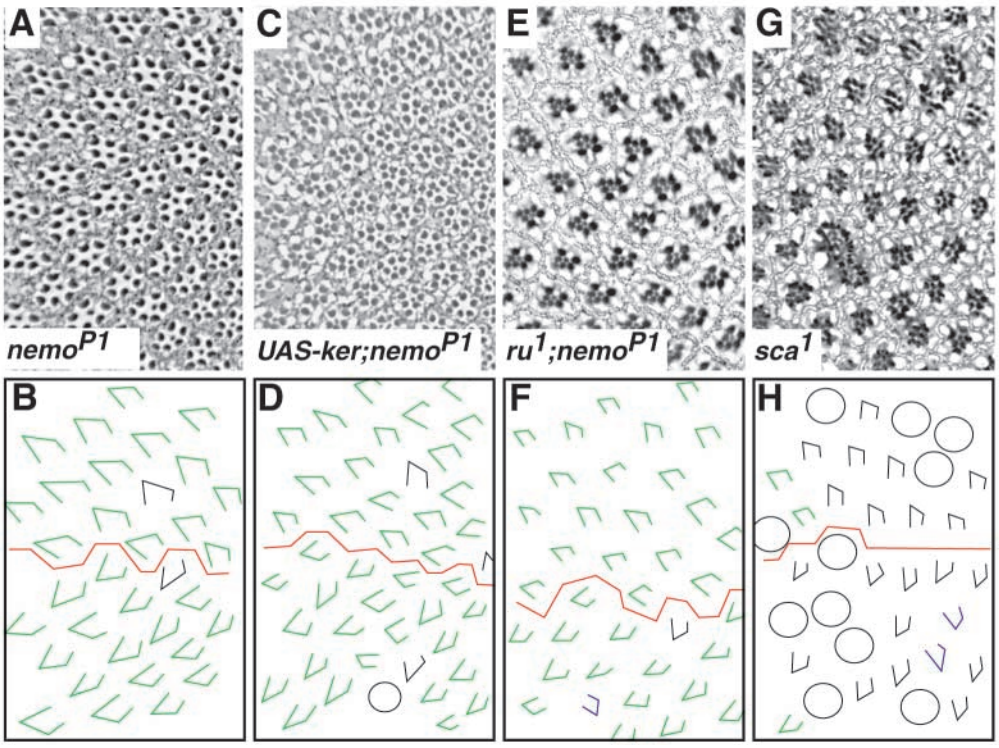
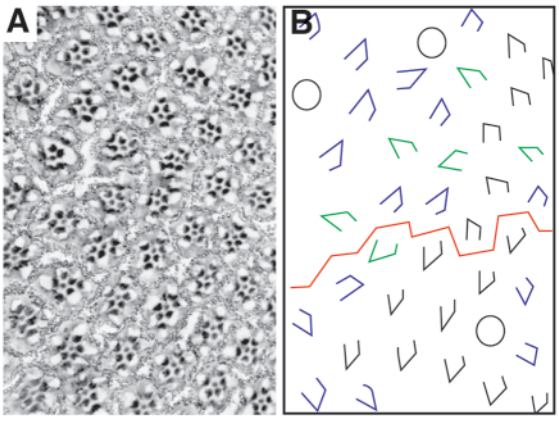


Fig. 6. Genetic interactions with other rotational genes. (A-F) Disrupting Egfr signalling has no effect on the *nemo*^{P1} phenotype. Top panels show sections through adult eyes; bottom panels are schematics of these images. (A,B) *nemo*^{P1}. All ommatidia are arrested at approximately 45°. (C,D) *sev-Gal4, UAS-keren/+; nemo*^{P1}. (E,F) *ru*^{1}, *nemo*^{P1}. Conditions of both overactive (C) and underactive (E) Egfr signalling fail to modify the *nemo*^{P1} phenotype. (G,H) *sca*^{1} mutants show relatively minor defects in ommatidial rotation in the adult eye. Most correctly specified ommatidia are orientated at 90° to the equator, with only a few being misrotated. Colour coding in schematics is as previously (black, correctly orientated; green, underrotated; blue, overrotated; black circles, mis-specified ommatidia; red line, equator).



Genotype	No.	%misrotated	%misrecruited
S/+	5	5.09±3.64	5.15±2.92
S/shg^{IG29}	5	**20.40±4.46**	8.97±3.09
S/shg^{k03401}	5	**15.17±4.29**	5.86±1.84
S/shg^{2}	5	**38.08±8.30**	**20.64±2.43**
S/wb^{09437}	6	8.95±3.73	5.94±3.78
mys^{1}/+;S/+	7	8.62±4.57	14.96±10.70

Fig. 7. E-cadherin interacts genetically with Egfr signalling. (A,B) Rotational defects in *S^{5671}/shg^{IG29}* adult eye sections. Rotational defects of *S^{5671}/+* eyes are significantly enhanced by halving the dose of E-cadherin (compare Fig. 7A,B with Fig. 2C,D). (C) Table showing interactions between *Star* and *shotgun*, *wing blister* and *myospheroid*. 'No.' indicates the number of eyes scored for each genotype. Only *shotgun* alleles significantly enhance the rotational defects of the *Star* heterozygote. Significant differences from *S/+* are indicated in bold type (P<0.05).

Discussion

We have discovered a new role for Egfr signalling in *Drosophila* eye development, and this has led us to identify a previously unrecognised process in normal eye patterning. Our results show that both over- and underactivation of the Egfr pathway cause defects in the rotation of ommatidia, suggesting a function for the pathway in the control of the co-ordinated rotation that occurs during the third instar eye disc. This rotational function is not a secondary effect of disrupting recruitment, but instead represents direct control of rotation by the Egfr. To our surprise, however, the rotational defects are not the result of perturbing the initial rotational movements, because these occur normally when Egfr signalling is disrupted. Instead, abnormally orientated ommatidia only become apparent in pupal stages. This indicates that the Egfr prevents the disruption of the previously established pattern. Our evidence suggests that cadherin-based adhesion participates in this Egfr-dependent protection mechanism.

Egfr signalling is already known to play several important roles in eye development, including cell recruitment, ommatidial spacing, cell proliferation and survival (Baker and Yu, 2001; Baonza et al., 2001; Bergmann et al., 1998; Domínguez et al., 1998; Freeman, 1996; Kumar et al., 1998; Kurada and White, 1998; Spencer et al., 1998). The identification of a further function – in ommatidial rotation – emphasises the pleiotropic effects of one signalling pathway in the development of a single tissue, and highlights the question of how such diverse successive effects are coordinated. One answer is that the signal itself does not specify the cellular consequences. Instead it is the developmental state of the receiving cell – mechanistically, its repertoire of signal-responsive transcription factors – that determines the outcome of signalling (Flores et al., 2000; Freeman, 1997; Xu et al., 2000). We suspect that another important factor in regulating reiterative signalling in the eye is the use of two different activating ligands: Spitz, which triggers cell recruitment and mitosis (Baker and Yu, 2001; Domínguez et al., 1998; Freeman, 1996; Tio and Moses, 1997), and Keren, which is inferred to control ommatidial spacing and survival (Wasserman et al., 2000), and which we hypothesise here to participate in rotational control. Importantly, however, there is no evidence that the different ligands produce different 'qualities' of signal; on the contrary, all current results support the idea that the ligands activate exactly the same effector pathways (see Gabay et al., 1997). Rather, we imagine that multiple activating ligands could allow for a more precise and complex regulation of the initiation of signalling. These important issues, however, will only be fully resolvable when *keren* mutants are isolated.

The fact that over- or underactivating the Egfr pathway has similar effects on rotation indicates that it is either the precise levels of signalling or the spatial distribution of signal activation that is important for controlling orientation. In the latter hypothesis, correct ommatidial rotation depends on asymmetric Egfr signalling in a specific subset of cells within each ommatidium. Therefore, global hyperactivation or loss of signalling would have similar effects because both conditions would disrupt the asymmetry required for function. The planar polarity receptor Frizzled shows this kind of dependency on asymmetric activation – loss and gain of Frizzled function in the eye show the same type of defects (Strutt et al., 1997; Tomlinson et al., 1997), because Frizzled must be preferentially active in R3 but not R4 in order to exert its effects (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999).

Perturbing Egfr signalling appears to be different from all other known rotation mutants, in that it exerts its effects on rotation after the normal process has been completed. In *nemo* and *scabrous* mutants, defects can be seen in the disc, while the ommatidia are still rotating (Choi and Benzer, 1994; Chou and Chien, 2002). This is also the case for Frizzled and other PCP components, which affect rotation at early stages (Zheng et al., 1995). Conversely, under conditions in which Egfr signalling is disrupted, ommatidia rotate and stop rotating precisely as they should, and yet the adult eyes show significant defects in ommatidial orientation. These observations imply that Egfr signalling is acting in a distinct process from other known components, that of maintaining ommatidial orientation after rotation is complete. Despite this evidence for a new aspect of rotational control affecting pupal eye development, our data show that Egfr signalling is actually required during the third larval instar, during or immediately after the second 45° rotation – if the pathway is disrupted at this time, rotational defects are seen in the adult eye. It would appear, therefore, that there is a delay between the time at which Egfr signalling is required and the time at which the phenotype becomes apparent.

A model that might account for these results is that the role of Egfr signalling is to establish a 'locking' mechanism that ensures that ommatidia remain in their final orientation. Such a mechanism might be necessary to protect the ommatidia against positional disruption during later events in eye development. Signalling would therefore be required during or at the end of normal rotation in order to set in place this hypothetical 'lock', although defects might not arise until significantly later than this, when processes occur that would cause ommatidia to reorientate in the absence of such a lock.

What might such processes be? During pupal development, the eye undergoes significant changes (Cagan and Ready, 1989; Wolff and Ready, 1993) (see Fig. 8). Additional cell types – primary, secondary and tertiary pigment cells – are recruited into the ommatidium from approximately 12 hours post-pupariation. Also at about this time, the eye disc everts, an event involving significant morphogenetic movement. Later, there is a phase of apoptosis starting at approximately 24 hours of pupal life, which is preceded by a reorganisation of interommatidial cells into a tight lattice network surrounding each cluster (Cagan and Ready, 1989). Later still, a further stress on the tissue might be rhabdomere morphogenesis, which initiates at approximately 37 hours and involves substantial cellular gymnastics (Longley and Ready, 1995). Any of these events could result in morphogenetic stresses on the eye tissue that could disrupt the precise rotational organisation of ommatidia. In this model, the presence of an Egfr-controlled lock functions to prevent such rotational disruption. The fact that loss of Egfr signalling has no effect in *nemo* mutant ommatidia might imply that there is a Nemo-dependent change in the adhesive properties of the cells when ommatidia commence the second 45° rotation; before this point, they are not sensitive to later disruption. The observation that *shotgun* mutants specifically enhance rotational defects of *Star* heterozygous eyes is consistent with this kind of model: Egfr signalling would result in a change of the adhesive properties of the cells, thereby restricting their motility with respect to their neighbours. Significantly, E-cadherin and Egfr signalling are associated in several other morphogenetic processes in *Drosophila* development (Dumstrei et al., 2002; Fulga and Rorth, 2002; James et al., 2002).

In addition to this potential Egfr-dependent lock, our observations point to a second mechanism in refining ommatidial orientation. In WT discs, approximately 5% of ommatidia are out of alignment with their neighbours, some of these being overrotated beyond 90° (see Fig. 4E, arrowheads) (K.E.B. and M.F., unpublished observations). By the adult, however, all ommatidia are perfectly orientated with respect to the equator. This implies that there is a correction mechanism later in development that repositions ommatidia that are initially improperly rotated. Further evidence for this comes from the discrepancy between the phenotype of *scabrous* mutants in the disc versus the adult. Ommatidia at the back of the third instar disc become overrotated (Chou and Chien, 2002), but in the adult, only a small proportion are incorrectly orientated, and some are underrotated. There is therefore a qualitative change in the phenotype between the larval disc and the adult; moreover, this change appears to lead to an improvement in ommatidial orientation. Because *scabrous*$^{-}$ eyes show significant recruitment defects, we cannot rule out the possibility that most of the incorrectly recruited ommatidia are also overrotated, and therefore that the correctly orientated or underrotated ommatidia actually represent the minority. However, similar adult phenotypes were also observed in eyes in which *nemo* was overexpressed (K.E.B. and M.F., unpublished), and these do not show problems with recruitment but do have equivalent overrotation defects in the disc (Chou and Chien, 2002). Together, these results all strongly imply the existence of an error-correcting mechanism that refines the initial rotational pattern laid down in the third instar.

The *Drosophila* eye provides a striking example of the precision with which developmental patterning can occur. Ommatidia in the adult eye are precisely orientated in essentially 100% of cases. Our results suggest that this precision is not simply a consequence of an initial rotational process, but also critically depends on at least two further aspects. First, there is an Egfr-dependent mechanism protecting the eye against disruption of the original pattern presumably caused by the morphogenetic and cellular upheavals that occur in pupal stages. Second, we have evidence for a refinement and error-correcting mechanism, whose molecular basis is unknown. Another aspect of fly eye patterning, cell recruitment, also depends on a two-stage process of initial patterning followed by refinement. In this case, too many cells are originally produced, presumably to ensure there are enough to form all necessary cell types. This is followed by specific apoptotic removal of superfluous cells in pupal life (Cagan and Ready, 1989; Miller and Cagan, 1998; Wolff and Ready, 1991). We suspect that it may prove to be a general property of pattern

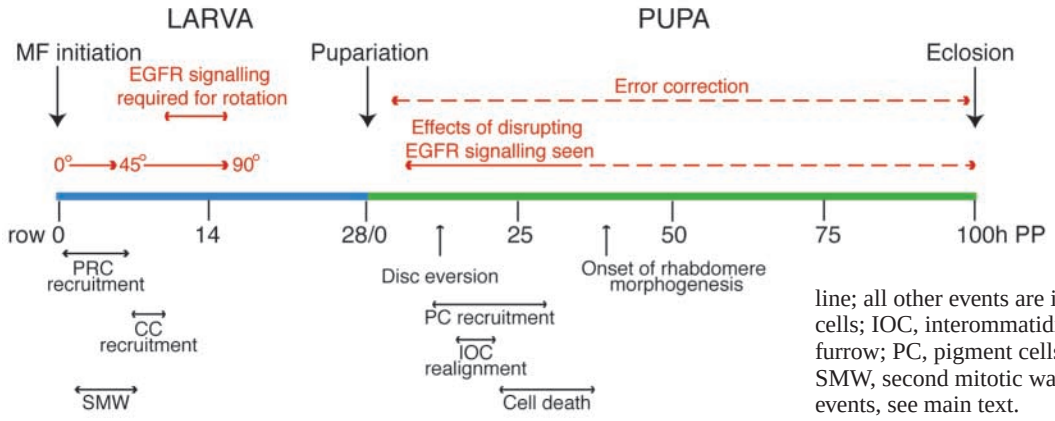


Fig. 8. Timeline showing significant events during *Drosophila* eye development. Larval time (blue) is scaled as number of ommatidial rows; pupal time (green) as hours post-pupariation. Events directly concerning rotation are shown in red above the line; all other events are in black below the line. CC, cone cells; IOC, interommatidial cells; MF, morphogenetic furrow; PC, pigment cells; PRC, photoreceptor cells; SMW, second mitotic wave. For precise timings of pupal events, see main text.

formation – especially when great precision is required, as in the case of a visual system – that refinement and active maintenance functions are programmed into the overall patterning process.

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