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 Egf receptor (Egfr). Experiments using a conditional Egfr mutation (Egfr\textsuperscript{tsla}) suggested that Egfr does not regulate Atonal restriction, whereas experiments using Egfr\textsuperscript{null} mosaic Minute\textsuperscript{c} clones suggested that it does. Here, we have re-examined both approaches. We report that the lesion in Egfr\textsuperscript{tsla} is a serine to phenylalanine change in a conserved extracellular ligand-binding domain. We show by biochemical and genetic approaches that the Egfr\textsuperscript{tsla} 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\textsuperscript{c} 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 Egfr\textsuperscript{P160} has reduced numbers of R8 cells; Ras pathway signals...
promote the expression of the Rough transcription factor in the cells that do not become R8s; and Ras pathway loss-of-function mosaic clones show reduced precision in the array of R8 cells (Baker and Rubin, 1989; Zak and Shilo, 1992; Dokucu et al., 1996; Kumar et al., 1998; Spencer et al., 1998; Baonza et al., 2001; Yang and Baker, 2001; Yang and Baker, 2003). Of these the most direct and elegant evidence is the last (Ras pathway loss-of-function mosaic clones).

Egfr null clones are very small because of an early proliferation defect (Xu and Rubin, 1993). Thus, we generated a conditional Egfr loss-of-function mutation (Egfr\textsuperscript{tsla}, a temperature-sensitive allele) and used it to remove Egfr function at specific times, by-passing the proliferation defect. We found that while this abolishes pMAPK in the intermediate groups, it does not affect the establishment or spacing of the Atonal-positive R8/founder cells (Kumar et al., 1998). We also observed that the high-level pMAPK antigen in the intermediate groups is predominantly cytoplasmic and proposed that there is a block to pMAPK nuclear translocation (‘MAPK cytoplasmic hold’) (Kumar et al., 1998; Kumar et al., 2003).

A second approach to overcome the proliferation defect was to use the Minute technique to give Egfr null cells a growth advantage (Baonza et al., 2001; Yang and Baker, 2001). In these clones, the single Atonal-positive cells are irregularly arranged rather than accurately spaced. This observation was made independently by two groups and led to the conclusion that Egfr signaling has a primary role in ommatidial spacing (Baonza et al., 2001; Yang and Baker, 2001).

How may these two views be reconciled? If Egfr signals do not regulate R8 spacing, then the pMAPK pattern may be explained by cytoplasmic hold, the Egfr\textsuperscript{tsla} phenotype may be an indirect consequence of a gain-of-function mutation, and the regulation of Rough expression in the non-R8 cells could be through a direct Egfr function in these cells, not due to any Egfr function in the R8s [indeed, a rough null has normal early ommatidial development (Tomlinson et al., 1988)]. However, if Egfr signals do regulate R8 cell spacing then the pMAPK pattern may be explained by a direct function, the Egfr\textsuperscript{tsla} phenotype may be due to over-inhibition of R8 cell formation and Rough expression in the non-R8 cells may be a direct result of Egfr signaling.

The two apparently direct loss-of-function experiments give different results: Egfr\textsuperscript{tsla} temperature shift versus Egfr-null Minute mosaics. We have now re-examined these two experiments and have tested them for four possible flaws. (1) The Egfr\textsuperscript{tsla} protein could be temperature-sensitive only during synthesis, so that temperature shift the previously synthesized protein remains active and provides sufficient function for normal R8 patterning (a form of perdurance). (2) The Egfr\textsuperscript{tsla} mutation might not be null at the restrictive temperature, and residual function could support normal R8 patterning (a form of perdurance). (3) At the time of the temperature shift, the furrow in Egfr\textsuperscript{tsla} eyes might arrest, freezing the Atonal pattern so that it appears to be wild type. (4) Minute\textsuperscript{tsla} clones may have non-cell autonomous effects on Atonal patterning.

Here, we report the characterization of Egfr\textsuperscript{tsla}, including its mutant lesion, the effects of temperature on its activity and stability, and the relocation of the protein from the cell surface into the cell. We show by biochemical and genetic means that Egfr\textsuperscript{tsla} is temperature-sensitive for activity and that it is functionally null at the restrictive temperature. We also report that, indeed, the Minute technique artificially 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

Egfr\textsuperscript{tsla/Df(2R)Egfr}\textsuperscript{tsla} and cn bw.

Egfr\textsuperscript{tsla} Minute\textsuperscript{tsla} clones

\[ w^{1118} ey:FLP; P(ry^{+}=neoFRT)42 D P(w^{+mc}=Ubi:GFP,ns)2R1P \]

Egfr\textsuperscript{tsla} Minute\textsuperscript{tsla} clones

\[ w^{1118} ey:FLP; P(ry^{+}=neoFRT)42 D P(w^{+mc}=Ubi:GFP,ns)2R1P \]

Egfr\textsuperscript{tsla} M(2)53 clones

\[ P(ry^{+}=hsFLP)1 w^{1118}; P(ry^{+}=neoFRT)42 D P(ry^{+}=tsla)42 D P(w^{+mc}=arm-lacZ)2V15 D M(2)53. \]

Egfr\textsuperscript{tsla} M(2)53 clones

\[ P(ry^{+}=hsFLP)1 w^{1118}; P(ry^{+}=neoFRT)42 D P(ry^{+}=tsla)42 D P(w^{+mc}=arm-lacZ)2V15 D M(2)53. \]

Egfr\textsuperscript{tsla} M(2)65i clones

\[ P(ry^{+}=hsFLP)1 w^{1118}; P(ry^{+}=neoFRT)42 D P(ry^{+}=tsla)42 D P(w^{+mc}=arm-lacZ)2V15 D M(2)53. \]

Egfr\textsuperscript{tsla} M(2)65i clones

\[ P(ry^{+}=hsFLP)1 w^{1118}; P(ry^{+}=neoFRT)42 D P(ry^{+}=tsla)42 D P(w^{+mc}=arm-lacZ)2V15 D M(2)53. \]

Wild-type M(2)53 clones

\[ P(ry^{+}=hsFLP)1 w^{1118}; P(ry^{+}=neoFRT)42 D P(ry^{+}=tsla)42 D P(w^{+mc}=arm-lacZ)2V15 D M(2)53. \]

**Sequencing of Egfr\textsuperscript{tsla} DNA**

PCR primer pairs were as follows.

Exon 1, type I: forward primer (fp), ATAGCTTGGAAGGGCTTTGATT; reverse primer (rp), TGGCCAGCACAATCGTACCC.

Exon 1, type II: fp, TTGACTAGCCACAACACGCCAC; rp, CAATATTATGTGCACTTCAG;

Exon 2: fp1, GGGTCAACCAACAAACACACACACAC; fp2, TGCATCAGCCACCTAAATTCGG; rp1, TGACTGGATATAGTGTATCAGTATAAGCT;

Exon 3: fp1, CTACAATTACCTGCGGAGCACC; fp2, CCACAGTGGCCAGAAGTCTGC; fp3, CGGCCATGGCGCAATGACTACAT; fp4, GTCTACATGCTGGCGCAATACAT; fp5, GGAACCCACC-CGCAGTCCCGGAAAT; fp6, TGCCGCGCGCTTCAGAAGAG; rp1, GACATGACGCGACAAATGTT; rp2, TCTATTATGCTG-GATGACAC; rp3, GATCTCTTCCTACGGTGAGAA; rp4, GGAGCAGCGTCCATCCAGGG; rp5, CTTCTTGGCATACGCCATCGTC;

Exon 4: fp1, GAGAAAAATGGAACCCATTGTGCTC; fp2, GACATGGATCCGAGCAAAATGTT.

Exon 5: fp1, GTCTGCAGCGAGACTGC; fp2, GAA-CAGGTGTGCTCAACAGT; fp3, CGCAGTCCGGCCACCAAT; fp4, GAACTGATCTGCGAGGTGCG; rp1, GCACTGATCCGCGAAATGTT; rp2, TCTATTATGCTG-GATGACAC; rp3, GATCTCTTCCTACGGTGAGAA; rp4, GGAGCAGCGTCCATCCAGGG; rp5, CTTCTTGGCATACGCCATCGTC; rp6, ACATCGGCTGCTGCGGAGC.

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ACT; rp3, AATGGCCAGATAGCGACCCTC; rp4, CACACAAAA-GGCCAGACATC; rp5, ACCCTCCGGACACCAAAACGC; rp6, CACCAAGACCCACAGTGTAGTGTAGTCGCCCAGACACTCGT.

Sequencing was done at the University of Iowa facility.

**Antibodies and histochemistry**

Two rabbits were injected with an Egfr C-terminal peptide [(C)QRELQLPHRNTRTETR] (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). Secondary 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-189), mouse anti pMAPK [1:500 for blots, 1:100 for immunohistochemistry], Sigma M-8159 (Gabay et al., 1997), rabbit anti-MAPK [1:1000, Promega 23783], 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-mouse HRP (1:40, 115-175-003), goat anti-rabbit TRITC (1:150, 115-175-003), goat anti-rat HRP (1:8000, 65-6120) was from Zymed. Syto-24 was from Molecular Probes (1:1000). 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-189), mouse anti pMAPK [1:500 for blots, 1:100 for immunohistochemistry], Sigma M-8159 (Gabay et al., 1997), rabbit anti-MAPK [1:1000, Promega 23783], 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-mouse HRP (1:40, 115-035-003), goat anti-guinea pig TRITC (1:150, 116-025-003). Goat anti-rabbit-HRP (1:8000, 65-6120) was from Zymed. Syto-24 was used to stain DNA (1:10000, Molecular Probes S-7559).

**Tissue culture**

The Egfr+ plasmid pMTEgfrType I was a gift of N. Baker (Lesokhin et al., 1999). The Egfrtsla plasmid pMTEgfrtsla Type I was derived from pMTEgfrType I using a Stratagene ‘QuickChange’ Site-Directed Mutagenesis Kit. The secreted Spitz plasmid is pMTSpitz, a gift of B.-Z. Shilo (Schweitzer et al., 1995). Schneider’s line 2 (S2) cells were maintained in Drosophila Schneider’s medium (Invitrogen 11720-034) with 10% heat-inactivated Fetal Bovine Serum at 25°C. Transfections were performed with Cellfectin (Invitrogen 10362-010) in serum-free medium for 4 hours 25°C. DNA concentrations used for transfections were 2-3 μg/ml for wild-type and mutant Egfr plasmids, or 12 μg/ml for pMTSpitz. Egfr transfections were incubated at 18°C for 15-16 hours in serum + medium, then for 24 hours in serum-free medium with 100 μM CuSO4. Fresh medium containing 10 μg/ml cycloheximide (CHX) was added and the cells were then treated as described in the text. We tested cycloheximide between 0 and 100 μg/ml, as assayed by 35S-methionine incorporation (data not shown). Spitz transfections were incubated at 25°C, following the same protocol except for the use of 0.7 mM CuSO4, Spitz supernatants were validated by immunoblot (data not shown). Cells were lysed with RIPA buffer as described previously (Schweitzer et al., 1995). Gel blots were performed by standard protocols (BioRad). Immunoprecipitation was performed as previously described (Schweitzer et al., 1995).

For surface biotinylation, the transfections and temperature shifts were as above, the cells were then incubated with 0.5 mg/ml EZ-Link™ sulfo-NHS-Biotin (Pierce, 21217) at 4°C for 30 minutes and 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 antigen 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.

**Results**

**Egfrtsla lesion**

We sequenced Egfrtsla and found a single coding change relative to the parent chromosome: 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 (EgfrMT2) 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), whereas Egfrtsla does not.

As a second test for receptor activity we measured autophosphorylation. Again, we transfected S2 cells at 18°C for Egfr+ or Egfrtsla expression. The cells were grown at either 18°C or 30°C for one hour and then treated with ligand (Fig. 1D). We assayed anti-Egfr immunoprecipitates for phospho-Tyrosine (Fig. 1E,F). We detected phosphorylated Egfr for both...
Egfr\(^{+}\) and Egfr\(^{tsla}\) 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\(^{tsla}\) at 30°C, perhaps through instability (see below).

We previously reported that Egfr\(^{tsla}\) mutants loose pathway activity within 30 minutes at 29°C in vivo (Kumar et al., 1998). We therefore determined whether Egfr\(^{tsla}\) 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\(^{tsla}\) antigen after the temperature shift. We always detected robust levels of MAPK phosphorylation driven by both Egfr\(^{+}\) and Egfr\(^{tsla}\) at 18°C. However, Egfr\(^{tsla}\) lost this activity within 15 minutes at 30°C in all experiments (Fig. 2B-D).

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

**Egfr\(^{tsla}\) 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\(^{tsla}\) 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. 4E,F). In contrast, transfected cells expressing Egfr\(^{+}\) show robust levels of Egfr and pMAPK (Fig. 4G,H).

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**Fig. 1.** Time course of Spitz induction of Egfr signaling in S2 cells. (A) Protocol used in B,C, see text. (B) Blot probed for Egfr. Second, non-specific band (asterisk) serves as a loading control. The DNA used and the times after Spitz induction are indicated above. (C) Blot for pMAPK shows pathway activation (same gel as is shown in B); asterisk indicates the level of pMAPK activation after 5 minutes. Note, the pMAPK intensity at 0 minutes (no time with Spitz induction) is indistinguishable from that of the untransfected controls. Also note, there is no detectable pMAPK (above controls) in the Egfr\(^{tsla}\) transfections, whereas there is detectable Egfr antigen. (D) Protocol used in E-H, see text. Lysates were prepared by immunoprecipitation using anti-Egfr. (E,F) Blot probed for phospho-Tyr (pTyr). Note that, at 18°C (E), the anti-Egfr precipitable pTyr signal is detectable over control (black asterisk) for the Egfr\(^{+}\) and Egfr\(^{tsla}\) transfections (white asterisks), whereas at 30°C (F), Egfr\(^{+}\) yields detectable anti-Egfr precipitable pTyr signal (white asterisk), but Egfr\(^{tsla}\) does not (black asterisk). (G,H) The same gels as in E,F, re-probed for Egfr antigen.

**Fig. 2.** Egfr\(^{tsla}\) is rapidly made inactive by a shift to 30°C. (A) Diagram of the protocol, see text. (B) Blot probed for Egfr. The DNA used and the time incubated at 30°C are indicated. (C) Blot probed for pMAPK shows pathway activation (same gel as is shown in B). Note, pMAPK levels appear constant over 60 minutes at 30°C for wild-type Egfr. However, for Egfr\(^{tsla}\), although pMAPK is detected at 18°C, after the shift to 30°C, the level falls rapidly down to background levels. Asterisks in B and C indicate examples of the 60-minute time-point bands used in the quantification shown in Fig. 3. (D) Same blot as in C, re-probed for total MAPK (loading control).
Transfected cells express elevated levels of Egfr antigen (Fig. 4E-P). Cells that express additional Egfr at 30°C (white arrowheads in Fig. 4E-H) and 18°C (not shown), and Egfr at 18°C (Fig. 4I-L), drive increased levels of nuclear pMAPK antigen (arrows in Fig. 4F,H,J,L), but cells expressing Egfr at 30°C do not (Fig. 4N,P).

All of these biochemical and cell biological experiments show that Egfr is a mutation that is temperature sensitive for activity. The level of ligand-dependent Egfr 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 Egfr mutants rapidly lose pMAPK antigen from the morphogenetic furrow (Kumar et al., 1998). We conclude that Egfr is indeed a rapidly acting mutation that is temperature sensitive for activity. Egfr is effectively wild type at 18°C and effectively null at 30°C.

Egfr 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 Egfr at 30°C (see above). It could be that after temperature shift, the Egfr 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 to total Egfr (Fig. 5B) to total Egfr antigen (see asterisk in Fig. 5B). We detected biotinylated Egfr at all time points from cells transfected with Egfr. Biotinylated Egfr 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 Egfr 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 Egfr homzygous clones (data not shown). We stained wild-type eye discs and
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 ommatidial (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
Development

**Egfr** mutations are very small and are similar to **Egfr** total area of their last day (Fig. 7B), or continuously at 30°C (Fig. 7C). The clones continuously at 18°C (Fig. 7A), at 18°C and then at 30°C for one day (although they are reduced towards the posterior side). However, the clones are very much smaller after continuous growth at 30°C. As previously reported (Domínguez 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 a null. 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
eight Egfr alleles in trans to wild type (Egfrh, Egfr2
and
Egfrtsl, 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 Egfr2
in trans to Minute(2)53 (Baonza et al., 2001), and we placed
Egfrtsl in trans to Minute(2)56i (Yang and Baker, 2001). We
also placed Egfrh in trans to both Minute(2)53
and
Minute(2)56i. All four clones 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 Egfrh 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, Egfrh 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
mutations 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 recombinant. 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
dominant synthetic interaction between the Minute
mutations and Egfr.

It may be that cell competition effects between the Minute+
and Minute heterozygous territories produce the Atonal
patterning defects. Such competition effects have been reported in
other Minute
experiments (de la Cova et al., 2004). Another
possibility is that the dying Minute+ cells release developmental
signals that affect retinal patterning in the surrounding tissues.
Indeed, it has been reported that dying cells release both
Wingless and Dpp (Perez-Garjio et al., 2004; Ryoo et al., 2004).
If either of these possibilities is true, then we would expect to
see defects in the Atonal expression pattern when clones are
induced from Minute heterozygous flies, even without any Egfr
mutation present in trans. In other words, fully wild-type clones
adjacent to Minute heterozygous territories would have Atonal
defects similar to those seen in the Egfr mutant clone-containing
discs. We stained such wild-type clones for Atonal expression,
and do indeed see just such defects, both within and outside of
the clones (Fig. 9P-R). We observed a total of fifteen such
cloned in the Atonal expression domain, taken from five
imaginal discs. Indeed, the Atonal pattern in these Minute
(alone) clones is indistinguishable from that in clones
for the same Minute with Egfr (compare Fig. 9Q to 9E-H).

Our data suggest that the Atonal patterning defects seen in
mosaic clone experiments, when the Minute technique is used,
may be due to a genetic effect of the Minute, and not of the
Egfr, mutation.

Discussion

We characterized the lesion in Egfrh 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 (Egfrtsla) for three phenotypes (Kumar et al., 1998). Here, we have shown in addition that Egfrtsla mutants at 30°C are phenotypically indistinguishable from nulls (Egfrtsla2 and Egfrtsla0), 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 morphogenetic furrow and the morphogenetic furrow.

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

Fig. 9. Two different Minute mutations affect Atonal expression, whereas Egfr alleles do not. Eye discs stained for Atonal are shown in A-C,E,F,H,I,K,L,N,O,R. Clones are negatively marked by β-gal (white in D,G,J,M,P, blue in F,I,L,O,R) and are outlined. (A-C) Egfr alleles in trans to wild type; note normal Atonal expression. (D-R) hs:FLP-induced clones induced in trans to Minute chromosomes. (D-O) β-gal-positive territories are heterozygous for the Egfr allele and for the Minute mutation. Black (β-gal negative) territories are homozygous for the Egfr allele and are wild type for the Minute mutation. Black (β-gal negative) territories are heterozygous for the Minute mutation. Black (β-gal positive) territories are heterozygous for the Minute mutation. Black (β-gal negative) territories are genetically (but not phenotypically) wild type. No Minute+ homozygous cells survive. Mutations are indicated on the left. Note, the Atonal pattern is not wild type in either the Egfr homozygous (black in D-O) or the Egfr heterozygous territories (compare the β-gal-positive regions to the heterozygous examples shown in A-C). Also, the Atonal pattern is not wild type in either the wild-type territories (white arrows in P-R) or the adjacent Minute heterozygous cells (black arrows in P-R). Note that Minute(2)53 and Minute(2)56i have different, but self-consistent dominant effects on Atonal expression. Atonal expression in the Egfrtsla Minute+ clones is indistinguishable from that in the two Egfr null alleles. Scale bar: 10 μm.
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 effect 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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