Genetic and biochemical analysis of the role of Egfr in the morphogenetic furrow of the developing Drosophila eye

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Summary

A key event in patterning the developing Drosophila compound eye is the progressive restriction of the transcription factor Atonal in the morphogenetic furrow. The Atonal pattern evolves from expression in all cells to an over-dispersed pattern of single founder cells (the future R8 photoreceptors). This restriction involves Notch-mediated lateral inhibition. However, there have been inconsistent data on a similar proposed role for the Egfr receptor (Egfr). Experiments using a conditional Egfr mutation (Egfrtsla) suggested that Egfr does not regulate Atonal restriction, whereas experiments using Egfr-null mosaic Minute clones suggested that it does. Here, we have re-examined both approaches. We report that the lesion in Egfrtsla is a serine to phenylalanine change in a conserved extracellular ligand-binding domain. We show by biochemical and genetic approaches that the Egfrtsla protein is rapidly and completely inactivated upon shift to the non-permissive temperature. We also find that on temperature shift the protein moves from the cell surface into the cell. Finally, we report a flaw in the Egfr-null mosaic Minute clone approach. Thus, we demonstrate that Egfr does not play a role in the initial specification or spacing of ommatidial founder cells.

Key words: Egfr, Morphogenetic furrow, Drosophila, eye, Atonal, Photoreceptor

Introduction

The Drosophila Epidermal growth factor receptor homolog (Egfr) is similar to vertebrate ERBB1 and to Let-23 in C. elegans, and is a type 1 transmembrane protein with intrinsic tyrosine kinase activity (Bogdan and Klambt, 2001; Shilo, 2003). In the developing fly eye, the major positive ligand is Spitz (Freeman, 2002). Like vertebrate EGFR, upon ligand binding the fly protein dimerizes, is activated and trans-autophosphorylates. Inside the cell, the signal is propagated via Ras and a kinase cascade with the final step being the fly homolog of P42/44 mitogen activated protein kinase (MAPK):Rolled (Marshall, 1994; Freeman, 2002; Shilo, 2003). Activated MAPK (pMAPK) translocates to the nucleus, where it can modulate the activities of transcription factors such as Pointed and Anterior open (Yan) in the fly eye (Brunner et al., 1994; O’Neill et al., 1994; Treisman, 1996; Freeman, 2002; Voas and Rebay, 2004).

Patterning in the Drosophila eye imaginal disc begins in the third larval instar when the morphogenetic furrow begins to move from posterior to anterior (Ready et al., 1976). This furrow produces the spaced array of ommatidia, with a new column made roughly every two hours (Ready et al., 1976; Basler and Hafen, 1989). The first photoreceptor cell to be specified and to differentiate is R8 (Ready et al., 1976; Tomlinson and Ready, 1987). R8 later induces the remaining cells of the facet and is thus also known as the ommatidial founder cell (Tomlinson, 1988; Freeman, 1997).

Coincident with the process of R8/founder cell specification is the expression of the proneural basic helix-loop-helix transcription factor Atonal; in atonal loss-of-function mutants, R8 cells do not form (Jarman et al., 1993; Jarman et al., 1994; Jarman et al., 1995). The level of Atonal first rises in all cell nuclei, then, deep in the furrow, it is then lost from some cells to leave spaced groups (the ‘intermediate groups’). Finally, Atonal is restricted to the single founder cells, where it persists for a few columns (Baker et al., 1996; Dokucu et al., 1996). R8/founder cell specification and spacing involves the Notch and Hedgehog pathways, through the regulation of atonal transcription (Cagan and Ready, 1989; Baker and Zitron, 1995; Baker et al., 1996; Dominguez, 1999; Suzuki and Saigo, 2000; White and Jarman, 2000; Baonza and Freeman, 2001; Baker, 2004).

In the developing eye, Egfr signaling is required for late R8 cell maintenance, the induction of all cells following the R8/founder cell, proliferation, ommatidial rotation and cell survival (Freeman, 1994; Tio et al., 1994; Freeman, 1996; Freeman, 1997; Tio and Moses, 1997; Dominguez et al., 1998; Kumar et al., 1998; Kurada and White, 1998; Halfar et al., 2001; Brown and Freeman, 2003; Firth and Baker, 2003; Kumar et al., 2003; Strutt and Strutt, 2003; Yang and Baker, 2003). It has also been suggested that Egfr signaling has a primary function in the initial specification and spacing of the Atonal-positive intermediate groups and/or the R8/founder cells based on four observations: pMAPK is strongly expressed in the intermediate groups; the gain-of function mutation EgfrE69 has reduced numbers of R8 cells; Ras pathway signals...
Development

If Egfr signals do regulate R8 cell spacing then the pMAPK regulation of Rough expression in the non-R8 cells could be an indirect consequence of a gain-of-function mutation, and the Minute mosaics. We have now re-examined these two experiments and have tested them for four possible flaws. (1) The Egfrtsla protein could be temperature-sensitive only during experiments and it is functionally null at the restrictive temperature. We also report that, indeed, the Minute technique artifically disturbs ommatidial spacing in the Egfr null mosaic experiments. We conclude that Egfr signaling does not normally function in the initial spacing of the R8/founder cells.

Materials and methods

Drosophila stocks and mosaic analysis

Genomic DNA

Egfrtsla M(2)53 clones

Egfrtsla Minute+ clones

Egfrtsla Minute- clones

Egfrtop-18A M(2)56i clones

Egfrtsla M(2)56i clones

Wild-type M(2)53 clones

Sequencing of Egfrtsla DNA

PCR primer pairs were as follows. Exon 1, type I: forward primer (fp), ATAGCTTGGAA-GAGGCCCTTGAATC; reverse primer (rp), TGGGCACCGCA-TGCAAGCCAGA.

Exon 1, type II: forward primer (fp), ATGCTTGGAA-GAGGCCCTTGAATC; reverse primer (rp), TGGGCACCGCA-TGCAAGCCAGA.
ACT; rp3, AATGGCCAGATAGCGACCCGG; rp4, CACACAAAAAAG-GCCAGACATC; rp5, ACCCTCCGGCACCACAAGGCC; rp6, CACCAGAACGACCAGTGATGTTGATCGGCCAGACACTG-GT.

Sequencing was done at the University of Iowa facility.

**Antibodies and histochemistry**

Two rabbits were injected with an Egfr C-terminal peptide [(C)QRELQPLHRNRNTETR] (Lesokhin et al., 1999), coupled to keyhole limpet hemocyanin. Sera were affinity purified (by Zymed). For imaging, S2 cells were cultured on cover slips after transfection and prepared as previously described (Lee et al., 2001). Eye discs (except those used for the anti-Egfr stains) were prepared as previously described (Tomlinson and Ready, 1987), modified as described by Tio and Moses (Tio and Moses, 1997), mounted in Vectashield (Vector Labs, H-1000) and imaged by confocal microscopy. For Egfr staining, the fix was 4% paraformaldehyde in PBS for 30 minutes at room temperature; eye discs were then washed and blocked as above, then incubated with primary antibody overnight at room temperature (Moberg et al., 2004). Primary antibodies: rabbit anti-Egfr [1:3000 for blots, gift of N. Baker (Lesokhin et al., 1999)], rabbit anti-Egfr (1:100) for immunoprecipitation, 1:500 for immunohistochemistry, rat anti-Spitz [1:20 (Schweitzer et al., 1995)], mouse anti-pTyr (PY20, 1:500, Santa Cruz Biotechnology SC-035-003), goat anti-guinea pig TRITC, (1:150, 106-025-003). Goat anti-rat Cy5 (1:200, 112-175-003), goat anti-mouse HRP (1:40, 115-035-003), goat anti-guinea pig TRITC, (1:150, 106-025-003). Goat anti-rabbit-HRP (1:8,000, 65-6120) was from Zymed. Syto-24 was mainly from Jackson ImmunoResearch: goat anti-mouse Cy5 (1:500, 115-175-003), goat anti-rabbit TRITC (1:250, 111-025-003), goat anti-rat Cy5 (1:200, 112-175-003), goat anti-rabbit HRP (1:40, 115-035-003), goat anti-guinea pig TRITC, (1:150, 106-025-003). Goat anti-rabbit-HRP (1:8,000, 65-6120) was from Zymed. Syto-24 was used to stain DNA (1:10,000, Molecular Probes S-7559).

**Tissue culture**

The Egfr+ plasmid pMtEgfrType I was a gift of N. Baker (Lesokhin et al., 1999). The Egfrtsla plasmid pMtEgfrtslaType I was derived from pMtEgfrType I using a Stratagene ‘QuickChange’ Site-Directed Mutagenesis Kit. The secreted Spitz plasmid is pMTsSpitz, a gift of G. Mardon (Nolo et al., 2000), mouse anti-β-gal (1:1000, Promega 23783), guinea-pig anti-Hrs [1:100, gift from Ursula Weber (Lloyd et al., 2002)], mouse anti-Armadillo [1:10, DSHB N2 7A1 (Riggleman et al., 1990)], guinea-pig anti-Senseless [1:1000, gift of G. Mardon (Nolo et al., 2000)], mouse anti-β-gal (1:1000, Promega 23783), guinea-pig anti-Hrs [1:100, gift from Ursula Weber (Lloyd et al., 2002)], mouse anti-Armadillo [1:10, DSHB N2 7A1 (Riggleman et al., 1990)]. Secondary antibodies were mainly from Jackson ImmunoResearch: goat anti-mouse Cy5 (1:500, 115-175-003), goat anti-rabbit TRITC (1:250, 111-025-003), goat anti-rat Cy5 (1:200, 112-175-003), goat anti-rabbit HRP (1:40, 115-035-003), goat anti-guinea pig TRITC, (1:150, 106-025-003). Goat anti-rabbit-HRP (1:8,000, 65-6120) was from Zymed. Syto-24 was used to stain DNA (1:10,000, Molecular Probes S-7559).

**Results**

**Egfrtsla lesion**

We sequenced Egfrtsla and found a single coding change relative to the parent chromosomal: the transition C1,754T [using the numbering system of Clifford and Schüpbach (Clifford and Schüpbach, 1994)] produces a missense S511F mutation (in the type I isoform). The change lies in the extracellular, conserved L2 domain, which is a leucine-rich repeat that functions in ligand binding (Clifford and Schüpbach, 1994; Burgess et al., 2003). S511 is not conserved in either vertebrate or nematode Egfr homologs, but lies in a variable region that functions in ligand specificity (Clifford and Schüpbach, 1994; Burgess et al., 2003). Three other temperature-sensitive alleles of Egfr have been reported, although, unlike Egfrtsla, none have strong phenotypes at the restrictive temperature. Two of these lesions lie in the intracellular kinase domain, and one (EgfrM2T) lies in the L1 domain [L206Q in the type I isoform (Clifford and Schüpbach, 1994)].

**Egfrtsla protein is thermo-labile for activity**

To determine if the Egfrtsla protein is temperature sensitive only during its synthesis or for its activity, we developed a tissue culture assay system using Drosophila Schneider line 2 cells (S2 cells) [Fig. 1A, assay adapted from Schweitzer et al. and Lesokhin et al. (Schweitzer et al., 1995; Lesokhin et al., 1999)]. These cells express low levels of endogenous Egfr, so all of the experiments were done in parallel with untransfected controls. We transfected cells to express additional Egfr+ or Egfrtsla at 18°C (the permissive temperature). One day later, we shifted the cells to 30°C and added cycloheximide to inhibit new protein synthesis. One hour later, we added conditioned media containing Spitz and harvested the cells at different time points. The levels of Egfr+ antigen remained fairly constant after 60 minutes with cycloheximide, suggesting that the wild-type protein has a much longer half-life than one hour under these conditions; Egfrtsla antigen was also detectable, although less so than wild type (Fig. 1B). In this assay, Egfr+ drives a ligand-dependent, high level of pMAPK within 5 minutes (asterisk in Fig. 1C). To measure total band intensities (integrated over a standard area) for Egfr+, the extract was precipitated with streptavidin beads (Pierce 2349) as described previously (Salazar and Gonzalez, 2002). Quantification was by the following steps. (1) Densitometry from non-saturated films to measure total band intensities (integrated over a standard area) for Egfr+ from the untransfected control cells and from those transfected for Egfr+ and Egfrtsla, and for pMAPK. (2) Track background (measured below each band) was subtracted from each total band density. (3) The untransfected control value was subtracted from each experimental transfection (Egfr+ and Egfrtsla). (4) The pMAPK value was divided by the Egfr+ antigen value (for each track) to give the activity per receptor. (5) Values were standardized to make the activity of Egfr+ at 30°C 100; all other values are percentages of wild type. (6) Three replicates were used for the mean and the standard error of the mean.
Egfr+ and Egfr\textsuperscript{tsla} at 18°C, but only for Egfr+ at 30°C (white asterisks). Egfr antigen was detected under all conditions (Fig. 1G,H); however, we detected reduced levels of Egfr\textsuperscript{tsla} at 30°C, perhaps through instability (see below).

We previously reported that Egfr\textsuperscript{tsla} mutants lose pathway activity within 30 minutes at 29°C in vivo (Kumar et al., 1998). We therefore determined whether Egfr\textsuperscript{tsla} becomes inactive in this system in a similar time frame after temperature shift. S2 cells were treated as described above, shifted to 30°C (for 0, 15, 30 or 60 minutes, Fig. 2A) and then treated with ligand. We detected reduced levels of Egfr\textsuperscript{tsla} antigen after the temperature shift. We always detected robust levels of MAPK phosphorylation driven by both Egfr+ and Egfr\textsuperscript{tsla} at 18°C. However, Egfr\textsuperscript{tsla} lost this activity within 15 minutes at 30°C in all experiments (Fig. 2B-D).

From these experiments, we conclude that Egfr\textsuperscript{tsla} protein is wild type at 18°C for ligand-dependent signaling activity. However, at 30°C, Egfr\textsuperscript{tsla} becomes inactive within 15 minutes. Furthermore, this is due to the temperature sensitivity of the protein after synthesis and this effect is rapid.

**Egfr\textsuperscript{tsla} activity at the restrictive temperature is not detectably different to zero**

We quantified the experiments in Fig. 2, at the 0- and 60-minute time points. Each condition was repeated in triplicate and two such experiments were quantified per receptor at 30°C (see Materials and methods and Fig. 3). From the two experiments, we conclude that the activity of Egfr\textsuperscript{tsla} is wild type at 18°C but that at 30°C its activity is very low, and is indistinguishable from no activity at all.

Furthermore, we directly visualized the activity of Egfr in individual cells by immunofluorescence (Fig. 4). We examined cells at the 60-minute time point, as above (Fig. 2A). Untransfected cells show low background levels of Egfr antigen (Fig. 4A,D) and a low level of pMAPK antigen (Fig. 4A,D) and a low level of pMAPK antigen (Fig. 4A,D).
Egfr and the morphogenetic furrow

4B,D). Transfected cells express elevated levels of Egfr antigen (Fig. 4E-P). Cells that express additional Egfrtsla at 30°C (white arrowheads in Fig. 4E-H) and 18°C (not shown), and Egfrtsla at 18°C (Fig. 4I-L), drive increased levels of nuclear pMAPK antigen (arrows in Fig. 4F,H,J,L), but cells expressing Egfrtsla at 30°C do not (Fig. 4N,P).

All of these biochemical and cell biological experiments show that Egfrtsla is a mutation that is temperature sensitive for activity. The level of ligand-dependent Egfrtsla activity at the permissive temperature is not distinguishable from that of the wild type, and the activity at the restrictive temperature is indistinguishable from no activity at all. In addition, these experiments show that the effect of the temperature shift is rapid, occurring within 15 minutes. These results are consistent with the in vivo study we published previously, which showed that Egfrtsla mutants rapidly lose pMAPK antigen from the morphogenetic furrow (Kumar et al., 1998). We conclude that Egfrtsla is indeed a rapidly acting mutation that is temperature sensitive for activity. Egfrtsla is effectively wild type at 18°C and effectively null at 30°C.

Egfrtsla antigen is rapidly removed from the cell surface after a shift to 30°C

We detected reduced levels of Egfr antigen in cells transfected with Egfrtsla at 30°C (see above). It could be that after temperature shift, the Egfrtsla protein is removed from the cell surface and targeted for degradation. To study this, we labeled transfected S2 cells with a non-membrane permeable biotinylation reagent to separate superficial from intracellular Egfr (Fig. 5A). We compared biotinylated Egfr antigen (see asterisk in Fig. 5B) to total Egfr antigen (see asterisk in Fig. 5C). We detected biotinylated Egfr at all time points from cells transfected with Egfr+. Biotinylated Egfrtsla is also seen at 18°C, but is rapidly lost so that none is detectable after 60 minutes at 30°C (Fig. 5B), and a low level of total antigen remains (Fig. 5C). These results strongly suggest that Egfrtsla protein undergoes a ligand-independent conformational change at 30°C, leading to rapid internalization.

To investigate this relocalization in vivo, we raised a new anti-Egfr serum [to the same C-terminal peptide as previously reported (Lesokhin et al., 1999)]. We tested it for specificity by staining eye imaginal disc Egfr2 homozygous clones (data not shown). We stained wild-type eye discs and

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Fig. 3. Quantification of the temperature-shift Egfr-activity data from S2 cells. Histograms for two experiments shown in Fig. 2, showing 0-minute at 18°C and 60-minute at 30°C time points (see text). Data are shown as a percentage of wild type at 30°C. Error bars indicate s.e.m.

Fig. 4. Nuclear pMAPK following Spitz activation in S2 cells. S2 cells were stained for Egfr antigen, pMAPK antigen and DNA as indicated. Conditions are as in Fig. 2A and Fig. 3, see text. Transfected DNA and temperature are indicated on the left. Transfected cells express elevated Egfr antigen (arrowheads), untransfected control cells do not. Note that cells transfected with Egfrtsla, at 30°C, do not express pMAPK over background levels. Arrows indicate increased levels of pMAPK antigen. Scale bar: 5 μm.
saw an Egfr expression pattern as has been previously described (data not shown) (Lesokhin et al., 1999). We see the wild-type pattern in Egfrtsla homozygous retinal clones raised at 18°C (Fig. 6A,B). In the furrow, we observed antigen concentrated in a subset of the Armadillo-positive cell junctions and the first two columns of ommatidial preclusters (see white arrowhead in Fig. 6B) (Takahashi et al., 1996; Ahmed et al., 1998). This junctional stain occurs around all sides of the cells deep in the furrow (see white arrowhead in Fig. 6B), but, at the edges of the furrow, it becomes lost from the faces of the cells that lie away from the furrow. The junctions between the cells within the precluster are heavily stained (see white arrowhead in Fig. 6B, inset); however, the junctions between the precluster cells and the surrounding cells are not (see black arrowhead in Fig. 6B, inset). We also saw numerous cytoplasmic granules in the assembling ommatidia (see arrows in Fig. 6A,B).

Thus, we find that Egfr antigen is normally strongly localized in the furrow to a specific subset of cell junctions: essentially the cells held in G1 cell-cycle arrest (Ready et al., 1976; Wolff and Ready, 1991; Thomas et al., 1994). This observation is intriguing, but the pattern does not correlate precisely with known Egfr signaling activity (from pMAPK staining). Egfr junctional staining is seen within and between the Atonal-positive intermediate groups, whereas pMAPK staining is seen only within them (Gabay et al., 1997; Kumar et al., 1998; Spencer et al., 1998). Thus, the biological significance of this pattern is presently unclear.

When we shift these discs to the non-permissive temperature, the junctional antigen in the furrow and early preclusters is rapidly converted to larger granules: partially after 15 minutes at 30°C (and then up to 15 minutes dissection time at room
temperature, Fig. 6D and inset), and completely by 30 minutes (and then up to 15 minutes dissection time at room temperature, Fig. 6F). Under these conditions Armadillo staining is unaffected (Fig. 6G). Thus, although Egfr antigen may co-localize with Armadillo in some of the Armadillo-positive junctions (furrow and early preclusters), Armadillo antigen distribution is not dependent on Egfr function (at least not in the short term). In mammals, ligand bound EGFR has been shown to pass through an endocytic pathway (Carpenter, 2000), and the Drosophila Hepatocyte Growth factor regulated tyrosine kinase substrate (Hrs) protein affects trafficking so that active Egfr is degraded (Lloyd et al., 2002; Weber et al., 2003). We co-stained Egfr tsla homozygous retinal clones after 30 minutes at 30°C for Egfr and Hrs antigens (Fig. 6H) and found no significant co-localization. This suggests that the Egfr antigen is removed from the Armadillo-positive junctional domains following temperature shift, but that this is not via a pathway that involves Hrs. These results are consistent with the biotinylation data described above. The simplest interpretation is that the Egfr tsla protein undergoes a conformational change upon temperature shift, leading to ligand-independent internalization via a non-signaling pathway.

Egfr tsla is null by genetic criteria

Egfr null mosaic clones are small (Xu and Rubin, 1993), so we compared the sizes of Egfr tsla clones to Egfr null clones. We used ey:Flp to induce Egfr tsla clones and raised the animals continuously at 18°C (Fig. 7A), at 18°C and then at 30°C for their last day (Fig. 7B), or continuously at 30°C (Fig. 7C). The total area of the Egfr tsla clone territory was roughly equal in size to the total area of wild-type twin-spots when raised continuously at 18°C and after one day at 30°C (although they are reduced towards the posterior side). However, the clones are very much smaller after continuous growth at 30°C. Likewise, Egfr homologous null clones made without Minute mutations are very small and are similar to Egfr tsla homologous clones after continuous growth at 30°C (compare Fig. 7C and 7D).

Egfr is required for late ommatidial development, so we stained Egfr tsla clones (raised at 30°C for 24 hours) for a late R8 marker, Senseless (Fig. 7E,F) (Frankfort et al., 2001), and a neural marker, Elav (Fig. 7G,H) (Robinow and White, 1991). As previously reported (Dominquez et al., 1998; Kumar et al., 1998; Spencer et al., 1998; Baonza et al., 2001; Yang and Baker, 2001), we find that R8 cells do form, although at later stages they are sometimes abnormally close (Fig. 7E,F). Elav expression is lost from the non-R8 photoreceptors (Fig. 7G,H) and also from most of the R8s, because of a late maintenance requirement (Kumar et al., 1998).

Thus, by these proliferation and differentiation defects, Egfr tsla (at the restrictive temperature) is indistinguishable from nulls.

Egfr tsla does not affect the rate of furrow progression

Thus far, we have tested for the first two of the four possible artifacts defined above: Egfr tsla is temperature sensitive for activity (there is no perdurance of activity), and it is biochemically and genetically indistinguishable from nulls. The third possible artifact was that the normal Atonal expression pattern in temperature-shifted Egfr tsla eye discs might result from a rapid arrest of furrow movement with no further change in the Atonal pattern. To test this, we stained Egfr tsla homozygous clones that were raised at 18°C and held at 30°C for 24 hours for Atonal. In this experiment, the Egfr tsla territories abut Egfr+ twin spots. If the loss of Egfr function causes the furrow to arrest with ‘frozen’ Atonal expression, we would see the furrow about twelve columns more advanced in the wild-type tissue than in the mutant clones. As before (Kumar et al., 1998), we saw normal Atonal expression in this mutant tissue. We also saw that the position of the furrow was equally advanced in the mutant clones and in the wild-type twin spots (Fig. 8A-C). Thus, we conclude that Egfr does not regulate the rate of furrow progression. We also confirm again that Egfr tsla has no effect on R8/founder cell initial spacing, neither with a 24-hour shift (Fig. 8A-C), nor when raised continuously at the non-permissive temperature (Fig. 8D-F).

Two different Minute mutations in mosaic clones have dominant and non-cell autonomous effects on Atonal expression

Having investigated the possible artifacts of the Egfr tsla studies,
we turned to the fourth possibility: some artifact in the Egfr
null Minute mosaic experiments. It could be that the Egfr
mutations used in these experiments have some dominant
effects on Atonal expression under these conditions (24 hours
at 30°C). Therefore, we stained eye discs heterozygous for
three Egfr alleles in trans to wild type (Egfrtsla, EgfrF2
and Egfrtop-18A, Fig. 9A-C), and we found that there was some
variation in the Atonal antigen staining intensity in different
specimens, but that this does not correlate with the three Egfr
genotypes. In all three cases, Atonal expression was normal.

It could be that Minute mutations have dominant and/or non-
cell autonomous clone effects on Atonal expression and R8/founder cell patterning. Indeed, it has been reported that a Minute
mutation affects gene expression and cell growth in competing, wild-type clones (de la Cova et al., 2004). To test
for this and other possible artifacts, we repeated the Egfr null
experiments, exactly as previously reported, using stocks
kindly supplied by those investigators. We placed EgfrF2 in
trans to Minute(2)53 (Baonza et al., 2001), and we placed
Egfrtop-18A in trans to Minute(2)56i (Yang and Baker, 2001). We
also placed Egfrtsla in trans to both Minute(2)53 and
Minute(2)56i. All four genotypes were raised at 18°C until the
early second instar and then clones were induced with hs:FLP
(90 minutes at 37°C). Thereafter, the animals were raised at
30°C and eye discs were stained for Atonal. In all four cases
(Fig. 9D-O), we found that the pattern of Atonal expression
was not wild type (compare with Fig. 9A-C). In these cases, the
Egfrtsla clones were indistinguishable from the clones of the
two Egfr null alleles made with the same Minute mutations
(compare Fig. 9D-F with 9G-I, and compare 9J-L with 9M-O).
Thus, by this phenotype also, Egfrtsla raised at 30°C is indistinguishable from the nulls.

Furthermore, we find that the pattern of Atonal expression
is different to that of wild type, both within the Egfr
homozygous mutant territories and outside of them (in the Egfr
Minute heterozygous cells). These abnormalities include the
occasional twinning of Atonal-positive cells (Fig. 9E,F),
reduced differentiation of the intermediate groups (all four
cases) and reduced Atonal expression in some cases (arrows in
Fig. 9K,L,N,O). Taken together, these data suggest that some
factor in both Minute experiments causes dominant and non-
cell autonomous defects in the pattern of Atonal expression and
R8/founder cell/ommatidial spacing.

How could the use of the Minute mutations produce these
effects? The simplest possibility is that Minute(2)53 and
Minute(2)56i have a dominant effect on Atonal patterning on
their own. Indeed, as well as affecting body size, developmental
time and bristle morphology, some Minute
mutants are reported to have dominant rough eye phenotypes
(Plough and Ives, 1934; Dunn and Mossige, 1937; Brehme,
1939; Kalisch and Rasmuson, 1974; Sinclair et al., 1981).
However, neither Minute(2)53 nor Minute(2)56i show a
dominant rough eye phenotype. Furthermore, we stained the
heterozygous stocks for Atonal expression and saw no defects
(data not shown). Thus, the Atonal defects are not due to a
simple dominant effect of the Minute mutations.

It could be that the phenotype is due to a dominant synthetic interaction between the Minute mutations and Egfr. Again, this
is not probable, as the Egfr mutant clones are Minute+ after
the somatic recombination. We stained animals that were Egfr
mutant in trans to the two Minute mutations (without inducing
clones) for Atonal expression and again saw no defects (data
not shown). Thus, the Atonal defects are not due to a

Discussion

We characterized the lesion in Egfrtsla and found that it is a
missense mutation in the conserved ligand-binding,
extracellular L2 domain (Burgess et al., 2003). Our biochemical and localization data suggest that the Egfrtsla protein functions normally at 18°C as a ligand-activated receptor. However, after shift to the non-permissive temperature, Egfrtsla rapidly becomes inactive and is removed from the cell surface, probably via a non-signaling endocytic pathway (with or without ligand). It may be that the Egfrtsla protein is conformationally unstable at 30°C and is degraded. Human EGFR is normally only internalized in response to ligand binding (Burke et al., 2001), but it has been reported that inhibition of PKA leads to internalization of unbound EGFR (Salazar and Gonzalez, 2002). Our mutation in the extracellular L2 domain suggests that this domain may be involved in mediating the stability of Egfr in the membrane.

Atonal expression and R8/founder cell spacing are normal in Egfrtsla eye discs incubated at the non-permissive temperature. We examined three possible artifacts that could have invalidated this observation. (1) Egfrtsla could be temperature sensitive for synthesis but not activity. If so, then protein made before the shift to 30°C might continue to supply sufficient function at the non-permissive temperature to support normal R8/founder cell development. However, we have shown that Egfrtsla is in fact temperature sensitive for activity, and that activity is lost within minutes of the temperature shift, while Atonal expression and R8/founder cell formation continues normally for 24 hours. (2) Egfrtsla could be leaky (i.e. not null) at 30°C, and some residual activity might supply sufficient function at the non-permissive temperature to support normal R8/founder cell development. We have shown before that Egfrtsla mutants (at the non-permissive temperature) are genetically indistinguishable from a null (Egfr224) for three phenotypes (Kumar et al., 1998). Here, we have shown in addition that Egfrtsla mutants at 30°C are phenotypically indistinguishable from nulls (Egfr22 and Egfrtop-18A), in Minute+ mosaic clones, as well as in their growth deficits in clones made without Minute mutations. Furthermore, we have undertaken quantitative biochemical experiments using S2 cells to show that Egfrtsla at 30°C is indistinguishable from a null in its ability to drive both the phosphorylation of MAPK and its own autophosphorylation. (3) It could be that, at 30°C, the furrow just freezes in Egfrtsla mutant eyes, leaving an arrested but apparently normal furrow. Here, we have shown that in adjacent Egfrtsla and Egfr+ territories, the furrow advances at the same rate after the temperature shift to 30°C. Thus, we conclude that our observation of normal Atonal and R8/founder cell patterning in Egfrtsla mutant eyes is not invalidated by any of these three possible artifacts.

Next, we turned to the possible problems associated with the Minute+ mosaic method used to make the same observations with the Egfr nulls (Baonza et al., 2001; Yang and Baker, 2001). We replicated the Minute+ Egfr null experiments exactly as previously reported, using the same Drosophila stocks, except to run them at 30°C. In parallel, we did the same
experiments with Egfrtsla. If the known Egfr nulls were to have a different phenotype to Egfrtsla, we would be forced to conclude that Egfrtsla is not behaving as a null in this assay. If, however, the two Egfr nulls have the same phenotype in this assay as Egfrtsla does, then we could conclude that the temperature-sensitive allele is indistinguishable from the nulls at 30°C, and that the difference is due to the Minute technique and not to Egfr. Indeed, we did find that the Egfrtsla and Egfr null phenotypes are indistinguishable, and thus we conclude that the Egfrtsla phenotypes assayed without the Minute technique are valid and that the discrepancy stems from some aspect of the use of the Minute mutations. We went on to stain Minute clones made without any Egfr mutation present and obtained the very same Atonal expression defects. Taken together, these data suggest that the spacing defects previously reported by others, and replicated by us here, are genetically dependent on the presence of the Minute clones, and are not an affect of the Egfr mutations.

Therefore, we conclude that Egfr has no primary role in R8/founder cell spacing and also that Egfrtsla is a rapidly acting temperature-sensitive mutation that is functionally null at the non-permissive temperature.

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