Multiple functions of the EGF receptor in *Drosophila* eye development

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Background: During animal development, cells need to make spatially and temporally regulated fate decisions. These decisions are largely controlled by intercellular signalling, often through receptor tyrosine kinases. One of these, the epidermal growth factor receptor (EGFR), regulates multiple cell fate decisions. Its importance in the recruitment of photoreceptors in the developing fly eye, a useful model for neural development, has already been reported. Other EGFR functions in the eye have not been characterised.

Results: We have examined the consequences of removing or activating the EGFR at different stages of eye development. The earliest stages of assembly occurred normally within *EGFR*$^-$ clones – the morphogenetic furrow was unimpeded and the R8 photoreceptor was specified. All subsequent photoreceptor recruitment was blocked. *EGFR*$^-$ clones had a characteristic shape indicating that they had undergone substantial cell death posterior to the furrow, where the differentiation program is normally activated; consistent with this, excess apoptosis was detected. We found that the receptor also regulates cell proliferation in the disc, has an early function at the disc margin (where the morphogenetic furrow initiates) and contributes to the regulation of spacing of the R8 precursors. Finally, we found that activation of the receptor is sufficient to trigger non-R8 photoreceptor development, even in cells in front of the furrow or in the absence of the proneural gene *atonal.*

Conclusion: At least five distinct functions of EGFR signalling need to be integrated during fly eye development. These include roles in cell proliferation, survival and differentiation.

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Background

Cell diversity and pattern during animal development is generated largely by the control of intercellular signalling. Cells interpret their positions by receiving signals from their neighbours and differentiate accordingly. The *Drosophila* eye has proved a useful model for studying the signalling processes that control the differentiation of the nervous system. The individual ommatidia of the *Drosophila* compound eye develop sequentially in a wave that moves across the larval eye imaginal disc — the epithelial sheet that differentiates into the eye [1]. A furrow starts at the posterior of the disc and sweeps anteriorly over the period of about two days. The individual ommatidia start to develop within this 'morphogenetic furrow' and mature in its wake [2,3].

There are two phases of photoreceptor development in each ommatidium. The first is the determination of the initial photoreceptor, the R8 cell, in a regularly spaced array along the furrow [3–5]. This process involves the progressive singling-out of cells that maintain the expression of Atonal, a proneural basic helix–loop–helix protein that specifies the R8 fate [6–9]. This singling-out process is controlled by lateral inhibition and resembles the determination of other sensory organs in the fly. The second phase of photoreceptor determination is the serial recruitment of the other seven photoreceptors (R1–R7) into a precise cluster around each R8 [5]. This recruitment is triggered principally by the activation of the epidermal growth factor receptor (EGFR) in the uncommitted cells surrounding each R8 cell [10,11]; the EGFR ligand Spitz is produced initially by R8 alone, and later by each of the pairs of already recruited cells, thereby triggering the addition of more cells to the growing cluster.

In mammals, the EGFR regulates cell proliferation and differentiation: the receptor is a crucial element of growth control [12,13]. In *Drosophila*, however, most is known about the role of the EGFR in triggering cell differentiation [14,15]; its role in proliferation is not clear. In the fly eye, the pivotal role of the EGFR in cell recruitment has been demonstrated with clones of cells lacking the receptor [16] and with a dominant-negative form of the receptor [10]. For technical reasons, neither of these approaches could dissect all the functions of the receptor in eye development; major issues therefore remained about the

complete role of the EGFR and about the relationship of the two phases of photoreceptor determination. For example, the role of the receptor in R8 determination has been unclear. It has been reported that in the absence of the EGFR there are no R8s, but the principal activating ligand, Spitz, is not required for normal R8 formation. The role of the morphogenetic furrow has also been uncertain. In addition to providing a mechanism for spacing the R8s appropriately, does it also impart competence on cells to be recruited by the EGFR? Another question is whether the receptor is needed for cell survival: *EGFR*$^-$ clones do not survive to adulthood [16,17], but it is not known whether this is a direct or a secondary effect. Finally, *EGFR*$^-$ clones are always much smaller than controls [16,18], raising the possibility that the EGFR may directly regulate cell division in flies [16], as it does in mammals. This idea is supported by the observations that overactivation of the Ras pathway, through which the EGFR signals, leads to excess proliferation in imaginal discs [19], as does the loss of *yan*, which acts negatively in the Ras pathway [20].

We have overcome the earlier difficulties of generating *EGFR*$^-$ clones in the developing eye by using the *Minute* technique [21]. By giving the cells surrounding the clone a growth disadvantage, we are able to generate large mutant clones. We have also examined the consequences of ectopically expressing an activated form of the receptor. This has allowed us to identify and dissect multiple roles of the EGFR during eye development. Our results indicate that there are at least five distinct functions for the EGFR: these include roles in the margin of the disc (where the furrow initiates), the control of cell proliferation, the singling-out of the correct number of Atonal-expressing cells, cell survival and cell recruitment. This work illustrates the complexity of spatial and temporal regulation that is required to control a growth factor receptor that influences such a variety of cell decisions.

Results

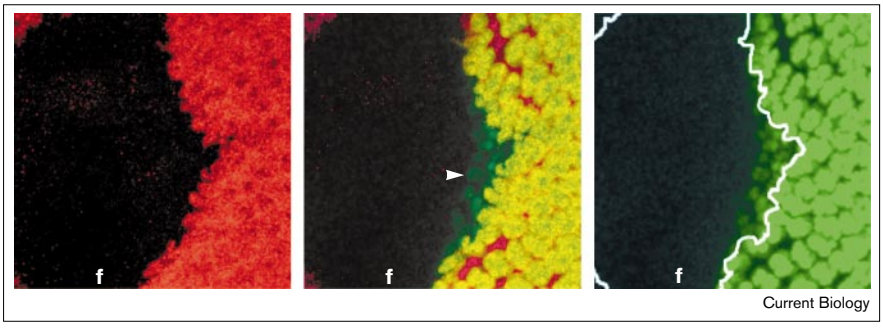
R8 cells develop in *EGFR*$^-$ clones

To understand the role of the EGFR in early neuronal specification in the eye, we generated clones of cells lacking the receptor. Because *EGFR*$^-$ clones have a growth and/or survival disadvantage compared with wild-type cells [16–18], we induced them in a *Minute* mutant background. Using this system, all cells except those that are homozygous for the *EGFR* mutation are heterozygous for a dominant growth-impeding mutation [21]. This gives the cells in the clone a competitive advantage. Within clones of cells mutant for a null allele of the *EGFR* (see Materials and methods), we found cells that expressed the neuron-specific antigen Elav behind the furrow, where photoreceptor differentiation normally occurs (Figure 1). This indicates that photoreceptor-like cells can differentiate in the absence of all EGFR function. The Elav-expressing cells were usually isolated although there are occasional groups of two or three cells. The cells were often more weakly stained than cells in adjacent wild-type tissue; also, unlike their neighbours, their spacing was abnormal.

All Elav-expressing cells within the clones also expressed Boss, a marker specific for the R8 cell (Figure 2a–c) [22]. This implies that R8 photoreceptors, but no others, are able to initiate development in the absence of EGFR function. The *EGFR*$^-$ cells that expressed Elav and Boss were also R8-like in their competence to recruit neighbouring wild-type cells into ommatidial clusters (Figure 2c). It has been reported previously that *EGFR*$^-$ clones in the eye do not survive to adulthood [16,17]; consistent with this we found that the *EGFR*$^-$ R8 cells eventually lost their expression of Elav and Boss and were no longer detectable in the posterior of the disc (the oldest region).

We obtained similar results for clones mutant for Ras1, through which the EGFR signals (data not shown). R8s also formed in *Minute*$^+$ *spitz*$^-$ clones (Figure 2d) but there

Figure 1



EGFR$^-$ cells can differentiate as neurons. *EGFR*$^-$ clones were double-labelled with an antibody to the nuclear neuronal marker Elav (green) and a second antibody to β-galactosidase (red). Mutant clones are recognised by their lack of red staining (left panel). The merged image is shown in the centre panel. Single cells positive for Elav (example indicated by the arrowhead) developed within the *EGFR*$^-$ cells located posterior to the furrow. In the extreme right panel, the white line indicates the border of the mutant clone. In this and the following figures, all panels show late third instar eye imaginal discs. Anterior is always to the left and the approximate position of the morphogenetic furrow is marked with an f.

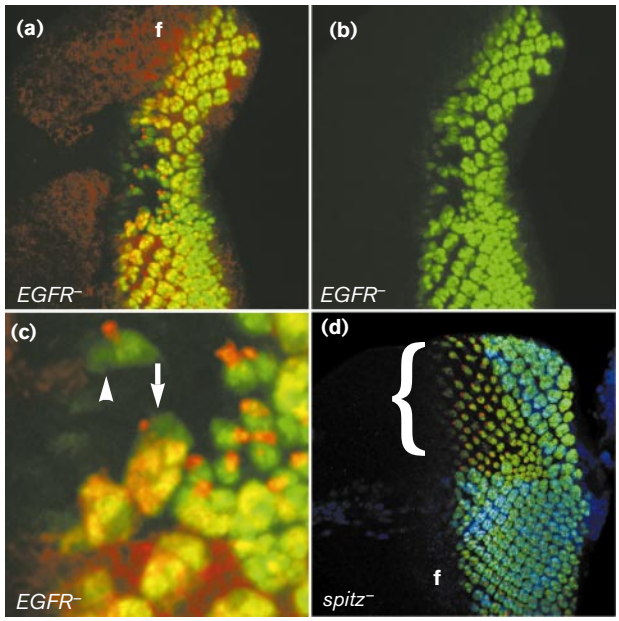
was a clear difference in the phenotype compared with *EGFR*$^-$ clones; in *spitz* clones, R8s survived much longer and their spacing was wild type [23].

When *EGFR*$^-$ clones extended to the margins of the eye disc, a different phenotype was observed. In these cases, loss of EGFR caused impaired disc growth and excess cell death (data not shown). We have not examined this phenomenon in detail, but it indicates a very early function for the receptor at the margins of the disc, the regions where the morphogenetic furrow initiates. The rest of the results described here concern the phenotype of interior clones, those that do not extend to the margins.

Rough expression is abolished in *EGFR*$^-$ clones

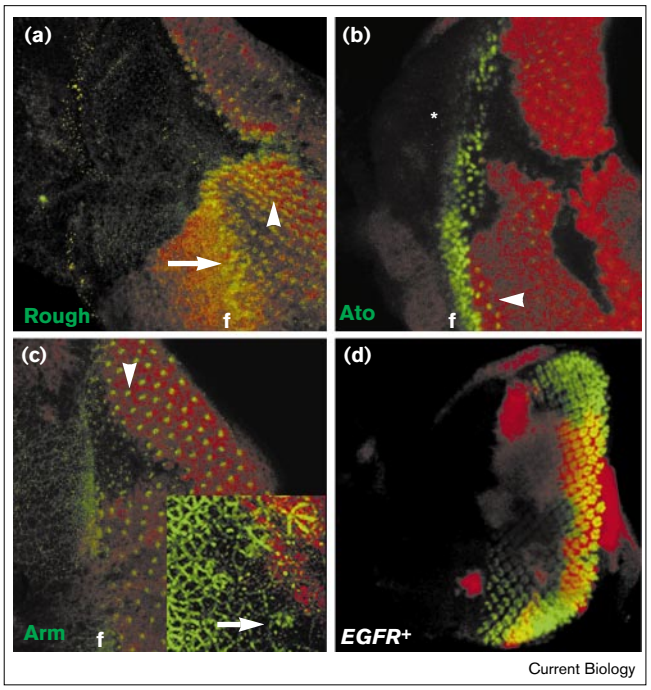
To support the observation that only R8s initiate development within the *EGFR*$^-$ clones, we looked at the expression of the homeobox protein Rough, which is expressed earlier than Elav, specifically in the outer photoreceptors R2, R5, R3 and R4 (although it is also expressed more widely earlier in the furrow) [24]. No Rough-expressing cells were seen within clones (Figure 3a) consistent with the conclusion that only R8s can form; unexpectedly, we found that the whole early domain of Rough expression in the furrow was also abolished. In wild-type discs, Rough expression in the furrow is complementary to Atonal: it is expressed in all the cells of the furrow that are outside the proneural groups [9]. When the proneural groups are

Figure 2



Only R8 photoreceptors develop in the absence of *EGFR*. **(a–c)** Eye disc with an *EGFR*$^-$ clone labelled with anti-β-galactosidase antibody (diffuse red); unstained tissue corresponds to the clone. The disc is also labelled with an antibody to the R8-specific marker Boss (bright red spots) and an antibody to Elav (a neuronal cell marker; green). The merged image is shown in (a) and the Elav expression in (b). All the Elav-expressing cells also expressed Boss, which stains the apical cell membranes of R8. (c) Enlargement of part of (a). Most Elav-expressing and Boss-expressing cells were isolated (arrowhead), but when these cells were at the clone border they could recruit neighbouring wild-type cells into irregular clusters (arrow). **(d)** Eye disc with a *spitz*$^-$ clone (region indicated with white bracket), stained as in (a–c), except that the β-galactosidase-negative marking of the clone is shown in blue. Loss of *spitz* blocked the recruitment of R1–R7 cells as previously reported but, in contrast to *EGFR*$^-$ clones, the *spitz* mutant R8 cells were arranged in a regular pattern.

Figure 3



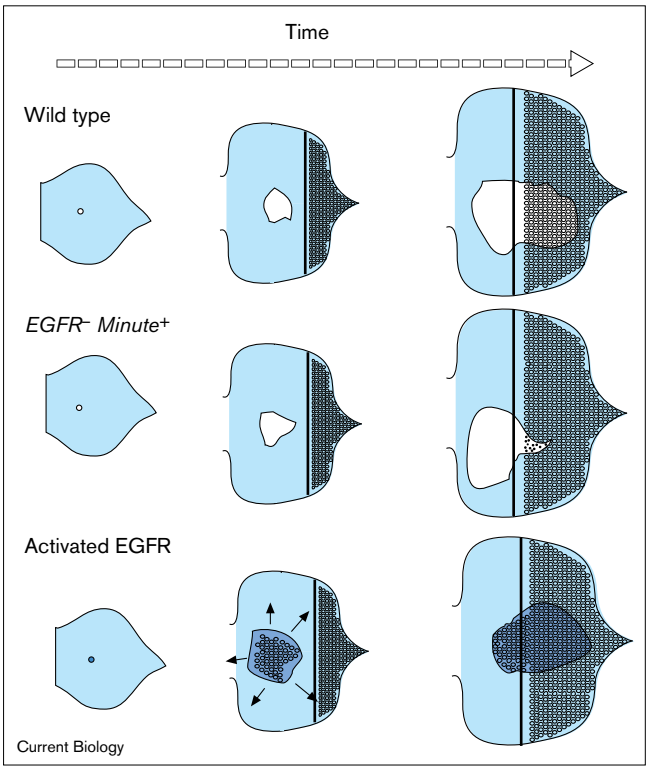
Requirement for EGFR in the morphogenetic furrow. In all panels, *EGFR*$^-$ clones are identified by the lack of red β-galactosidase staining. **(a)** The loss of EGFR within the furrow resulted in loss of Rough expression (seen as green staining). Note that both expression of Rough in the furrow (arrow) and in the R2, R5, R3 and R4 precursors (example indicated by arrowhead) is abolished within the mutant clone. **(b)** Atonal expression (Ato; green) in the furrow was largely unaffected in an *EGFR*$^-$ clone, but in parts of the furrow most distant from wild-type tissue, the expression of Atonal was reduced (asterisk). There were excess R8 precursors and they were less organised than in wild-type tissue (compare the Atonal-expressing cells within the clone with those outside it, marked with an arrowhead). **(c)** Eye disc with an *EGFR*$^-$ clone stained with anti-Armadillo antibody (Arm; green), to label apical cell profiles. The cells have undergone apical constrictions associated with the morphogenetic furrow and, in wild-type tissue, ommatidial rosettes formed at the posterior edge of the furrow (arrowhead); photoreceptor precursors in these rosettes showed increased Armadillo staining. In *EGFR*$^-$ clones, the furrow was normal but only single cells had enhanced Armadillo staining (example indicated by the arrow in the inset); also the rosettes were not maintained and non-R8 *EGFR*$^-$ cells lost their apical constrictions. **(d)** *Minute*$^+$ wild-type clones in an eye imaginal disc. These clones were irregular in shape and did not taper away behind the furrow like *EGFR*$^-$ clones (compare the clone shapes in (d) with those in (a–c)).

refined to a single R8 precursor, Rough is expressed in all cells except R8 and then fades away; later in development expression is reinitiated in R2–5. It has been proposed that Rough represses Atonal expression, consequently regulating the selection of R8 [9]. Our result demonstrates that the EGFR positively regulates Rough expression in the furrow (although we do not know how direct this control is) and may therefore function to control the singling-out of R8; this is supported by the abnormal spacing of R8s and Atonal-expressing cells (see below) that we saw in the clones.

The progress of the furrow is unimpeded by *EGFR*⁻ clones

The loss of Rough in *EGFR*⁻ clones suggests a role for the receptor within the furrow. To examine this more thoroughly, we looked at the expression of Atonal, the earliest marker for the furrow. In wild-type discs, Atonal, the proneural gene for R8, is initially expressed in all cells at the anterior edge of the furrow; its expression is progressively restricted to the evenly spaced single cells that will differentiate as R8s [7–9]. We found that early aspects of this expression pattern were largely unaltered in *EGFR*⁻ clones (Figure 3b): mutant cells expressed Atonal and the stripe of expression passed through clones with little or no delay. Most *EGFR*⁻ cells expressed wild-type levels of Atonal but in parts of the furrow most distant from wild-type tissue, expression was reduced (though never abolished; Figure 3b, asterisk). Posterior to the furrow, Atonal-expressing cells were mostly isolated, implying that the restriction of Atonal to a single cell has occurred normally in the majority of cases. The normal regular spacing of Atonal-expressing cells was disrupted, however, consistent with our observation that the R8s within a clone are not evenly spaced. Furthermore, there were more Atonal-expressing cells than in wild-type tissue, suggesting that excess R8 cells are selected in the absence of the EGFR.

Figure 4



Summary of clone phenotypes. The evolution of wild-type, *EGFR*⁻ and activated EGFR clones of cells at different developmental stages. After the induction of the clone, the mutant cells divide during the second and third instar larval stage; those expressing the activated receptor undergo excess proliferation (see later). After the morphogenetic furrow (black line) has passed through a wild-type or *EGFR*⁻ clone, ommatidial clusters or single R8 cells, respectively, form within the clones. As neurogenesis begins, the *EGFR*⁻ clones taper away due to excess cell death. Precocious photoreceptor determination was observed in clones expressing the activated EGFR (see later); posterior to the furrow these clones showed excess photoreceptor recruitment.

As cells enter the furrow they undergo dramatic morphological changes that can be detected by examining their apical profiles using an antibody against the Armadillo protein [25]. In wild-type discs, the apical profiles of cells become constricted as they enter the furrow and then all cells except those in ommatidial 'preclusters' release their apical constriction [4]. Within the preclusters, photoreceptor precursors are distinguished by enhanced Armadillo staining. In *EGFR*⁻ clones, we found that cells constricted normally and without delay as the furrow passed through the clone but preclusters were not maintained. Instead, only single cells (presumably R8s) showed increased Armadillo staining (Figure 3c, inset).

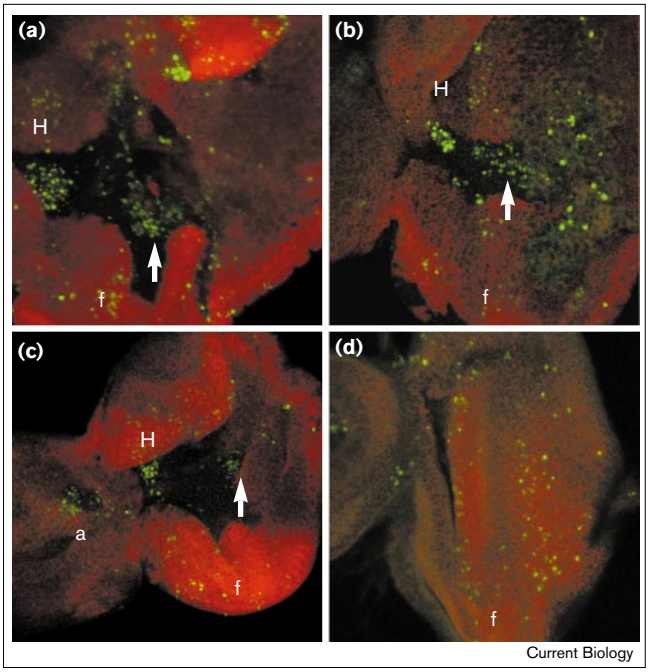
The EGFR is required for cell survival behind the morphogenetic furrow

We noticed that *EGFR*⁻ clones in the developing eye disc had an unusual and characteristic shape. They could grow quite large, but they invariably tapered away behind the furrow, leaving only small, narrow regions in the area of the disc where photoreceptor recruitment occurs (for example, see Figures 1,2a,3a–c). The change of shape coincided with the onset of Elav expression. No such narrowing occurred in control *EGFR*⁺ clones generated in the same *Minute* background (Figure 3d). To understand this phenomenon it is necessary to consider the development of the disc. The clone is induced early in development, long before the furrow starts to sweep across the disc. Clones induced early therefore reach a substantial size before the furrow reaches them (Figure 4). The shape change we saw implies that when cells in a clone are reached by the furrow they become unable to survive. The *spitz*⁻ clones did not taper behind the furrow (Figure 2d) suggesting that the absence of Spitz does not induce the same kind of early-onset cell death as absence of the EGFR.

To determine whether the change in clone shape coincides with abnormal cell death we looked for apoptosis in *EGFR*$^-$ clones by terminal deoxynucleotidyl transferase mediated dUTP–biotin nick end labelling (TUNEL) [26] (see Materials and methods). We found that there was indeed excess cell death associated with *EGFR*$^-$ clones (Figure 5a–c). As predicted from the shape of the clones, this occurred predominantly posterior to the furrow, where large clusters of TUNEL-positive cells were often seen. Anterior to the furrow there were occasional scattered cells that stained but not the large clusters seen in posterior regions. In addition to the clusters of dying cells in clones behind the furrow, we also saw other regions of the eye–antennal disc that were TUNEL-positive when the EGFR was removed, suggesting that the cell survival function of EGFR signalling is not limited to the retina. These regions included a characteristic domain in the tissue anterior to the retina that is the primordium of part of the head, and several parts of the antennal portion of the disc, whose fate we do not know. Although control discs that are heterozygous for the *Minute* mutation showed an overall slightly increased level of apoptosis compared with wild-type discs, we never saw the clusters of dying cells that we found associated with *EGFR*$^-$ clones (Figure 5d). Moreover, as the *EGFR*$^-$ cells are actually homozygous wild type for the *Minute* mutation, almost all the cell death in them must be due to the loss of the EGFR — wild-type discs show very little apoptosis at this stage [27].

The observations that *EGFR*$^-$ clones did not shrink until the furrow had passed and that TUNEL staining in the retina was largely restricted to behind the furrow both suggest that EGFR signalling becomes essential for cell survival only in the differentiating tissue. Our observations support the proposal of Xu and Rubin [16] that the small size of *EGFR*$^-$ clones in eye discs is caused by a proliferation defect in *EGFR*$^-$ cells, rather than substantial cell death ahead of the furrow.

Figure 5



Loss of EGFR causes increase of cell death in the differentiated region of the disc. **(a–c)** Clusters of TUNEL-positive apoptotic cells (green) could be seen (arrows) in *EGFR*$^-$ clones (which lack red β-galactosidase staining), but only in the region behind the morphogenetic furrow. Anterior to the furrow, isolated cells within the clones were TUNEL positive. Not all clones were TUNEL positive, although it appears that TUNEL-negative clones may have already undergone extensive cell death, as judged by their tapered shape. Other regions of the eye–antennal disc are also TUNEL positive in *EGFR*$^-$ clones, including a characteristic cluster in the primordium of the head capsule (H) and domains within the antennal region (marked a). **(d)** A control *Minute* heterozygous disc with no clone. There was more cell death than in wild-type discs (data not shown), but no clusters were seen. Note that the *EGFR*$^-$ clones in (a–c) are homozygous wild type for the *Minute* mutation, so all excess cell death within the clones was caused by EGFR loss.

EGFR activation ahead of the furrow triggers neural development

One implication of the results described above is that EGFR signalling is required for cells to be recruited as non-R8 photoreceptors. This agrees with previous work using a dominant-negative form of the receptor [10]. We wanted to test whether EGFR activation was also sufficient to trigger photoreceptor differentiation in cells that have not undergone any of the developmental history associated with the passage of the morphogenetic furrow. To this end, clones of cells were induced that expressed an activated form of the EGFR [28]. As expected, clones posterior to the furrow had extra photoreceptor recruitment (data not shown). Ahead of the furrow, clones have irregular clusters of cells which express Elav, implying that EGFR activation there is indeed sufficient to trigger the neuronal fate (Figure 6a). We also found cells expressing markers of the cone cell fate; some of the clusters also had cells that expressed Boss, indicative of the R8 fate (Figure 6b,c).

Atonal-independent photoreceptor determination

These large clones do not allow us to distinguish whether photoreceptor recruitment is a primary response to EGFR activation, or a secondary effect in which cells are recruited by cells determined earlier. To resolve this, activated EGFR was also expressed in *atonal*$^-$ eye discs. Normally, in the absence of Atonal, no photoreceptors develop [6]. When we expressed the activated EGFR in *atonal*$^-$ discs (under the control of the optomotor-blind (omb) promoter driving expression of the *Gal4*-encoded transcriptional activator; omb–Gal4), neuronal differentiation was observed (Figure 7a–c). In these cases, no Boss-expressing cells were found. No Elav expression was seen in discs before the time the endogenous furrow would normally start

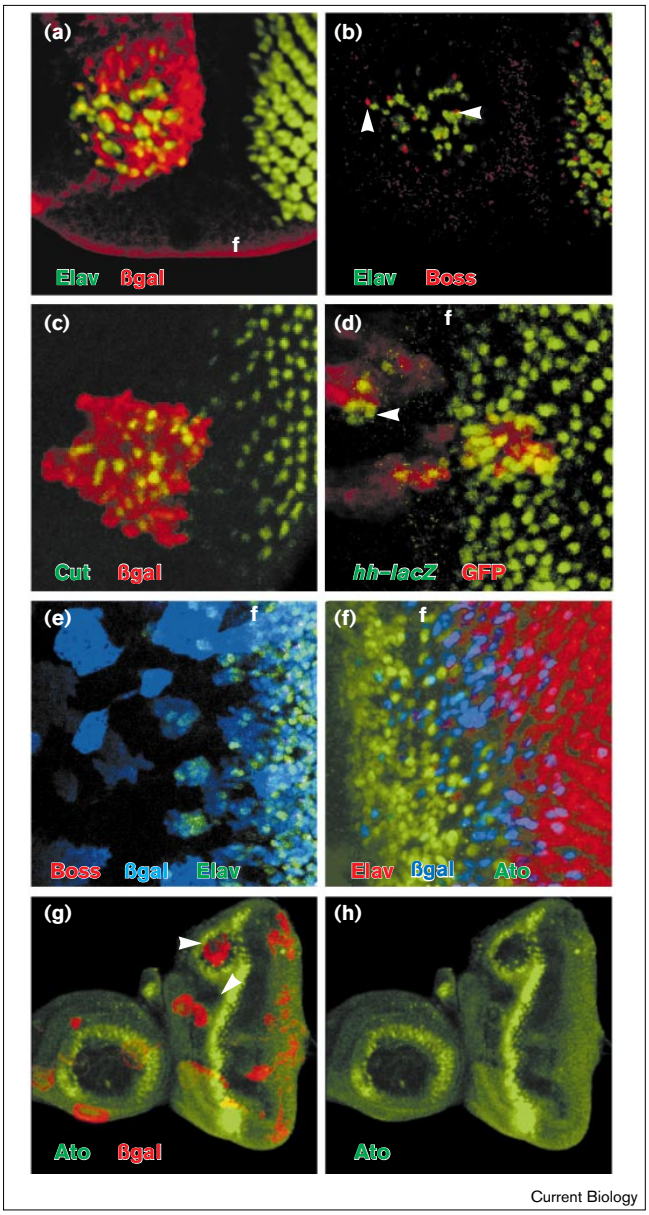


Figure 6

Activation of *EGFR* is sufficient to trigger neuronal differentiation. Discs with marked clones of cells expressing the activated form of the EGFR. **(a)** Ectopic expression of the activated receptor (red; βgal) induced Elav-expressing cells (green) in a large clone anterior to the furrow. **(b)** The same clone showing that some of the Elav-expressing cells also expressed the R8 marker, Boss (red; examples indicated by arrowheads). **(c)** A similar clone with cells expressing the cone-cell marker, Cut (green). **(d)** Some cells within clones expressing the activated EGFR also expressed a *hedgehog* enhancer trap (*hh–lacZ*; green) anterior to the furrow (example indicated by arrowhead); all photoreceptors posterior to the furrow express the *hedgehog* enhancer trap. **(e)** Multiple small clones of activated EGFR-expressing cells (blue) induced late in eye development. Only clones near the furrow expressed Elav (green); none ahead of the furrow expressed Boss (red). Boss staining was seen only in R8 cells posterior to the furrow. **(f)** Multiple small clones of activated EGFR-expressing cells (blue); again, only those close to the furrow express Elav (red) after 12–24 h. None of the clone cells expressed Atonal (Ato; green), although ectopic Atonal-expressing cells were seen adjacent to cells expressing the activated receptor. **(g)** Atonal expression (green) was absent from clones expressing the activated EGFR (red), but such clones were often surrounded by Atonal-expressing cells (arrowheads). **(h)** The same disc, showing only the Atonal expression. The lack of Atonal within clones is clear. As in (e,f), this demonstrates that Atonal is induced in cells next to cells triggered to differentiate by the EGFR, but not in those cells themselves.

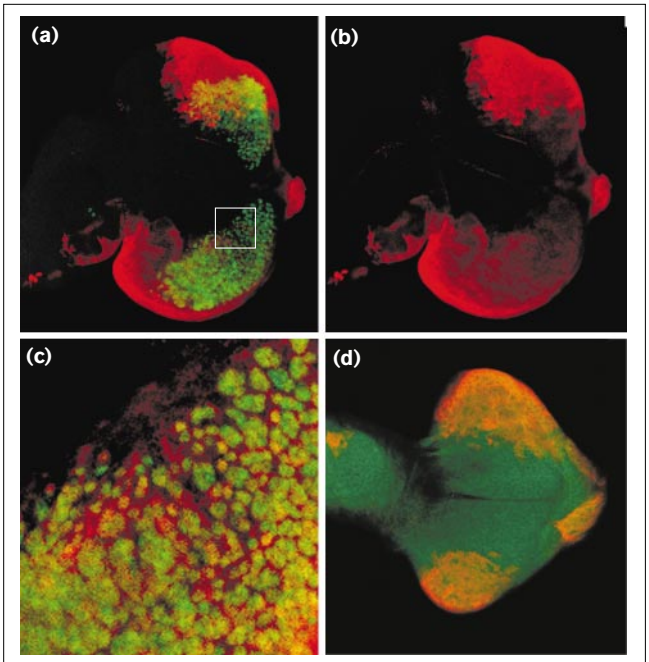
(Figure 7d). This implies that after the beginning of the third instar, activation of the receptor is sufficient to trigger the differentiation of non-R8 photoreceptors in the absence of Atonal. Conversely, R8s require Atonal but not the EGFR. Therefore, the R8 cells seen in large clones of activated EGFR must have been secondarily induced by the directly induced photoreceptors. We confirmed this by inducing clones late and looking at the response of cells after only 12–24 hours. Cells in these small clones of approximately 1–4 cells expressed Elav but not Atonal (Figure 6e,f). We did, however, see Atonal induced in neighbouring wild-type cells. Similarly, although there was a striking lack of Atonal expression within large clones, they had a halo of Atonal in surrounding wild-type cells (Figure 6g,h). Once Atonal was expressed in wild-type cells, the normal cascade of ommatidial development was triggered and we saw an ectopic morphogenetic furrow. When clones were close to the endogenous furrow, it accelerated around them. We also found that some cells within activated-EGFR clones also expressed Hedgehog (Figure 6d). Hedgehog induces Atonal directly, thus controlling furrow progression [25]. Presumably this is the signal responsible for the secondary induction of R8 cells and the initiation of an ectopic furrow.

These results demonstrate that the photoreceptors comprise two classes: R8, which requires Atonal but not the EGFR, and non-R8, in which EGFR signalling is necessary and sufficient to trigger their development. This answers one of the questions that we posed above: it is clear that the prior expression of Atonal and the passage of the morphogenetic furrow is not required to make cells competent to respond to EGFR activation.

A temporal gradient of competence to differentiate

Although our results imply that activation of the EGFR in a cell anterior to the morphogenetic furrow is sufficient to trigger non-R8 photoreceptor determination, it is clear that not all cells are equally poised to respond to EGFR signalling. We never saw ectopic neuronal differentiation in young eye discs in which the furrow has not yet initiated; before furrow initiation, the clones of cells expressing activated EGFR proliferated but did not differentiate. In fact, we often saw hyperproliferation associated with these clones (data not shown). When late clones were induced, only those near the furrow started to differentiate and express Elav (Figure 6e,f). Cells more distant from the furrow were nevertheless competent to undergo neuronal

Figure 7



EGFR triggers differentiation of non-R8 photoreceptors in *atonal*$^{-}$ discs. **(a–c)** Expression of the activated form of the EGFR under the control of the omb–Gal4 driver induced neuronal differentiation in a complete loss-of-function *atonal* mutant. Elav-expressing cells (green) can be seen within the omb–Gal4 domain. Cells within this domain are marked with a *lacZ* transgene coupled to the upstream activator sequence to which Gal4 binds (*UAS–lacZ*; red). The merged image is shown in (a), and the omb–Gal4 staining alone in (b). This disc was also stained for Boss protein but no expression could be detected, indicating that in the absence of Atonal, no R8s can be determined, even by the activated EGFR. (c) Higher-magnification view of the boxed region in (a). Some irregular ommatidial clusters can be seen. **(d)** In younger discs, before the normal onset of the morphogenetic furrow, no Elav expression (green) was seen when the activated EGFR was expressed in the omb–Gal4 domain.

differentiation, as can be inferred by the existence of anterior clones with well advanced ectopic furrows (Figure 6a–c); these must have been initiated when the endogenous furrow was at a considerable distance. These observations lead us to conclude that cells become competent to differentiate as photoreceptors at about the time the endogenous furrow initiates, but that subsequently they acquire an increased disposition to differentiate in response to EGFR signalling as the furrow approaches them.

Discussion

We have examined the consequences of removing or overactivating the EGFR during the development of the *Drosophila* eye. These data, coupled with previous results, allowed us to identify five distinct functions for this receptor in regulating the differentiation of the retina. The earliest detectable requirement is in regulating cell proliferation in the eye imaginal disc (this work and [16]). Next, there is a growth control function at the edges of the disc, implied by the defects seen when *EGFR*$^{-}$ clones extend to the margins. We have not analysed this phenomenon in detail and other than identifying it as one of the EGFR functions in eye development, we do not address it here. Next, the EGFR is a positive regulator of the Rough protein in the furrow, where it regulates spacing of R8s. Fourth, we have identified an intriguing requirement for the receptor in cell survival in the differentiating region of the disc. Finally, the EGFR is the principal, and sufficient, trigger of cell recruitment of the non-R8 photoreceptors, the cone cells and the pigment cells (this work and [10]). These experiments also identify processes the EGFR is not involved in. Most notably, there is no requirement for the EGFR in either morphogenetic furrow progression or in the differentiation of the R8 photoreceptor. As we have obtained similar results with clones of *ras1*$^{-}$ cells (data not shown), signalling by another receptor tyrosine kinase through the Ras pathway is also ruled out in R8 determination or furrow progression. These results combine to produce a more complete picture of the different phases of eye development, the multiple parts played by the EGFR, and the integration of these functions in pattern formation.

The EGFR and the control of cell proliferation

In mammalian cells, the role of the EGFR in proliferation control is well studied. In flies the role of the receptor in differentiation is well characterised but its role in proliferation has not been examined in detail. *EGFR*$^{-}$ clones in the wing are much smaller than controls [18] and Xu and Rubin found the same of eye discs [16]. In this study, we have given the *EGFR*$^{-}$ clones a competitive growth advantage over the surrounding cells. In this condition, we could induce large clones, containing many hundreds of cells, indicating that there is no absolute requirement for the EGFR in cell division within the eye disc. Nevertheless, the shape of *EGFR*$^{-}$ clones and our observation that there was little abnormal cell death in the retina ahead of the furrow imply that the reduced size of *EGFR*$^{-}$ clones is not primarily caused by cell death but by a role for the receptor in regulating proliferation. Consistent with this, we have frequently found excessive tissue growth in the discs in which the activated EGFR is expressed (our unpublished observations). This coincides with the observations that increased signalling by the Ras pathway also causes excess proliferation [19,20] and that too many cells behind the furrow enter S phase in EGFR gain-of-function mutations [17].

The EGFR and the control of neuronal selection in the morphogenetic furrow

Neither the EGFR nor Ras1 is required for the progression of the furrow, or in the determination of R8 photoreceptors. The latter results conflict with the results published by Xu and Rubin [16], in which they were unable to find any neuronal differentiation in *EGFR*$^{-}$

clones. The reason for this discrepancy is unclear, although it is possible that by allowing the clones to proliferate more readily (by making them in a *Minute* background) they may be 'healthier' than those made previously. Xu and Rubin used an antibody to a different marker for neuronal differentiation, horseradish peroxidase (HRP); it is possible that HRP is expressed too weakly to be seen in the clones. We used several markers and although they are sometimes expressed more weakly than in wild-type cells, it is clear that R8s do form. Current models of furrow progression suggest that secreted factors like Hedgehog are produced by differentiating photoreceptors and diffuse anteriorly to induce Atonal expression and thereby 'push' the furrow forward (reviewed in [29]). It is therefore surprising that clones of *EGFR*$^-$ cells, in which there are only a small number of photoreceptors (all R8s), do not slow down the furrow. Perhaps the furrow is only pushed by factors secreted by R8, or possibly there is a redundant mechanism that allows the furrow to progress even in the absence of most photoreceptors.

The only clear requirements for the EGFR in the furrow are for the normal expression of Rough and the correct spacing of R8 cells. The exact role of Rough is not understood but its expression pattern complementary to Atonal, coupled with the effects of its removal or overexpression, have led Dokucu *et al.* [9] to propose that it acts to delimit the number of single Atonal-expressing cells that will become R8s. In *rough*$^-$ mutants, Atonal expression is not restricted to single cells; instead, two to three cells per cluster retain its expression. We sometimes saw a similar phenotype in *EGFR*$^-$ clones, which lack detectable Rough, and there are clearly excess singled-out Atonal-expressing cells. These results are consistent with the proposal that Rough is a repressor of proneural selection and that EGFR activation participates in the singling-out process, although they also imply some redundancy in the system, as most of the R8s in *EGFR*$^-$ clones are single. R8s in *spitz*$^-$ clones are regularly spaced (this work and [23]), so the ligand responsible for activating the receptor in the furrow is unknown.

The dependence of *rough* expression on the EGFR and the role of the receptor in spacing could provide an explanation for the *Ellipse* mutations, a long-standing puzzle. These are gain-of-function EGFR mutations, yet they lead to the determination of too few R8 cells and thus very reduced eyes [17]. They therefore have the opposite effect to that predicted for a receptor with a principal function of recruiting photoreceptors. Our data suggest that ubiquitous overactivity of the receptor in *Ellipse* mutations might cause a failure of R8 determination because of *atonal* repression in the furrow. Consistent with this idea, Atonal expression is never seen in cells expressing the activated EGFR (for example, see Figure 6f,h).

The EGFR and cell survival

Programmed cell death is an important element of tissue patterning and is known to occur in wild-type eye development [30]. In the eye there is very little death until all ommatidial recruitment is complete in the pupa, at which time there is a concerted burst of cell death that removes all undifferentiated cells [27,31]. The EGFR has recently been implicated in promoting survival of cells at this late stage [32]. Our data demonstrate a new role for the receptor in promoting survival of cells much earlier in development. Before the passage of the furrow, loss of the EGFR does not cause many cells to die in the retina, but soon after the furrow has passed, lack of EGFR function causes large amounts of cell death. This is most clearly seen by the dramatic shape changes seen in clones and is supported by the large clusters of TUNEL-positive cells that we found in clones posterior to the furrow. This suggests that once the program of differentiation has been initiated by the furrow, cells die by default unless they have the EGFR. This phenomenon is distinct from the much later cell death seen in many mutants in which ommatidia fail to differentiate. For example, no similar death is seen in *spitz*$^-$ clones, nor in *Ellipse* mutants, in which very few ommatidia form. In both these cases there is cell death, but not until much later, first seen in the posterior of the eye disc.

For non-R8 cells, survival and differentiation are both controlled by the EGFR. The decision to differentiate is controlled by the ligand, Spitz [10,23,33,34], but as there is no early cell death in *spitz*$^-$ clones, it cannot be responsible for the survival function of the receptor. Although there could be an unknown ligand responsible for activating the receptor in cells that need it for survival, it is possible that low levels of ligand-independent signalling might be sufficient.

The phenomenon of a developmental onset of a requirement for EGFR-mediated cell survival is novel and could be an important general mechanism to regulate patterning. We see other regions in the eye–antennal disc complex where excess cell death is triggered by loss of EGFR, suggesting that EGFR-mediated survival also occurs elsewhere. As in the retina, this is not a universal requirement — the cell death only occurs in clones in certain regions, and we speculate that these regions also coincide with domains where differentiation programs are being executed.

The EGFR and the recruitment of photoreceptors

We have shown that activation of the EGFR is sufficient to trigger the neuronal differentiation in cells ahead of the furrow, which are developmentally naive. It has previously been proposed that photoreceptor subtype specification is determined by the developmental history of cells at the time the EGFR is activated [11]. The current work suggests that the basic decision to differentiate neuronally is programmed into a cell very early — at about the time the

morphogenetic furrow initiates at the posterior of the disc. We do not know what triggers the acquisition of this basic competence: possibilities include an intrinsic timing mechanism, intercellular signalling within the disc, or a response to a hormone. The ability of the EGFR to trigger non-R8 photoreceptor determination ahead of the furrow, even in the absence of Atonal, allows us to consider the two phases of photoreceptor determination as being quite separate. This simplifies our view of ommatidial development. The furrow provides a mechanism for determining and spacing R8 cells but does not confer competence on other cells to differentiate in response to EGFR signalling. Once the R8s are correctly determined they become the first source of Spitz that initiates the recruitment of the later photoreceptors from the pool of other cells — all of which are already competent to respond and have no requirement for Atonal.

Conclusions

The EGFR has at least five distinct functions in the developing eye. In temporal order they are as follows. First, it controls cell proliferation. Second, early in development, it is needed at the disc margins. Third, it regulates Rough expression in the furrow, thereby affecting the spacing of R8 cells. Fourth, it is needed to promote survival of cells once the differentiation program has begun behind the furrow; this identifies a new developmental phenomenon. Fifth, it is the necessary and sufficient trigger of recruitment of photoreceptors other than R8. This work elaborates our understanding of eye development in general, and specifically of the role of the EGFR. In at least one way it simplifies the story; the morphogenetic furrow can now be seen as simply a mechanism for establishing the correct R8 spacing pattern. Even before its passage, cells are competent to respond to EGFR activity by differentiating as photoreceptor neurons.

Materials and methods

Drosophila *stocks*

The following alleles were used: $EGFR^{1K35}$, $ras1^{\Delta C40b}$ (gift of N. Perrimon) and spi^{1}[33,34]. $EGFR^{1K35}$ is a null allele which results from a premature termination near the amino terminus of the protein [35]; $hh^{P30}$is a *lacZ* enhancer trap line in the *hedgehog* locus [36]; ato^{3} is a null allele of *atonal* (kindly provided by A. Jarman); the *Df(3R)* p^{13}, *e/TM6B* (from the Bloomington Stock Center) disrupts or deletes the *atonal* locus. Flies of the genotype $ato^{3}/Df(3R)$ p^{13} are semiviable; adults lack all photoreceptors.

Immunohistochemistry

Eye imaginal discs from third instar larvae were stained as described by Gaul *et al.* [37], except that fluorescent secondary antibodies were used. The following antibodies were used: monoclonal rat anti-Elav antiserum [38] (1:100), mouse anti-Cut [39] (1:15), mouse anti-Armadillo [40] (1:100) and mouse anti-Rough [24] (1:15) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa; rabbit anti-β-galactosidase (1:2000, Cappel); mouse anti-β-galactosidase (1:250, Promega); mouse anti-Boss [41] (1:2000, gift of L. Zipursky); rabbit anti-Atonal [42] (1:5000, gift of A. Jarman). Texas Red, FITC and Cy5 conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:500. Rough protein was detected using biotinylated anti-mouse followed by Alexa 594-streptavidin (Molecular Probes). Discs were mounted in Fluoromount G (Southern Biotechnology). Images were collected on a Biorad MRC 1024 confocal microscope.

TUNEL assay

Apoptotic cells were detected using the ApopTag system (ONCOR) according to the manufacturer's directions. Briefly, discs were fixed for 20 min in 4% paraformaldehyde in PBS and then permeabilised with 0.2% Triton X-100 in PBS. No protease digestion was performed. DNA ends were labelled with digoxigenin 11-dUTP for 1 h at 37°C and then detected with FITC-conjugated anti-digoxigenin.

Generation of mitotic clones

Mitotic recombination was induced using the FRT–FLP technique [16] in combination with the *Minute* technique [21]. The genotype of the larvae in Figures 1, 2a–c, 3a–c and 5a–c are *y w hsp70-flp;* FRT^{42D} $EGFR^{1K35}/FRT^{42D}$*arm-lacZ, M(2)53* (the *FRT M(2)* chromosome was the gift of Roger Phillips). In these experiments the homozygous mutant tissue is marked by the absence of β-galactosidase staining. The genotype of the disc shown in Figure 3d is *y w hsp70-flp;* FRT^{42D}*hsp70-πMyc/*FRT^{42D}*arm-lacZ, M(2)53.* The genotype of the disc shown in Figure 2d is *y w hsp70-flp;* spi^{1} FRT^{40A}*/M(2)24F αtub-nuclear-lacZ* FRT^{40A}. The α-tub–nuclear lacZ construct is described in Harrison and Perrimon [43] and was transposed from cytological position 67B,C to 28 as described [44]. In all cases FLPase activity was induced with a 1 h heat shock at 38°C. Clones were induced in first or early second instar larvae.

Ectopic expression of activated EGFR

To activate the EGFR pathway ectopically, we placed an activated form of the receptor (tor^{4021}*-EGFR*) downstream of the GAL4–UAS sequence (R.J. Howes, personal communication; [28,45]). Using flies containing an actin<FRT<y+<FRT<Gal4 transgene [46] onto which either UAS–nuclear lacZ, UAS–CD2 or UAS–GFP had been recombined we induced marked clones of cells ectopically expressing the activated receptor. Ectopic clones were induced for 20 min at 38°C, 2–4 days after egg laying (Figure 6a–d,g,h). Small clones (Figure 6e,f) were induced 12–24 h before dissection.

Rescue of photoreceptor determination in atonal⁻ eyes

Flies of the genotype omb–Gal4; ato^{3}/TM6B were crossed to $UAS–EGFR^{act}$ *UAS–lacZ/CyO; Df(3R)* p^{13}, *e/TM6B*. Tb^{+} (thus $ato^{3}/Df(3R)p^{13}$), were stained with anti-β-galactosidase to identify larvae carrying the $UAS–EGFR^{act}$ *UAS–lacZ* chromosome, and double-stained with anti-Elav, anti-Boss or anti-Cut antibodies to assess cell fates in the mutant eyes.

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