Role of the EGFR/Ras/Raf pathway in specification of photoreceptor cells in the *Drosophila* retina

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SUMMARY

The *Drosophila* EGF receptor is required for differentiation of many cell types during eye development. We have used mosaic analysis with definitive null mutations to analyze the effects of complete absence of EGFR, Ras or Raf proteins during eye development. The *Egfr*, *ras* and *raf* genes are each found to be essential for recruitment of R1-R7 cells. In addition *Egfr* is autonomously required for MAP kinase activation. EGFR is not essential for R8 cell specification, either alone or redundantly with any other receptor that acts through Ras or Raf, or by activating MAP kinase. As with *Egfr*, loss of *ras* or *raf* perturbs the spacing and arrangement of R8 precursor cells. R8 cell spacing is not affected by loss of *argos* in posteriorly juxtaposed cells, which rules out a model in which EGFR acts through *argos* expression to position R8 specification in register between adjacent columns of ommatidia. The R8 spacing role of the EGFR was partially affected by simultaneous deletion of *spitz* and *vein*, two ligand genes, but the data suggest that EGFR activation independent of *spitz* and *vein* is also involved. The results prove that R8 photoreceptors are specified and positioned by distinct mechanisms from photoreceptors R1-R7.

Key words: EGF receptor, Ras, Raf, *Drosophila*, eye, Photoreceptor cell

INTRODUCTION

Receptor tyrosine kinases have important roles in the specification of cell fates during development, as has been revealed in part from studies of the developing compound eye of *Drosophila*. Receptors act singly or in combination to specify retinal cell fates, including those of the R1, R2, R3, R4, R5, R6 and R7 photoreceptor cells (Freeman, 1997; Simon, 2000; Zipursky and Rubin, 1994). Downstream of receptors, a cascade of Ras/Raf/mitogen-activated protein (MAP) kinase activation has been identified (Hafen et al., 1994; Wassarman et al., 1995). Downstream signaling has been much studied using genetic enhancement and suppression assays because Ras, Raf and MAP kinase are thought to function in many events prior to cell fate specification. For example, cells that lack YAN, an ETS-domain transcription factor that is one of the MAP kinase targets, have altered proliferation and fail to enter any retinal pathway (Rogge et al., 1995). The role of receptor tyrosine kinases in R8 photoreceptor specification is still not fully understood. We have used mosaic analysis to look directly at early R8 differentiation in cells that lack the EGFR receptor or downstream effectors.

Each unit of the compound eye, also known as an ommatidium, is constructed by local cell interactions within the eye imaginal disc, an epithelium that differentiates into the eye at metamorphosis. Each ommatidium is founded by initial specification of a single cell, the precursor of the R8 photoreceptor cell, which is required for subsequent induction of the other 18 precursor cells for each ommatidium (Jarman et al., 1994; Tomlinson and Ready, 1987). The best understood induction is that of the R7 photoreceptor cell, which requires activation of two receptor tyrosine kinases. Sevenless is activated in a cell neighboring R8 by the transmembrane protein BOSS expressed on the R8 cell surface. Sevenless signals through consecutive activation of Ras, Raf and Rolled/MAP kinase to release barriers to differentiation in the nucleus of the R7 cell precursor (Hafen et al., 1994; Li et al., 1997; Tang et al., 1997; Wassarman et al., 1995). The equivalence group of cells competent to respond to Sevenless activation is establised in part by prior activation of another kinase, the EGFR receptor (Xu et al., 2000).

In most ommatidial cells, except for R7, the EGFR receptor plays the final role in triggering differentiation, acting through a similar signal transduction pathway to Sevenless (Freeman, 1997). R8 activates the EGFR agonist Spitz (Spi) by expressing *rhomboid*, which processes Spi from an inactive precursor, so activating EGFR in neighboring cells (Freeman, 1997). Successive episodes of EGFR activation by Spi are thought to recruit most or all of the remaining cell types to the ommatidium (Dominguez et al., 1998; Freeman, 1996; Kumar et al., 1998; Tio and Moses, 1997). The spatial extent of activation is thought to be limited at each step by secretion of an EGFR antagonist, Argos (Freeman, 1997; Jin et al., 2000). Another EGFR ligand gene, *vein* (*vn*), is expressed in the eye but ommatidia can develop normally in the absence of *vn* function (Spencer et al., 1998; Lesokhin et al., 1999). Competence to respond to EGFR activation and the specificity of response are thought to depend on signals from other, non-RTK pathways (Flores et al., 2000; Simon, 2000).
The specification of the founding R8 photoreceptor cells depends on the proneural bHLH protein encoded by atonal (ato; Jarman et al., 1994). Expression of ato begins in a continuous stripe ahead of the morphogenetic furrow in response to Hedgehog (Hh) and Decapentaplegic (Dpp) proteins diffusing from the more posterior, differentiating portion of the eye disk (Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000; Fig. 1). The first patterning occurs as initial ato expression is replaced by autoregulatory ato expression only in proneural intermediate groups of up to 10 cells (Jarman et al., 1995; Sun et al., 1998). Lateral inhibition by the Notch pathway then reduces each intermediate group to a single atonal-expressing R8 precursor cell when each new column 0 is defined (Baker et al., 1996). Intermediate groups are regularly spaced out-of-phase with the single R8 cells just to the posterior in column 0, indicating that each column acts as a template for positioning development of the next (Fig. 1; Baker et al., 1990). Ato autoregulates and defines cells as R8 precursors in conjunction with its bHLH

intermediate group cells and R8 precursors, precisely overlapping functional ato expression. Senseless expression is maintained in the differentiating R8 photoreceptor cells, although ato expression fades posterior to column 3.

The role of receptor tyrosine kinases in specifying the founding R8 cells of each ommatidium is unclear. A point mutant allele of the EGFR called Ellipse prevents formation of intermediate group or R8 cells, suggesting a role for Egfr in ato expression and R8 specification (Lesokhin et al., 1999). Interestingly, Egfr activity is required for ato expression in chordotonal organs, another part of the peripheral nervous system where ato is required (Lage et al., 1997; Okabe and Okano, 1997). Nevertheless several groups have concluded that EGFR activity is not required for R8 specification. R8 specification occurs normally when EGFR function is reduced using a temperature-sensitive allele, even though recruitment of other ommatidial cells fails (Kumar et al., 1998). In addition, ato expression and R8 specification occur in clones of cells homozygous for apparent null mutations of EGFR, although R8 spacing is aberrant (Dominguez et al., 1998; Lesokhin et al., 1999). These studies lead to the view that EGFR is not essential for specification of R8 cells, although involved in an aspect of R8 spacing and essential for subsequent recruitment of ommatidial cells after R8.

By contrast, others concluded that EGFR was essential for R8 specification. This conclusion was based on failure to specify R8s after expression of dominant-negative EGFR, after ectopic expression of Argos, and in clones of cells lacking both vn and rhomboid. In addition, ectopic activation of the EGFR pathway by several methods was reported to enhance Ato expression and promote ectopic R8 specification. A viable argos mutant genotype, aos+sp1, differentiated occasional ectopic R8 cells at positions intermediate between the normal ones, Spencer et al. posited a model in which R8 specification is initiated by EGFR activation, either by an unknown ligand or independent of ligands, to establish the atonal-expressing proneural groups (Spencer et al., 1998). Selection of single R8 cells by lateral inhibition within proneural groups is followed by expression of vn and activation of Spi in the single R8 precursor cell. The resulting local EGFR activation maintains Atonal expression in the proneural groups, is required for maintenance of R8 fate by the single R8 cells and also leads to Argos expression in the proneural groups. Argos creates an ‘exclusion zone’ within which further EGFR activation cannot occur, and contributes to the spacing of future columns of ommatidia by ensuring that EGFR activation to initiate future proneural groups occurs in the niches between Argos-secreting groups of the previously established row (Spencer et al., 1998).

How can these views be reconciled? One possibility is that one set of experiments or another has failed to eliminate EGFR activity. Another possibility is that there might be a third receptor tyrosine kinase in the Drosophila eye, acting redundantly with EGFR in R8 specification, somewhat like the dual role for EGFR and Sevenless in R7. If dominant-negative EGFR and Argos inhibit function of both the EGFR and the third kinase, they might reveal the redundant role for the two receptors. By contrast analysis of clones of cells mutant for the EGFR alone would not reveal functions performed redundantly by the putative other kinase.

Two pieces of experimental evidence support the theory of another receptor redundant with EGFR. First, although MAP kinase phosphorylation was reported to disappear rapidly following inhibition of EGFR function using a temperature-

Fig. 1. Aspects of eye disc development (Wolff and Ready, 1993). In this and the following figures, late third instar eye discs are shown with anterior towards the left. (A) Differentiation of the eye imaginal disc begins at the posterior margin (right) and progressively spreads anteriorly (left), one new column of ommatidia initiating every 90-120 minutes. (B) The proneural gene atonal is expressed by all cells in a stripe anterior to any differentiation (boxed area on the left). Expression is lost from some cells but maintained by autoregulation in regularly spaced ‘intermediate groups’ comprising ~10 cells each (gray nuclei). Within these groups, one cell will be specified as R8 (black), maintain ato expression longest, differentiate and recruit other photoreceptor cells (circles) by expressing ligands for the EGFR and BOSS receptor tyrosine kinases. In wild-type development the first column where R8 is the only cell retaining ato expression corresponds to column 0 in the nomenclature of Wolff and Ready (Baker and Zitron, 1995; Jarman et al., 1995; Wolff and Ready, 1993). The alternating phasing of the ommatidial columns implies that each column is an inhibitory template for the next. The spacing factor has variously been proposed to be scabrous (Baker and Zitron, 1995), argos (Spencer et al., 1998), hedgehog (Dominguez, 1999), or an unidentified factor expressed in response to MAP kinase activity (Chen and Chien, 1999).
sensitive allele, phosphorylated MAP kinase reappeared after 2 hours without EGFR activity. This suggests that a homeostatic mechanism, perhaps involving another receptor tyrosine kinase, can compensate for loss of EGFR function (Kumar et al., 1998). Second, it has been suggested that Raf function is required for ato expression (Greenwood and Struhl, 1999). As the Ras/Raf pathway acts downstream of many receptor tyrosine kinases, this is consistent with the notion that R8 specification requires Ras and Raf activation, but that EGFR is not the only receptor that can activate this pathway.

MATERIALS AND METHODS

Drosophila strains employed

top18A (Price et al., 1989); ras1∆40B (Hou et al., 1995); spsCl (Tio and Moses, 1997); vt5 (Schnepp et al., 1996); aosΔ5, aosΔ31 (Freeman et al., 1992); raf1-29 (Melnick et al., 1993); [armlacZ] transformants (Vincent et al., 1994) and [eyFLP] transformants (Newsome et al., 2000).

Generation of mutant clones

Clones of homozygous mutant cells were obtained using the FRT-FLP technique (Golic, 1991; Xu and Rubin, 1993). EGFR clones and Ras clones were generated using Minute technique to compensate for their inherent growth deficiencies (Morata and Ripoll, 1975). The genotype of the larvae shown in Fig. 2 is hsFLP; FRT42 top18A/FRT42 [armlacZ] M(3)S6i. The genotype shown in Fig. 3 is hsFLP; FRT82 ras1∆40B/FRT82 [armlacZ] M. For these Minute genotypes, heat shock (1 hour, 37°C) was given between 48 and 72 h after egg laying, at 25°C. The genotype of disc shown in Figs. 4 and 5 is raf1-29 FRT18A/ [armlacZ] FRT18A; hsFLP38. The genotype shown in Fig. 6 is hsFlp122; argosΔ FRT80B[armlacZ] FRT80B. For these genotypes, heat shock of larvae was given between 24 and 48 hours after egg laying. The genotype shown in Fig. 5 is [eyFLP]; spsCl FRT40/ [armlacZ] FRT40; vt5 FRT80B[armlacZ] FRT80B.

Antibodies

Antibody reactions were performed as described (Lesokhin et al., 1999). Monoclonal antibodies specific for β-galactosidase (mAb40-1a) and Elav (rat mAb7E8A1A0) were obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, Department of Biological Sciences, Iowa City IA52242, USA under contract N01-HD-7-2363 from the NICHD. Other antisera were rabbit anti-Ato (Jarman et al., 1995), rabbit anti-Boss (Kramer et al., 1991), guinea pig anti-Senseless (Nolo et al., 2000), anti-E(spl)m6 (mAb174; Jennings et al., 1994) and monoclonal mouse anti-dpERK (Sigma). Cy2-, Cy3- and Cy5-conjugated secondary antisera were from Jackson Immunoresearch. Confocal microscopy was performed using a Biorad MRC600 instrument.

RESULTS

Loss of the EGFR

Several groups have described eye development in clones of cells mutant for the EGFR (Domínguez et al., 1998; Lesokhin et al., 1999). Each has reported similar results, and concluded that R8 specification occurs in EGFR mutant clones, although the number and spacing of R8 cells differ from the wild-type pattern. The EGFR mutant alleles used are thought to lack all EGFR function based on genetic criteria, and top18A corresponds to a premature termination codon in the extracellular domain (Clifford and Schupbach, 1994). It is noteworthy, however, that three chain termination mutations in the EGFR extracellular domain each have different phenotypes, emphasizing the possibility that genetic background, translational readthrough or internal initiation, undetected alternative splicing or cryptic transcription from internal promoters might conceivably lead to expression of some form of EGFR protein from these alleles (Clifford and Schupbach, 1994). To eliminate such possibilities, we have examined clones of cells mutated for top18A, in which the entire EGFR open reading frame is deleted and absent from the genome (Price et al., 1989). Such cells cannot synthesize any form of EGFR protein.

As shown in Fig. 2, top18A homozygous cells develop as has been described previously for putative null alleles. Some Egfr-positive cells expressed the neural marker Elav but more weakly than wild-type cells. The neuronal cells were also reduced in number, and the number did not increase posterior to the furrow as occurs during normal recruitment. There appeared to be a delay in differentiation within Egfr clones (Fig. 2A-C).

In wild type, expression of the Senseless protein resolves to single R8 cells and reports Atonal activity (Nolo et al., 2000). In top18A clones Senseless expression was delayed, and was both initiated and maintained in more cells that in wild type (Fig. 2D-F). After several columns of multiple Senseless-expressing cells, the protein disappeared more posteriorly. Labeling the same specimens showed that all Elav-positive cells also expressed Senseless (Fig. 2G-J).

We could not detect MAPK activation in top18A clones (Fig. 1K-L). As spacing defects in R8 specification suggest loss of Notch signaling, expression of the N-responsive E(spl) genes was also examined. E(spl) expression was reduced in top18A clones. Loss of E(spl) expression was more complete in the center of the clones (Fig. 2M-O).

The development of the top18A clones was consistent with previous descriptions of other Egfr alleles (Domínguez et al., 1998; Lesokhin et al., 1999). Specification of an abnormal pattern of R8 precursor cells, often twinned, was not followed by recruitment of any other neuronal cell types, and posterior to the furrow the R8 cells disappear. The weak Elav expression and loss of Senseless expression presumably reflect incipient cell death, as they are not seen if baculovirus p35 is expressed in the eye disc (data not shown).

Others have reported that inhibition of EGFR activity using a temperature-sensitive allele led rapidly to loss of MAPK activation, as revealed by loss of dpERK staining (Kumar et al., 1998). However, dpERK staining reappeared within 2 hours, indicating compensation by and EGFR-independent pathway of MAPK activation. By contrast, dpERK staining was permanently lost from EGFR mutant clones (Fig. 2K-L). Our data show that compensation is not an autonomous response of eye disc cells to loss of EGFR function. Thus, R8 specification in top18A mutant clones cannot be attributed to this putative compensating pathway, and indeed dpERK levels were very low in top18A mutant clones.

Loss of Ras

The ras1∆40B allele corresponds to deletion of the Ras open reading frame (Hou et al., 1995). Loss of Ras function had similar effects on photoreceptor differentiation to loss of EGFR function. A few cells begin to differentiate and express Elav, albeit weakly compared with wild-type cells, and no
additional photoreceptors were recruited (Fig. 3A-C). The pattern of Atonal expression was used to assess R8 specification. In many ras clones the pattern of Atonal expression resembled that described for Egfr mutant clones (Dominguez et al., 1998; Lesokhin et al., 1999). The intermediate group pattern was abnormal and resolved incompletely, leading to specification of multiple R8 cells (Fig. 3D-F). Some ras clones were very large; these showed nonautonomous delay and loss of atonal expression in the central portions of the clone, when ras mutant territory
extended posterior to the morphogenetic furrow (Fig. 3G-I). Levels of Atonal expression were maintained in clones where cells posterior to the furrow were wild type (Fig. 3D-F). The pattern of R8 specification was also assessed by Senseless expression. There was a nonautonomous delay in Senseless expression, and the pattern of R8 cells was aberrant (Fig. 3J,K). After a few columns, Senseless protein disappeared and multiple R8 cells specified in an aberrant pattern (arrows). Senseless expression in raf clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappeared in the raf mutant clones. Elav-positive, Senseless-positive cells disappear...
cells normally but no further ommatidial cell types (Tio and Moses, 1997). Clones of cells mutant for both \( \text{spi} \) and \( \text{vn} \) developed similarly to \( \text{spi} \) mutant cells, but occasionally R8 spacing was affected so that twinned R8 cells were seen. The R8 spacing defect was much less prevalent than in \( \text{Egfr} \), \( \text{ras} \) or \( \text{raf} \) clones.

**Loss of \( \text{argos} \)**

The major effect described for most viable \( \text{argos} \) mutations is additional photoreceptor recruitment (Freeman et al., 1992). Disordered ommatidia in \( \text{aos}^{slP1} \) (Okano et al., 1992; Spencer et al., 1998) are consistent with ectopic R8 specification, although changes in proliferation, survival, or ommatidial rotation associated with changed numbers of R1-R7 cells might also be responsible. Null alleles of \( \text{argos} \) are embryonic lethal when homozygous and their effect on eye development has not been examined before.

We generated mosaic clones of cells homozygous for \( \text{aos}^{D7} \), caused by deletion of the start site for translation (Freeman et al., 1992). The pattern of Atonal expression was completely normal in many \( \text{aos}^{D7} \) clones, suggesting no role in R8 specification or spacing (Fig. 6A-C). Importantly, for the possible function of \( \text{argos} \) in spacing intermediate groups, loss of \( \text{argos} \) function from several ommatidia did not perturb the positioning of more anterior intermediate groups, which still arose equidistant from the ommatidia to the posterior that lacked \( \text{argos} \) function. A different result was seen in larger clones, \( \geq 10 \) ommatidia wide (Fig. 6D-F). The periodic spacing of intermediate groups was lost and the pattern of R8 specification disrupted. This confirmed that \( \text{argos} \) has a role in patterning R8 specification, but nonautonomy indicated that this role can be performed by Argos protein diffusing over a distance of several ommatidia, and does not communicate the position of ommatidia that are only in the next column.

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**Fig. 5.** \( \text{spi} \) \( \text{vn} \) double mutant clones. The \( \text{spi} \) \( \text{vn} \) double-mutant cells lacked \( \beta \)-galactosidase expression (red). Wild-type cells, or cells singly mutant for either \( \text{spi} \) or \( \text{vn} \), express \( \beta \)-galactosidase expression at various levels. (A,B) R8 cell specification (revealed by Senseless expression in green) occurred in \( \text{spi} \) \( \text{vn} \) mutant cells. There were occasional abnormalities in R8 spacing (arrows). (C-F) Elav expression (green) and Senseless expression (blue). All the Elav-positive neurons in \( \text{spi} \) \( \text{vn} \) clones also expressed the R8 marker Senseless. (G-J) Elav expression (green) and Boss expression (blue). All the Elav-positive neurons in \( \text{spi} \) \( \text{vn} \) clones also expressed the R8 marker Boss. Examples of twinned R8 cells are shown by arrows.

**Fig. 6.** Eye discs with \( \text{argos} \) clones (lack of red \( \beta \)-galactosidase staining). Green, Ato expression. (A-C) Null \( \text{argos} \) clones (\( \text{aos}^{D7} \)) including several ommatidia show no defects whatsoever in R8 precursor specification. Positioning of R8 precursors was unaffected by having \( \text{argos} \) mutant cells in posterior columns, demonstrating that \( \text{argos} \) was not required for positioning R8 specification with respect to posteriorly neighboring ommatidia. (D-F) A larger \( \text{argos} \) mutant clone showing aberrant \( \text{ato} \) pattern and R8 spacing in the clone interior (affected area between the pink markers). Clones of another \( \text{argos} \) allele, \( \text{aos}^{A5} \), associated with deletion of 5' untranslated sequences, showed normal R8 specification regardless of size, and we found that the lethality of this chromosome could be separated from a viable \( \text{argos} \) phenotype by meiotic recombination (data not shown).
DISCUSSION

It is thought that EGFR activity is required for recruiting R1-R7 photoreceptor cells to ommatidia, probably through Ras, Raf and MAPK but the role of this pathway in R8 specification has been less clear. Loss-of-function studies with putative \textit{Egfr} null clones or temperature sensitivity have suggested that EGFR is dispensable for R8 specification (although involved in R8 spacing; Dominguez et al., 1998; Kumar et al., 1998; Lesokhin et al., 1999); studies with dominant negative approaches have suggested that EGFR is essential for R8 specification (Spencer et al., 1998). There is also a particular class of \textit{Egfr} mutants, the \textit{Elp} alleles, that prevent R8 specification (Lesokhin et al., 1999), and there is evidence that R8 specification might depend on EGFR-independent Raf activation (Greenwood and Struhl, 1999). We have undertaken a study of null mutations in the EGFR/Ras/Raf pathway to resolve some of these issues.

Two prior studies of \textit{Egfr} mutant clones used the genetically amorphic point mutations \textit{flb1K35} and \textit{topCO} (Dominguez et al., 1998; Lesokhin et al., 1999). For \textit{topCO}, the molecular defect is unknown; \textit{flb1K35} corresponds to Gln267 in Ochre, which truncates the EGFR early in the extracellular domain (Clifford and Schupbach, 1994). Although it is a reasonable assumption that these are both null alleles, it is worth noting that another mutation encoding Gln430 in Amber (\textit{top}^{K8}) retains significant function, so the possibility of residual function in \textit{topCO} or \textit{top1K35} caused by readthrough, translational reinitiation or other mechanisms cannot be completely excluded (Clifford and Schupbach, 1994). These possibilities can be excluded, however, for the allele \textit{top}^{K8}, which deletes all EGFR-coding sequences from the genome (Price et al., 1989). We have found the phenotype of \textit{top}^{K8} clones to be similar to \textit{flb1K35} and \textit{topCO}. Greenwood and Struhl have also reported \textit{ato} expression in \textit{top}^{K8} clones (Greenwood and Struhl, 1999). We conclude that cells completely lacking EGFR-coding capacity can still differentiate R8 photoreceptor cells, although their patterning is abnormal and they later die. Cells that completely lack EGFR are not recruited as any other photoreceptor type.

By the late third instar, cells in mutant clones have lacked \textit{Egfr} gene function for approximately 120 hours. It is possible that cells might have a homeostatic mechanism (such as upregulation of another receptor) that compensates for sustained absence of \textit{Egfr} function, and that some processes that would be \textit{Egfr}-dependent in normal eye cells have been rescued in the clones. There is experimental evidence for such homeostasis from studies of the \textit{Egfr}^{ts2} allele. When EGFR function is interrupted, MAP kinase activation is lost from eye discs within 30 minutes, but levels of activated MAP kinase rebound within a few hours, even in the continued absence of \textit{Egfr} function (Kumar et al., 1998).

We examined MAP kinase activation within clones of \textit{Egfr} mutant cells. MAP kinase activation was undetectable. Thus, specification of R8 cells in \textit{Egfr} mutant clones is not associated with MAP kinase reactivation via an alternative pathway. This finding indicates that the restored dpERK staining seen in the \textit{Egfr}^{ts2} allele must depend nonautonomously on loss of \textit{Egfr} function in other cells. For example, loss of EGFR function from the whole animal may lead to changes in humoral signals that nonautonomously affect MAPK by some mechanism.

Genetic studies suggest that specification of most ommatidial cells depends on activation of Ras and Raf by EGFR (or by EGFR and Sevenless in the case of R7; Hafen et al., 1994; Zipursky and Rubin, 1994; Wassarman et al., 1995). R8 cell specification in the absence of \textit{Egfr} might indicate activation of Ras and Raf by another receptor. We have examined clones of cells null for \textit{Egfr} or \textit{Raf} to test this.

We found the null phenotype of Ras closely resembled that of EGFR. Ato expression initiated normally but patterning was affected and more cells than normal retained atonal expression posterior to the furrow. R8 cells were specified and expressed the R8 protein Senseless. No other Elav-expressing photoreceptor cells were recruited.

The phenotype of clones mutant for \textit{raf} was similar. R8 cell specification began relatively normally, as indicated by onset of Ato and Senseless expression. R8 cell precursors were improperly spaced, however. More posteriorly, \textit{raf} mutant R8 cells expressed the neural protein Elav only transiently.

These results also confirm directly that Ras and Raf are required for R1-R7 recruitment, and show that after these clones are induced in the first larval instar, Ras and Raf play no essential roles in the proliferation, survival or maintenance of eye disc identity of most eye disc cells.

As null clones for \textit{Egfr}, \textit{ras}, and \textit{raf} each permit R8 specification, although affecting R8 spacing, we conclude that R8 specification can occur independently of EGFR, and is also independent of any other receptor that acts through Ras and Raf. Although we have not tested the requirement for MAP kinase directly (as the MAP kinase gene \textit{rolled} maps proximal to all extant flip recombination target (FRT) sites), we found that MAP kinase activation was undetectable in \textit{Egfr-null} clones.

Is there any way that deletions of the \textit{Egfr} gene might still permit signal transduction? One might argue that if, in the absence of ligand, EGFR normally associates with signal transduction components in readiness for signaling, then deleting the \textit{Egfr} gene might release such components, permitting a higher baseline of ligand-independent signaling than if EGFR protein were present. Our results make this unlikely because the similar mutant phenotypes of \textit{Egfr}, \textit{ras} and \textit{raf} mean that each of the \textit{Egfr}, \textit{Ras} and \textit{Raf} proteins would have to be necessary to sequester this component, but there is biochemical evidence that Ras and Raf are not associated in the absence of extracellular ligands (Hallberg et al., 1994).

For both \textit{Egfr} and \textit{ras}, there was a nonautonomous delay of morphogenetic furrow movement and loss of \textit{ato}, especially in large clones with substantial areas of mutant cells posterior to the furrow. This suggests \textit{Egfr} and \textit{ras} are required for expression of factors that push the morphogenetic furrow across the eye disc. Two such factors are Hh and Dpp (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999). Hh is reported to be expressed by photoreceptor cells (Ma et al., 1993); therefore, fewer cells are expected to express Hh in \textit{ras} or \textit{Egfr} clones.

There were some differences between clones mutant for \textit{raf} and \textit{ras} clones mutant for \textit{Egfr}. Less Elav was detected in \textit{raf} mutant cells. In \textit{Egfr} or \textit{ras} mutant clones, Elav protein is detected in the mutant R8 cells, although at lower levels than in nearby wild type cells. In \textit{Egfr} mutant clones, normal levels of Elav protein are restored by expression of baculovirus p35, indicating that low Elav levels reflect commitment of \textit{Egfr} mutant cells to apoptosis (data not shown). It is possible that Elav was lost more rapidly in \textit{raf} mutant cells because of more rapid apoptosis than \textit{Egfr} or \textit{ras} mutant clones. Delayed furrow progression was not seen in \textit{raf} mutant clones, but this may be because they were too small.
The differences between raf clones and Egfr or ras clones could indicate ras-independent signaling to raf, as has been proposed to occur during the determination of the embryonic termini (Hou et al., 1995). Such signaling to permit Elav expression in more R8 precursor cells (or preserve R8 precursor cells from apoptosis for longer) would have to be independent of Egfr as well, whereas all raf activity in the embryonic termini is dependent on torso, the relevant receptor (Hou et al., 1995). An alternative explanation is that these apparent differences relate to the much smaller size of raf clones compared with Egfr and ras clones. For the autosomal Egfr and ras mutations the Minute technique was used to compensate for the growth disadvantage of the homozygous cells. This is not readily possible for the X-linked raf mutation. As a consequence, the raf clones examined were much smaller than the Egfr and ras clones, and grew at a reduced rate relative to neighboring wild-type cells. In the similar situation of Minute heterozygous clones growing slowly in wild-type backgrounds, nonautonomous interactions have been demonstrated, prolonging the cell doubling time of the slow-growing M/+ cells, and accelerating the doubling time of neighboring wild-type cells (Morata and Ripoll, 1975; Simpson and Morata, 1981). If changes in cellular properties are also induced by the differential growth of neighboring homozygous raf mutant and wild-type cells, it is possible that faster loss of Elav might not indicate additional roles for raf in differentiation or survival, but an indirect effect of competition by the nearby wild-type cells on the raf cells. At present, experimental evidence to distinguish these models is not available.

Our finding that Ato expression and R8 specification occur in the absence of raf differs from another study that reported raf to be required for atonal expression (Greenwood and Struhl, 1999). It is possible that the inferior clonal marker used in those studies hampered precise delineation of raf clone boundaries.

The common requirements for Egfr, ras and raf in R8 spacing are not shown by null mutations in spi, an EGFR ligand required for recruitment of R1-R7 (Tio and Moses, 1997). It was possible that spi was required redundantly with vn, another ligand with no essential role in ommatidium development. We have found that R8 precursor specification occurs in clones doubly mutant for both spi and vn. R8 spacing occurs almost normally, although there are rare cases of multiple R8 cells like those that occur more frequently in Egfr mutant clones. This raises the possibility that spi and vn do have redundant roles in R8 precursor spacing, but that if this is so, there must be another ligand, or ligand-independent process, that is also involved. Recently Wasserman et al. reported that the Drosophila genome sequence predicts another Spi-like protein (Wasserman et al., 2000). They further reported that cells doubly mutant for two putative ligand processing molecules encoded by rhomboid and roughoid resemble cells mutant for the EGFR. This suggests that rhomboid and roughoid redundantly process spi and spi-like, which act redundantly on EGFR in R8 spacing (Wasserman et al., 2000). The spi, spi-like double- and spi, spi-like, vn triple-mutant combinations that would directly test the relative contributions of all three ligands have yet to be examined.

The inhibitory ligand argos is also required nonautonomously for R8 spacing. It had been suggested that argos could diffuse from proneural intermediate groups, where it is expressed in response to EGFR activation, creating an ‘exclusion zone’ for further EGFR activation that will position future intermediate groups precisely out of phase (Spencer et al., 1998). We found, however, that argos function can be performed by protein secreted several ommatidia away, which questions whether argos conveys precise spatial information. Crucially, proneural intermediate groups are positioned normally even if immediately posterior regions are null mutant for argos, refuting the ‘exclusion zone’ model for argos action. Larger argos clones did affect R8 spacing distant from the clone boundary, suggesting that argos may be globally necessary in an unpatterned way to keep EGFR activity in check. An alternative is that argos is required indirectly through its effect on photoreceptor differentiation. Accordingly, ectopic photoreceptor cells in argos mutant territories might alter the expression of furrow progression signals such as Dpp and Hh.

Our main result is that R8 precursor specification occurs in cells null for Egfr, ras or raf. This is consistent with the proposed EGFR/Ras/Raf pathway of recruitment for photoreceptors R1-R7. Our results appear definitively to exclude essential roles for Egfr, ras, raf, spi or vn, in R8 specification (although they support roles in R8 spacing), and show that argos is dispensable for the proposed signaling by each pair of proneural intermediate groups that positions R8 specification in the next most anterior column. We think that R8 specification instead relies on autoregulatory transcription of the proneural ato gene promoted by two other DNA-binding proteins, daughterless and senseless that can occur without EGFR signaling. Defects in arrangement of R8 cell precursors show that the EGFR/Ras/Raf pathway nevertheless plays a role in patterning of R8 cells. The increased number of R8 cells in mutants indicates that EGFR normally activates Ras and Raf to suppress R8 specification in certain locations. The EGFR pathway might modulate Notch. The Egfr requirement for R8 spacing was found to be more autonomous than the Egfr requirement for E(spl) expression, however, raising the possibility of another target (Fig. 2). One candidate is the homeobox gene rough (Domínguez et al., 1998).

Our results do not support any redundant receptor acting in parallel with EGFR that could be affected when EGFR function was reduced by ectopic expression of dominant-negative EGFR, ectopic expression of argos, or in the Ellipse point mutant alleles of the EGFR (Lesokhin et al., 1999; Spencer et al., 1998). Our results rule out homeostatic compensation for EGFR loss by another related receptor. Instead it is possible that when experimental conditions perturb but do not eliminate EGFR function, R8 specification can be suppressed more generally than usual without achieving other normal EGFR outputs, such as specification of R1-R7 cells.

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