Development 125, 3875-3885 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5225

Dissecting the roles of the *Drosophila* EGF receptor in eye development and MAP kinase activation

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Accepted 16 July; published on WWW 7 September 1998

SUMMARY

A new conditional *Egfr* allele was used to dissect the roles of the receptor in eye development and to test two published models. EGFR function is necessary for morphogenetic furrow initiation, is not required for establishment of the founder R8 cell in each ommatidium, but is necessary to maintain its differentiated state. EGFR is required subsequently for recruitment of all other neuronal cells. The initial EGFR-dependent MAP kinase activation occurs in the furrow, but the active kinase (dp-ERK) is observed only in the cytoplasm for over 2 hours. Similarly, SEVENLESS-dependent activation results in cytoplasmic appearance of dp-ERK for 6 hours. These results suggest an additional regulated step in this pathway and we discuss models for this.

Key words: EGFR, MAP kinase, ERK, *Drosophila*, Retina, RTK, Ommatidium

INTRODUCTION

Receptor tyrosine kinases (RTKs) function in many eukaryotic signal transduction pathways to regulate the cell division cycle, cell fate, cell death, cell motility, axon guidance, neural crest migration and immune cell function (reviewed in Pawson and Bernstein, 1990; Ullrich and Schlessinger, 1990; Greenwald and Rubin, 1992; Perrimon, 1994; Burden and Yarden, 1997; Edery et al., 1997; Orioli and Klein, 1997). Like other RTKs, members of the EGF receptor family act through a pathway of cytoplasmic factors that includes the GTP-binding protein RAS, protein kinase cascades and other regulatory proteins (reviewed in McCormick, 1993; Perrimon, 1994; Schlessinger and Bar-Sagi, 1994). One of the best studied cytoplasmic cascades consists of a sequential activation of Raf1, MEK and the Mitogen-Activated Protein Kinase (MAPK), also known as ERK (extracellular signal regulated kinase, reviewed by Seger and Krebs, 1995). Inactive ERK is held in the cytoplasm probably by association with either cytoskeletal microtubules (Reszka et al., 1995), MEK (Fukuda et al., 1997) or both. When an activating signal passes down the pathway, MEK phosphorylates ERK, which then translocates to the nucleus where it can act on transcription factors. Nuclear translocation of active MAPK is an extremely rapid process (Chen et al., 1992). Targets outside the nucleus such as the EGF Receptor itself have also been identified (Northwood et al., 1991; Gonzalez et al., 1993). Indeed, the nuclear localization of many other proteins is regulated by phosphorylation (reviewed Jans and Hubner, 1996).

A single EGFR homologue has been identified in *Drosophila* (Livneh et al., 1985). Three different classes of mutations correspond to defects in this gene: maternal effect oogenesis mutations ($Egfr^{top}$), zygotic lethals ($Egfr^{flb}$) and homozygous viable, dominant rough eye mutations ($Egfr^{Elp}$, reviewed by Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). Three activating ligands are known: GURKEN acts only in oogenesis (Neuman-Silberberg and Schüpbach, 1993). SPITZ acts at several phases in the embryo (e.g. to regulate development of the ventral ectoderm, chordotonal organs and segment polarity and later in the developing eye and other imaginal tissues (Mayer and Nüsslein-Volhard, 1988; Rutledge et al., 1992; Schweitzer et al., 1995a; Golembo et al., 1996a; Tio and Moses, 1997). VEIN has so far only been reported to function during muscle attachment in the embryo (Yarnitzky et al., 1997) and in the developing wing disc (Schnepp et al., 1996). Finally, there is also one proposed inhibitory ligand: ARGOS (Freeman et al., 1992; Schweitzer et al., 1995b), which is induced by the EGFR pathway on most tissues upon its activation (Golembo et al., 1996b). This paper focuses on the roles of EGFR in the developing *Drosophila* retina.

The *Drosophila* compound eye is derived from a monolayer epithelium and ultimately contains only ten cell types. Each facet (ommatidium) includes three types of photoreceptor

neurons: the basal central R8, six outer cells (R1-6) and the apical central R7. There are also seven types of non-light-sensitive accessory cells (Ready et al., 1976). In early life, the eye primordium grows as an unpatterned monolayer columnar epithelium. In the final larval phase, a wave of pattern formation, cell-cycle regulation and cell-type specification sweeps across the field from posterior to anterior: the morphogenetic furrow (Ready et al., 1976; Heberlein and Moses, 1995). The furrow is associated with synchronization of the cell cycle (G_1 arrest), alterations of cell shape (shortening), changes in gene expression and the first allocation of cells to future ommatidia. As they emerge from the furrow, the ommatidial preclusters consist of five cells: the first photoreceptor cell (R8) and two pairs of future outer cells (R2 and R5 and R3 and R4, Tomlinson, 1988; Wolff and Ready, 1993).

Once the precluster is established, the remaining cells are recruited by means of local signaling. The best known of these steps is the recruitment of the final photoreceptor: R7 (reviews include: Banerjee and Zipursky, 1990; Dickson and Hafen, 1994; Simon, 1994; Wassarman et al., 1995). This recruitment depends upon a specific ligand BRIDE OF SEVENLESS (BOSS) on the surface of the R8 cell and a specific receptor tyrosine kinase SEVENLESS (SEV) on the receiving cell. Following ligand binding, the SEV receptor then acts through many of the same elements of the RAS/MAPK cascade as does EGFR signal (Simon et al., 1991; Diaz-Benjumea and Hafen, 1994). It has been proposed that, after the founding R8 cell is specified, all the other cells are recruited by similar mechanisms of ligands and receptors (via a 'combinatorial code', Tomlinson and Ready, 1987a; Tomlinson, 1988). The furrow lays down a new column of ommatidia approximately every 2 hours (Basler and Hafen, 1989), but the ommatidia within a column are not formed simultaneously. The first cluster in each column is formed at the eye midline, and subsequent clusters are formed dorsal and ventral to this at approximately 15-20 minute intervals (Wolff and Ready, 1991; Baker et al., 1996; Dokucu et al., 1996). These two temporal gradients (two hour and fifteen minute) were operationally important for this study as they have allowed us to time the molecular events in EGFR signal transduction very precisely.

The *Egfr* has been known to play a role in *Drosophila* retinal development since 1989, when Baker and Rubin showed that the dominant rough eye mutation *Ellipse* is a gain-of-function allele of *Egfr* (Baker and Rubin, 1989). Since then two models have been proposed for the function of the EGFR in the fly retina: (1) that it is the receptor for lateral inhibition in the furrow and hence controls cluster spacing (Baker and Rubin, 1989), and (2) that EGFR acts in the recruitment and specification of all the cell types of the ommatidium (Freeman, 1996). Recently, it has been suggested that this may occur via the regulation of two antagonistic transcription factors (briefly discussed in a conference review: Roush, 1997). In order to study the function of EGFR in the *Drosophila* eye, we derived a conditional (temperature-sensitive, = t.s.) mutation of *Egfr*. We can now circumvent the early EGFR function in cell proliferation (Xu and Rubin, 1993), and test its actions in the furrow and after, by direct and simple loss of function. We have used this mutation to test both models described above and to discover two novel functions.

An operational problem with the study of signal transduction in the *Drosophila* retina has been that the effects of genetic perturbations can only be observed indirectly, by their developmental consequences. Thus after a treatment such as the upshift of a temperature-sensitive mutations, the induction of the ectopic expression of genes or the production of loss-of-function mosaic clones (like those for Egfr itself, Xu and Rubin, 1993), sufficient time (hours or days) must be allowed for a developmental change to become visible. It can thus be difficult to distinguish primary from secondary effects. Recently a new reagent has become available for the direct visualization of the activity of the RAS/MAPK pathway: a monoclonal antibody specific for the active form of the MAP kinase (dp-ERK, Gabay et al., 1997a; Yung et al., 1997). This antibody has been shown to detect the *Drosophila* dp-ERK in situ (produced by the *rolled* gene, Biggs et al., 1994; Brunner et al., 1994) in cells at multiple stages of development, many of which are triggered by EGFR (Gabay et al., 1997a,b). We have utilized this reagent to test the activity of this critical signal transduction cascade within minutes of an experimental treatment.

We have used the dp-ERK antibody in conjunction with the new *Egfr* t.s. allele, and both gain and loss of *sev* function, to demonstrate directly when, and in which cells, these receptors signal through the RAS/MAPK pathway. Furthermore, we have also discovered a novel regulatory step in the pathway: in the furrow, dp-ERK appears early, but is detected in the cytoplasm for more than 2 hours before it is translocated into the nucleus in only a subset of cells.

MATERIALS AND METHODS

Drosophila genetics and temperature-shift regimes

Flies were cultured on standard cornmeal medium. $Egfr^{Elp1}$ is a gain-of-function allele and was a gift of Nick Baker (Baker and Rubin, 1989). $Egfr^{CO}$ was used as the standard null allele and a gift from Trudi Schüpbach (Clifford and Schüpbach, 1989). sev^{D2} was used as the *sev* null mutation and was a gift of Mike Simon. $sevE{:}sev^{S11}$ was used to supply activated SEV behind the furrow and was a gift of U. Banerjee (Basler et al., 1991). The screen parent stock was: *w*; *cn bw*; *P[(w, ry)D]3* (*P[(w, ry)D]3* is an insertion in polytene band 90E; a gift from R. Levis and G. M. Rubin). Mutagenized males were crossed to *w*; $Egfr^{Elp1}$/In(2LR)O females. Both of these stocks were first made isogenic for the second and third chromosomes. Mutations were isolated as dominant suppressors of $Egfr^{Elp1}$ and were recovered over In(2LR)O as revealed by the *cn* mutation. All putative *Egfr* mutations were tested for lethality in *trans* to $Egfr^{CO}$ at both 18°C and 28°C. We tested 133,171 mutagenized chromosomes and recovered 38 alleles of *Egfr*. Two of our *Egfr* alleles are lethal in *trans* to a null allele at 28°C but viable at 18°C, but only one satisfied our genetic criteria as a null at 28°C ($Egfr^{tsla}$, see text). In temperature-shift experiments, $Egfr^{tsla}/Egfr^{CO}$ larvae were raised at 18°C and then moved to 28°C, at the times and for the durations noted in the text (see Results below). For the experiment shown in Fig. 1D, the larvae were returned to 18°C and allowed to continue their development (see Results below). The EGFR gain-of-function genotype was $Egfr^{Elp1}/Egfr^{+}$.

Histology

Eye discs were prepared as described by Tomlinson and Ready (1987b), as modified by Tio and Moses (1997), mounted in Vectashield (Vector Labs, H-1000) and examined by laser-scanning confocal microscopy or stained with 0.5 mg/ml DAB with 1.5 mM $NiCl_2$ and 1.5 mM $CoCl_2$ and mounted in Vectashield. Primary

antibodies were: rat anti-ELAV (Iowa, Developmental Studies Hybridoma Bank, Bier et al., 1988; Robinow and White, 1991), mouse mAb 22C10 (a gift of Larry Zipursky and Seymour Benzer; Fujita et al., 1982), mouse mAb anti-BOSS (a gift of Larry Zipursky; Kramer et al., 1995), rabbit anti-ATO (a gift of Yuh-Nung Jan and Andrew Jarman; Jarman et al., 1994), and mAb anti-dp-ERK (Yung et al., 1997). Secondary antibodies were: Cy5-conjugated goat anti-mouse IgG (Jackson Labs, 115-175-003), FITC-conjugated goat anti-rabbit IgG (Jackson Labs, 111-095-003) and FITC-conjugated goat anti-rat IgG (Jackson Labs 112-095-003). DNA was detected using SYTO-24 (Molecular Probes, S7559). Cytoplasmic actin was detected with rhodamine-conjugated phalloidin (Molecular Probes, R-415). S-phase cells were detected by 5-bromo-2′ deoxyuridine incorporation, (BrdU, Wolff and Ready, 1991) using Sigma BrdU (catalog # B-5002) visualized with anti-BrdU (Becton Dickinson, 347-580). Acridine orange staining was performed as described by Spreij (1971). Scanning electron microscopy was performed as described in Moses et al. (1989). Embryonic cuticles were prepared as described in Wieschaus and Nüsslein-Volhard (1986).

RESULTS

Egfr^{tsla} is a tight temperature-sensitive allele

To study *Egfr* loss of function in the developing eye, we sought to avoid the problems inherent in the mosaic clone approach (Xu and Rubin, 1993) by deriving a conditional allele, so that function could be removed at a specific **time** in development in all cells, rather than continuously in a defined group of cells. To be useful in our studies of retinal development, it is important that the t.s. mutation be fully wild type at the permissive temperature (18°C), but fully null at the restrictive temperature (28°C). Three t.s. mutations of *Egfr* were previously recovered, but none of them satisfy these requirements (Clifford and Schüpbach, 1994). We conducted a genetic screen designed to produce a temperature-sensitive (t.s.) allele of *Egfr* (see Materials and Methods). Our screen was based on the fact that the dominant *Egfr^{Elp}* alleles are suppressed towards wild type when placed in *trans* to a null allele (compare Fig. 1A and B and see Baker and Rubin, 1989). This procedure makes null alleles dominant and permits a powerful F_1 screen. One of our mutations (henceforth called *Egfr^{tsla}*) fulfills these criteria: when placed in *trans* to a null allele its zygotic cuticle phenotype is indistinguishable from wild type at 18°C and from the homozygous null at 28°C (Fig. 1E-H). *Egfr^{tsla}* acts as a dominant suppressor of *Egfr^{Elp}* at 28°C (Fig. 1C) that is indistinguishable in degree from the suppression associated with a known null (Fig. 1B), but does not suppress *Egfr^{Elp}* at 18°C. We thus conclude that by these genetic criteria, *Egfr^{tsla}* is a tight t.s. allele and suitable for our purposes. Henceforth the *Egfr^{tsla}*/*Egfr^{-}* genotype will be referred to as '*Egfr^{ts}*'.

EGFR acts at four different stages in the developing eye, but not in cluster spacing

Subjecting *Egfr^{ts}* flies to the non-permissive temperature for 24 hours (beginning at a time when the furrow is about half way across the eye field) results in structural defects, many of which are observed in external views of the adult eye (Fig. 1D). Some similar defects can be seen with treatments at the non-permissive temperature as short as 1 hour. The most obvious defect is a physical scar that runs across the eye in a dorsal-to-ventral direction. Since new ommatidia are found anterior to the scar, we conclude that development can recover following restoration of EGFR function. To observe more directly the immediate effects of EGFR loss of function during eye development, we conducted further temperature-shift experiments with *Egfr^{ts}* and examined the developing late-larval eye discs. We raised *Egfr^{ts}* larvae at 18°C and then placed them at 28°C for 24 hour period prior to dissection (henceforth called the 'EGFR-TS' condition). EGFR-TS discs were compared with those from both wild type and *Egfr^{Elp}*/+ (gain-of-function genotype). In wild-type discs, phalloidin staining reveals cytoskeletal actin and, at an apical level, can show cluster formation in the furrow (Fig. 2A). *Egfr^{Elp}*/+ greatly

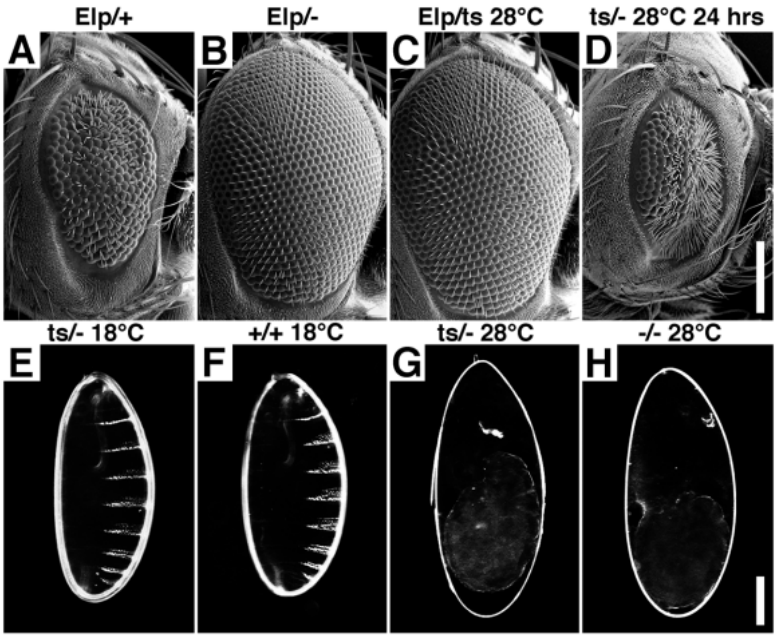

Fig. 1. A temperature-sensitive mutation of *Egfr*. (A-D) Scanning electron microscopy of adult compound eyes. Dorsal is up and anterior is to the right. Scale bar in D shows 100 μm. (A) *Egfr^{Elp}*/*Egfr^{+}*. (B) *Egfr^{Elp}*/*Egfr^{CO}*. (C) *Egfr^{Elp}*/*Egfr^{tsla}* raised continuously at 28°C. (D) *Egfr^{tsla}*/*Egfr^{CO}* raised at 18°C, moved to 28°C for 24 hours in late larval life and then returned to 18C. Note that the *Egfr^{tsla}* suppression of the dominant *Egfr^{Elp}* rough eye phenotype is indistinguishable from that of the null allele (*Egfr^{CO}*). (E-H) Embryonic cuticle phenotypes. (E) *Egfr^{tsla}*/*Egfr^{CO}* raised at 18°C. (F) Wild type raised at 18°C. (G) *Egfr^{tsla}*/*Egfr^{CO}* raised at 28°C. (H) *Egfr^{CO}*/*Egfr^{CO}* raised at 28°C. Anterior is up and ventral is to the right. Scale bar in H is 200 μm. Note that the *Egfr^{tsla}*/*Egfr^{CO}* embryonic phenotype is indistinguishable from that of the homozygous null allele (*Egfr^{CO}*) at the restrictive temperature and is indistinguishable from wild type at the permissive temperature.

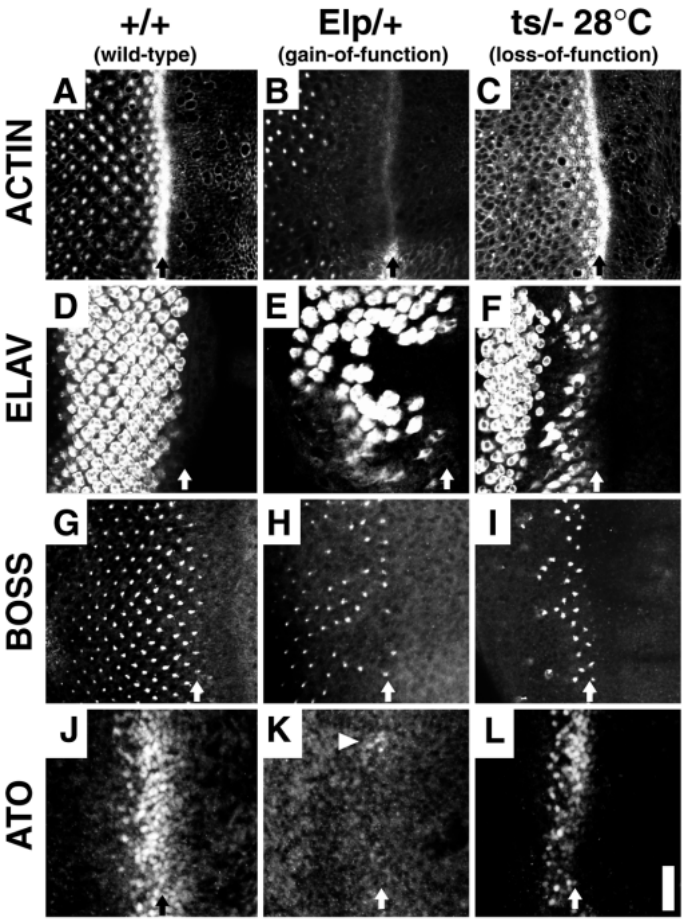

Fig. 2. Third instar eye disc confocal immunohistochemistry against neuronal markers. (A,D,G,J) Wild type; (B,E,H,K) $Egfr^{Elp}/Egfr^{+}$; (C,F,I,L) EGFR-TS condition (see text). (A-C) actin, (D-F) ELAV, (G-I) BOSS, (J-L) ATO (see Materials and Methods). Anterior is to the right. Scale bar in L is 20 μm. Arrows indicate position of furrow. Arrowhead in K indicates isolated developing cluster. Note that the EGFR-TS condition does not affect R8 founder cell or cluster formation in the furrow.

reduces cluster formation, consistent with its published deficits in cluster number (Fig. 2B, Baker and Rubin, 1992; Zak and Shilo, 1992). EGFR-TS discs show few effects on cluster formation in and close to the furrow but five or six columns posterior to the furrow the clusters are dysmorphic (Fig. 2C).

A neural-specific marker (ELAV) normally reveals the clusters as they assemble (Fig. 2D, Robinow and White, 1991). In $Egfr^{Elp}/+$ ELAV reveals a reduced numbers of clusters (Fig. 2E). However, the ELAV pattern in EGFR-TS condition is more complex: a nearly normal array of single neurons is seen close to the furrow for about four columns (8 hours), then there is an empty gap and finally a tightly packed array of clusters at the posterior side (Fig. 2F). Similar results are obtained with another neural marker (mAb 22C10, data not shown). We interpret the array of single neurons close to the furrow as isolated R8 founder cells, which are unable to induce the neural differentiation of their neighbors. This is entirely consistent with the phenotype seen with the loss of SPITZ (ligand) function (Tio and Moses, 1997). The ELAV gap corresponds to a loss of R8 neurons. This could be either cell death, excess cell division forcing the neurons apart or de-differentiation. We favor the last interpretation, as we did not observe excess cell death in this region with acridine orange staining (data not shown) nor did we see excess S-phase cells in this zone by BrdU incorporation (data not shown). Moreover R8 cells do **not** undergo this de-differentiation in *spitz* null mosaic clones (Tio and Moses, 1997). Thus this EGFR function cannot be SPITZ mediated. Following the gap, the clusters are seen in various stages of assembly. However these have too few neurons for their relative location in the disc. We interpret these as clusters that have slowed or stopped recruiting new cells at the time of the temperature shift.

These interpretations are supported by staining for the R8-specific marker BOSS (Fig. 2G-I). In EGFR-TS condition, the initial single neurons are BOSS-positive, and then this antigen is lost in the gap, and does not return. Furthermore, the initial columns of R8 (BOSS-positive) cells are evenly spaced. This result is not consistent with any model for EGFR function in cluster spacing.

We also examined the R8 founder cell proneural protein ATONAL (ATO). In wild type, ATO expression rises ahead of the furrow, then is focused progressively to just the R8 cells (Fig. 2J and Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996). Consistent with the deficit in cluster formation in $Egfr^{Elp}/+$, we observed a loss of ATO expression (Fig. 2K). However, in EGFR-TS condition, ATO expression and focusing are unaffected (Fig. 2L). It is interesting to note that the ELAV gap (the de-differentiation of the R8 cells in EGFR-TS) approximately correlates to the normal time and position of the downregulation of ATO in the first few columns after the furrow. It may be that the R8 cells are initially specified and supported by ATO, but later become supported by the RAS/MAPK pathway, like all the other photoreceptors.

In the experiments described above, eye disc development was observed 24 hours after *Egfr* function was removed. This is a shorter time than has been possible up to now (the mosaic clones used by others remove Egfr function 2 to 3 days before this time, Xu and Rubin, 1993). However, it is possible that within this 24 hour period secondary developmental effects obscure the primary defect caused by removing EGFR activity. Thus we wished to reduce this time as far as possible. We examined $Egfr^{tsla}/Egfr^{CO}$ eye discs after a series of shorter periods at the restrictive temperature (for 1, 2, 6 and 12 hours in Fig. 3 and for wild type and 24 hours in Fig. 2D and F). The results are consistent with those described above for 24 hour shifts (the 'EGFR-TS' condition): a requirement for EGFR in maintaining the R8 photoreceptor cells for about 8 hours (4 columns) after they first form and later defects in recruiting all additional neurons.

We shifted slightly younger $Egfr^{ts}$ larvae to 28°C, at a time when the furrow is not yet initiated and found a novel and unexpected EGFR function: it is required for the initiation of neural differentiation on the posterior margin. Normally the furrow initiates at the posterior margin and is negatively regulated by WINGLESS expression on the disc margins, in particular on the dorsal side. When *wingless* function is removed by temperature shift, there is ectopic furrow initiation from the dorsal margin (and less so from the ventral margin, Ma and Moses, 1995; Treisman and Rubin, 1995). In this early EGFR-TS condition, we see an inhibition of neural

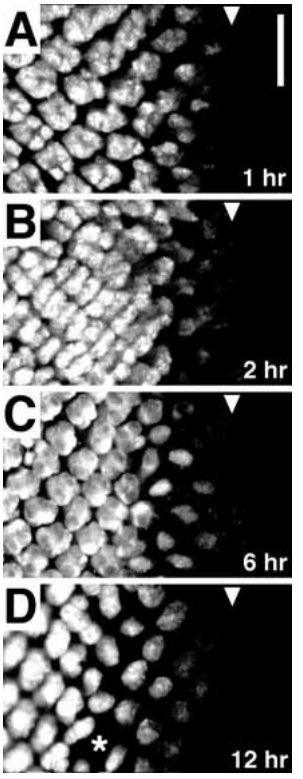

Fig. 3. Brief temperature shifts have reduced affects proportional to the time. (A-D) $Egfr^{tsla}/Egfr^{CO}$ raised at 18°C and shifted to 28°C for 1, 2, 6 and 12 hours, respectively, and then stained to show developing neurons (Elav). Fig. 2D shows wild type, which is indistinguishable from 0 hours. Fig. 2F shows 24 hours. (A) Note that no mutant phenotype can be seen in 1 hour discs. (B) At 2 hours, there is one additional column of single neurons; (C) at 6 hours, there are four additional columns of single neurons; (D) at 12 hours, there is also a small non-neuronal gap (marked by an asterisk), and at 24 hours (Fig. 2F), this gap is longer (see text). Anterior is to the right. Arrowheads indicate furrow. Scale bar in A is 20 μm.

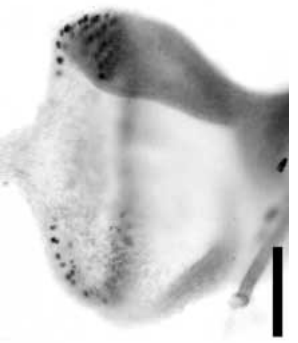

Fig. 4. EGFR is required for neuronal differentiation at the posterior margin. EGFR-TS third instar eye disc stained with ELAV after an early shift. Anterior is to the right. Scale bar is 50 μm. Note that neural differentiation is eliminated at the posterior margin, but not on the dorsal and ventral margins.

differentiation at the posterior margin, but not at the (mostly) dorsal margin (Fig. 4).

dp-ERK antibody reveals activated MAPK in the developing retina

To directly visualize RAS/MAPK pathway signaling downstream of EGFR we made use of a new reagent: a monoclonal antibody specific to the active, di-phosphorylated form of the MAP kinase (dp-ERK, Yung et al., 1997). In the developing eye dp-ERK was reported in large clusters of cells in the furrow (Gabay et al., 1997a). We examined the dp-ERK pattern in the eye disc at a higher resolution. In addition to the identification of do-ERK in large clusters of cells in the furrow (Fig. 5A,B and Gabay et al., 1997a), followed by smaller clusters in later columns. We also observed dp-ERK in additional positions. There is a low level of cytoplasmic antigen anterior to the furrow, then in the furrow the large clusters develop from the midline. Within one column in the furrow, the dp-ERK staining clusters are initially small, then larger, and then smaller again in a series of about ten. As clusters within a column are formed at 15-20 minute intervals, this phase of dp-ERK accumulation corresponds to more than 2 hours in time. The clusters ultimately focus to one or a few cells, in which dp-ERK can then be seen for about two columns (4 hours).

This development from the eye midline of small clusters, through large clusters and then back to one or a few cells is consistent with the expression of SCABROUS and with the proneural focusing of ATO in the founding R8 cells (Baker et al., 1990; Mlodzik et al., 1990; Baker and Zitron, 1995; Brown et al., 1995; Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996; Lee et al., 1996). Thus the developing dp-ERK pattern appears to correlate with the specification of the ommatidial precluster. We positioned the large dp-ERK clusters relative to the early steps of ommatidial formation by double staining for dp-ERK and cytoplasmic actin. We find that the large clusters dp-ERK correspond to a very early stage, one column anterior to the first clear ommatidial clusters, which appears to be the 'rosette' stage (Fig. 5C, Wolff and Ready, 1991).

dp-ERK accumulation in the furrow requires EGFR activity

Several observations confirm that the dp-ERK (and thus MAP kinase signaling) in the furrow is dependent on the activity of EGFR. The dp-ERK pattern broadens and fails to focus in the gain-of-function $Egfr^{Elp}/+$ genotype (not shown). In the $Egfr^{ts}$ genotype, the dp-ERK antigen is visibly reduced within 15 minutes of the shift up to the restrictive temperature and is barely detectable after 30 minutes (Fig. 6B). Interestingly, after about 2 hours at the restrictive temperature dp-ERK antigen begins to rise again in a broad band around the furrow and persists for at least 24 hours (Fig. 6C). This suggests that, as a secondary consequence of a failure of normal development, RAS/MAPK pathway signaling is induced by a tyrosine kinase receptor **other** than EGFR, or by another signaling pathway. This signaling is also unlikely to be mediated by SEV, which is not expressed this close to the furrow (Tomlinson et al., 1987). All of these events occur hours before it is possible to observe morphological changes in these cells, demonstrating the value of this direct in vivo output assay for signal transduction.

dp-ERK in the furrow is not cytoplasmic

It is important to note that, contrary to expectations, dp-ERK in the developing eye is primarily cytoplasmic. We stained wild-type discs to reveal dp-ERK and DNA and then colocalized these in confocal optical sections (Fig. 7). The field shown (Fig. 7A-C) was chosen as it is slightly angled, such that clusters near the top of the panel are cut apically (blue arrow), and those near the bottom of the panel are cut basally (where the nuclei lie at this stage, yellow arrow). The dp-ERK antigen is clearly mostly cytoplasmic in the large furrow clusters as we cannot detect dp-ERK in cell nuclei at this stage (Fig. 7D-F). At a later stage (more posterior in the same field), dp-ERK can be seen in occasional, apical nuclei (which have risen to join a cluster, yellow arrowhead, Fig. 7E,H,I). The time series of cluster formation in the furrow shows that detectable nuclear dp-ERK can follow

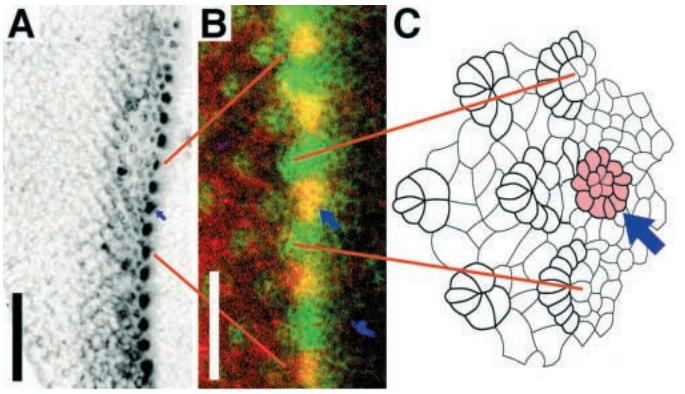

Fig. 5. dp-ERK is accumulated in rosette stage ommatidia in the furrow. (A,B) Flat views of dp-ERK in wild-type discs eye at different magnifications. (A) A low power view, stained only for dp-ERK for clarity. (B) A similar disc at higher magnification, stained for dp-ERK (red), actin (green) and DNA (blue). This allows the position of the dp-ERK containing clusters (red) to be compared to the early stages of ommatidial development (green). (C) Diagram (after Wolff and Ready, 1993) to show position of dp-ERK stain seen in A. Anterior is to the right. Blue arrows indicate large clusters of cells in the furrow that contain a high level of dp-ERK. Scale bars: (A) 50 μm and (B) 20 μm.

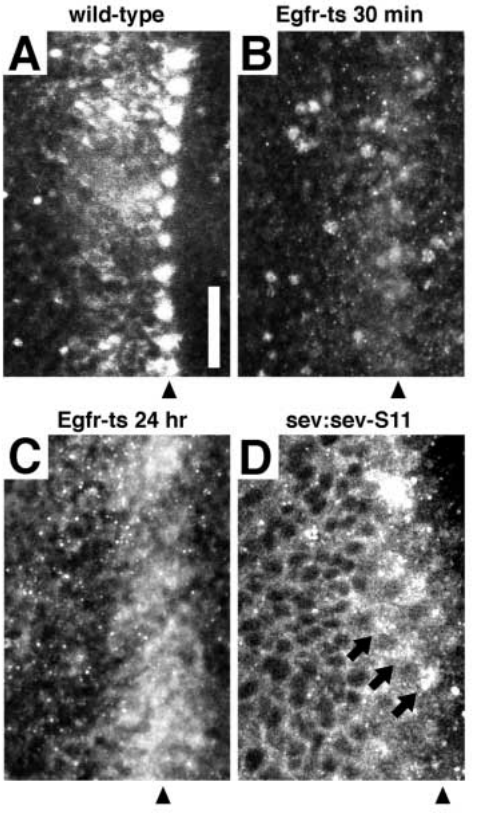

Fig. 6. dp-ERK in the furrow is normally regulated by *Egfr*. Third instar eye discs stained for dp-ERK. (A) Wild type; (B) $Egfr^{tsla}/Egfr^{CO}$ raised at 18°C, then moved to 28°C for 30 minutes in late larval life (15 minute is similar). Note that the regular clustered staining is lost. (C) $Egfr^{tsla}/Egfr^{CO}$ raised at 18°C, then moved to 28°C for 24 hours in late larval life (2 hours is similar). Not that broad and unpatterned band of staining has become established since the earlier time point. (D) $sevE:Sev^{S11}$. Note the multiple columns of dp-ERK staining clusters and the increased level dp-ERK in posterior ommatidia. Arrows show additional rows of large clusters (in D). Arrowheads indicate furrow. Anterior is to the right. Scale bar in A, 20 μm.

cytoplasmic phosphorylation by 2 hours or more. Later, we observe transient dp-ERK antigen in cell nuclei, such as the developing R3 and R4 cells, for much shorter times (approximately half an hour, arrowheads in Fig. 8A,B). The fact that we can detect dp-ERK in these later nuclei in the same specimen as the one in which we cannot detect dp-ERK in the large furrow clusters demonstrates that the observed changes in the subcellular partitioning of dp-ERK are unlikely to be artifactual.

dp-ERK antigen is regulated by SEV in the R7 cells

About nine columns (18 hours) after dp-ERK staining first appears in the furrow, it can be seen in the cytoplasm of the future R7 cell (not shown). This dp-ERK persists for about four columns (8 hours), and is genetically dependent on the activity of the *sev* gene as it is absent in discs derived from *sev* null mutant larvae (not shown). We are thus able to directly visualize signal transduction from this other receptor tyrosine kinase. It is interesting to note that dp-ERK is detectable in the cytoplasm of the future R7 for an extended period (about 8 hours).

Ectopic activation of the RAS/MAPK pathway increases the level of cytoplasmic but not nuclear dp-ERK

The results presented above demonstrate that accumulation of dp-ERK in the nucleus does not always follow activation of EGFR or SEV. We wanted to examine the issue further, by following dp-ERK localization after ectopic activation of the signaling pathway. Normally, SEV is expressed in many cells within the developing ommatidium (Banerjee et al., 1987; Tomlinson et al., 1987) but only one cell is destined to become the future R7 photoreceptor (Tomlinson and Ready, 1987a). A constitutively active SEV receptor has been shown to transform the remaining SEV-expressing cells into R7 neurons (Basler et al., 1991; Dickson et al., 1992). Increased activity of some of the components of the Ras/MAPK cascade such as *ras* (Fortini et al., 1992), *Dsor1* (Tsuda et al., 1993) and *rolled* (Brunner et al., 1994) also lead to the ectopic formation of R7 neurons.

We used a $sevE:sev^{S11}$ construct to express activated SEV in many cells posterior to the furrow (Fig. 6D, Basler et al., 1991). In $sevE:sev^{S11}$ discs, additional rows of large dp-ERK staining clusters are seen just posterior to the furrow (arrows in Fig. 6D). These clusters can be seen to interdigitate with the clusters within the furrow and these may correspond to cells within each ommaditium that normally express *sev* (R3, R4, R1, R6, R7 and cone cells, Tomlinson et al., 1987). The close proximity of the second row of staining clusters to the morphogenetic furrow is not surprising since *sev* expression is first seen in this second row in wild type discs (Tomlinson et al., 1987). In more posterior regions of the disc, the level of cytoplasmic dp-ERK is greatly elevated. Thus, ectopic activation of an RTK

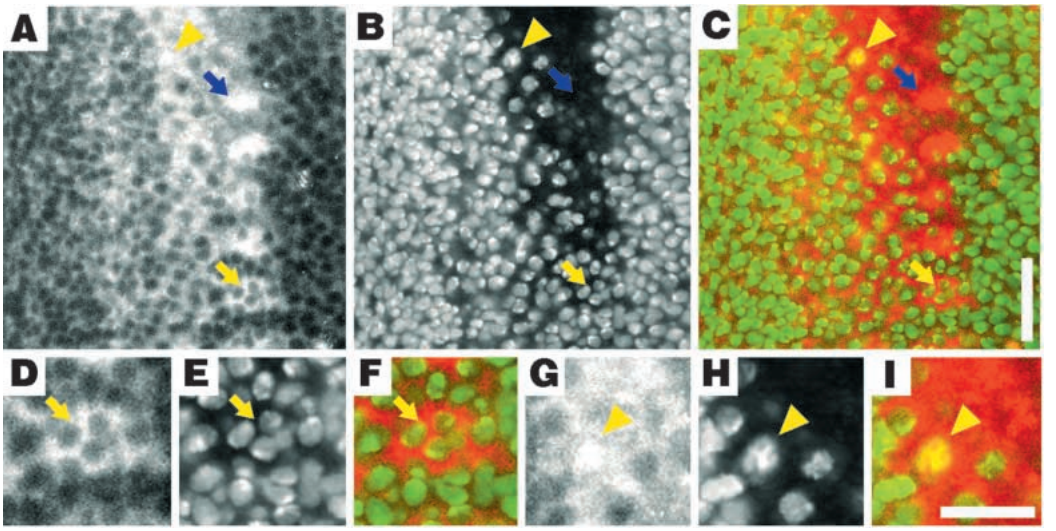

Fig. 7. Active MAPK antigen is detected in the furrow but is not nuclear. (A-I) Confocal images of a developing wild type eye disc stained for dp-ERK. (A,D,G) Stained for dp-ERK; (B,E,H) stained for DNA and (C,F,I) the corresponding merged images (dp-ERK in red and DNA in green). Yellow arrows mark a rosette stage cluster of cells seen in a basal region of the section, blue arrows mark a rosette-stage cluster of cells seen in an apical region and yellow arrowheads shows a cell nucleus located in more posterior regions of the disc. Anterior is to the right. Scale bar represents 10 μm in C and 10 μm in I. Note that dp-ERK in the rosettes is not detectable in cell nuclei.

pathway, similar to RTK pathways normally functioning in eye disc development, is not sufficient to induce the accumulation of dp-ERK in the nuclei.

DISCUSSION

The results presented in this report describe, for the first time, the true loss-of-function phenotype of EGFR in *Drosophila* compound eye development. We have derived a temperature-sensitive allele of *Egfr* that is fully wild type at the permissive temperature and fully null at the restrictive temperature. This allowed us to avoid the difficulties inherent in genetic mosaic analysis of genes with early or continuous function (such as *Egfr*) by removing the function at a specific time. We were thus able to test two previously published models for the function of EGFR in the developing eye: (1) EGFR is the receptor for cluster/founder cell spacing (Baker and Rubin, 1989) and (2) EGFR triggers differentiation of **all** cell types in the developing eye (Freeman, 1996). The EGFR-TS phenotypes that we observe are not fully consistent with either of these models.

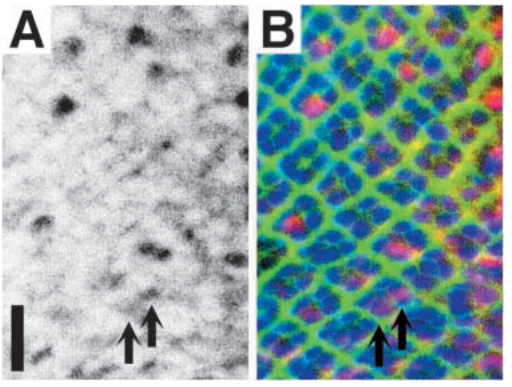

Fig. 8. Active MAPK is transiently detectable in the nuclei of developing outer photoreceptors. (A) Wild-type disc stained for dp-ERK. Arrows show nuclei of outer cells R3 and R4. Other nuclei that are positive for dp-ERK can also be seen. (B) Confocal merged image of the same field stained for dp-ERK (red), actin (green) and DNA (blue). Anterior is to the right. Scale bar in A represents 10 μm. Note that nuclear dp-ERK is very rare, indicative of very short periods of activation.

The first data to suggest that *Egfr* functions in *Drosophila* retinal development came from an analysis of *Ellipse* (*Egfr*^{Elp}) mutations. *Egfr*^{Elp} alleles appear to be dominant gain-of-function mutations as they are suppressed in *trans* to null alleles of *Egfr* (Baker and Rubin, 1989). In *Egfr*^{Elp} homozygotes, there are fewer ommatidia than normal and they are separated by increased space (Baker and Rubin, 1989, 1992; Zak and Shilo, 1992). This led Baker and Rubin to suggest an attractive model: that the preclusters and/or founder (R8) cells are normally spaced evenly by a short-range diffusible inhibitor (a mechanism known as 'lateral inhibition', Wigglesworth, 1940) and that the receptor for this inhibitor is EGFR (Baker and Rubin, 1989). Thus in *Egfr*^{Elp}, hyperactive EGFR leads to **increased** space between the clusters. This model made a testable prediction that loss of EGFR function should **reduce** the space between ommatidia. In this paper, we show that in the EGFR-TS complete loss-of-function condition a normally spaced array of single R8 cells is formed. This strongly suggests that EGFR has no role in cluster spacing. If there is any role for EGFR in establishing cluster and/or R8 cell spacing, it must be a minimal contribution and/or be highly redundant. By definition, any system that establishes lateral inhibition must act quantitatively – removing EGFR totally (with the t.s.) produces **no** detectable quantitative change in spacing.

The *ato* gene encodes a transcription factor that acts as the

proneural gene for the founding R8 cell (Jarman et al., 1994, 1995; Dokucu et al., 1996). ATO expression is initially broad in and anterior to the furrow, but is progressively focused to just the R8 cells, where it persists for 6-8 hours (three or four columns, Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996). The *rough* gene encodes another transcription factor required for the specification of a subset of the outer photoreceptor cells (Tomlinson et al., 1988). ROUGH is first expressed in the furrow (Heberlein et al., 1991) in a pattern that is reciprocal to that of ATO. Furthermore ROUGH and ATO are antagonistic (Dokucu et al., 1996). It has been proposed that *rough* is positively regulated in response to EGFR signaling and thus $Egfr^{Elp}$ phenotype may be explained as an over induction of ROUGH, a consequent loss of ATO and thus a deficit of clusters. However, we have shown that, in the EGFR-TS condition, ATO expression and proneural focusing are unaffected.

A second model has been proposed for the function of EGFR in the *Drosophila* retina: that 'Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye' (Freeman, 1996). This was suggested by the loss-of-function phenotype and overexpression of *spitz* and from dominant-negative EGFR mutant protein (Freeman, 1996; Tio and Moses, 1997). The fact that a regularly spaced array of R8 cells is formed in the EGFR-TS condition suggests that, at least as far as the R8 cell type is concerned, EGFR cannot be said to trigger the differentiation of all cell types in the eye. It is formally possible that EGFR does normally play a role in R8 cell specification, but that this role is redundant or dispensable (ie other RTKs can specify R8 cells in the absence of EGFR function). It is likely that all subsequent recruitment steps require EGFR however (Freeman, 1996, 1997). EGFR may act in combination with other receptors such as SEV to raise the level of RAS/MAPK pathway activity over some critical threshold (Greenwood and Struhl, 1997; Tio and Moses, 1997). Furthermore, the observation that dp-ERK staining initially disappears from the furrow, but later rebounds (without EGFR function) strongly suggests that other receptor tyrosine kinases are present in the furrow and can act there. Our $Egfr^{ts}$ data are similar but not identical to those obtained from the expression of a dominant-negative mutant protein (Freeman, 1996), presumably because induction of dominant-negative EGFR could not fully inactivate the endogenous activity.

We have found a novel function for EGFR in the developing fly retina: it is required to initiate neural differentiation at the posterior margin, but not at the dorsal and ventral edges of the eye field. It is interesting to note that *dachshund* (*dac*) shows a very similar phenotype in retinal mosaics and that *dac* is a dominant suppressor of $Egfr^{Elp}$ (Mardon et al., 1994). Furthermore, DAC is part of a complex that includes the EYES ABSENT protein to control the specification of the eye field at an early stage (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). Perhaps the EGFR is part of this system too. We and others have found that WINGLESS acts in the polar (mostly dorsal) margins to repress furrow initiation there (Ma and Moses, 1995; Treisman and Rubin, 1995; Royet and Finkelstein, 1996; Reifegerste et al., 1997; Royet and Finkelstein, 1997; Heberlein et al., 1998; Treisman and Heberlein, 1998). It may be that EGFR signaling plays a role in antagonizing that inhibition on the posterior margin at the time of furrow initiation. Indeed, the EGFR ligand SPITZ has recently been reported to counteract WINGLESS signaling in *Drosophila* epidermal development (O'Keefe et al., 1997; Szuts et al., 1997).

In summary, we can now enumerate four functions for EGFR in the developing eye. Others have shown that EGFR functions early in regulating cell proliferation (Xu and Rubin, 1993). We have shown that EGFR functions in furrow initiation at the posterior margin of the eye, in R8 cell maintenance and in the recruitment of the other cells. Furthermore, our data suggest that EGFR is unlikely to have any significant role in founder cell specification or cluster spacing.

dp-ERK in the eye disc

We have used a dp-ERK-specific antibody to detect RAS/MAPK pathway signal transduction in vivo in near real-time. This has allowed us to distinguish the primary from the secondary developmental effects of removing EGFR function by temperature shift. It has also allowed us to show that the major receptor controlling the activity of this pathway in the furrow is EGFR. We have discovered that nuclear accumulation of dp-ERK is not an obligatory consequence of RTK activation. Rather, accumulation of dp-ERK in the cytoplasm can be detected up to two or eight hours after activation of EGFR or SEV, respectively. Two models may account for these results. One possibility is that a novel regulated step in ERK/MAPK signaling: translocation to the nucleus is under separate and subsequent control to MAPK phosphorylation. In the furrow, dp-ERK is first held in the cytoplasm in large clusters of cells for more than 2 hours before it is detected in the nucleus. A similar, and even longer 'cytoplasmic hold', is later seen in the R7 cell downstream of the SEV receptor. An alternative possibility is that there is a very potent dp-ERK phosphatase activity in the nucleus and that the regulation of this activity results in the observed preponderance of dp-ERK in the cytoplasm at many stages. A number of MAPK phosphatases have been already identified and this is the primary avenue for the de-activation of MAP kinases (reviewed in Nebreda, 1994). At this time, we can offer no molecular model for regulation of the cellular sites for dp-ERK accumulation and this will be a focus of our future studies. Regardless of the underlying molecular mechanisms, the capacity to modulate the amount of nuclear dp-ERK provides an additional level for regulating the timing and consequences of RTK signaling. It remains to be seen if this regulatory step is used in other systems.

What is the developmental function of EGFR-dependent MAP kinase activation in the large clusters in the furrow? We have shown that these dp-ERK clusters correspond to the 'rosette' stage (Wolff and Ready, 1991) and are lost in the EGFR-TS condition. Even without EGFR function, we find that ommatidial clusters and R8 photoreceptor cells are formed normally, as seen by staining for cytoplasmic actin and the expression of ATO and BOSS. The first developmental defect seen after removing EGFR activity, is the failure of R2, R5, R3 and R4 to differentiate as neurons (i.e. the array of single R8 cells seen), as is seen with *spitz* loss of function (Tio and Moses, 1997). Thus, the only function of this earliest SPITZ to EGFR to MAPK/ERK signal may make the non-R8 cells of the precluster competent to develop later as neurons.

It is humbling to note that ten years after the publication of the combinatorial model for cell-type specification in the *Drosophila* retina (Tomlinson and Ready, 1987a) and the molecular isolation of the first retinal receptor to fit this model (Banerjee et al., 1987; Hafen et al., 1987), we have not yet identified the molecular signals that specify R8, all the outer photoreceptor cell types or the accessory cells. It is clear that RAS pathway activity alone cannot convey cell-type specificity, as activated pathway elements can produce multiple cell types in the eye (Basler et al., 1991; Fortini et al., 1992; Brunner et al., 1994; Karim et al., 1996), and because at least two receptors (EGFR and SEV) produce different results at different stages while signaling through many of the same elements (including RAS and MAPK). It has been suggested that timing alone, superimposed on periodic restimulation of the RAS/MAPK pathway can specify the precise array of retinal cells (Freeman, 1997). This is unattractive, as it is hard to see how timing regulation could be so spatially precise. It is likely that the true mechanism involves at least 14 differentially expressed transcription factors in the developing ommatidia (reviewed in Kumar and Moses, 1997). However, the signals that establish the precise mosaic distribution of these transcription factors are still unknown.

This work was supported by a grant to K. M. from the National Science Foundation (IBN-9507857) and a postdoctoral fellowship to J. K. from the National Eye Institute (1 F32 EYO6763-02). The confocal microscope was provided by a grant from the NSF (BIR-9419941). We thank M. Freeman for his interest, support and discussions, D. Yang and H. T. Tran for technical help with the screen, L. R. Bell, C. A. Brennan and R. Reifegerste for their comments on the manuscript, N. Baker, T. Schüpbach, S. Benzer, S. L. Zipursky, U. Banerjee, M. Simon, G. M. Rubin, A. Jarman and Y. N. Jan for their gifts of reagents, and N. Baker and R. Cagan for sharing their ideas and data with us freely prior to their publication.

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