The *Drosophila* TGF\(\alpha\) homolog Spitz acts in photoreceptor recruitment in the developing retina

Murni Tio† and Kevin Moses*

Department of Biological Sciences, University of Southern California, 825 W. 37th Street, Los Angeles, CA 90089-1340, USA

*Author for correspondence (e-mail: kmoses@mizar.usc.edu)

† Present address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511

**SUMMARY**

In vertebrates and *Drosophila*, the Epidermal Growth Factor Receptor (EGFR) signal transduction pathway is important in the regulation of cellular development. EGFR is bound by several activating ligands including Transforming Growth Factor-\(\alpha\) in vertebrates, and its homolog Spitz in *Drosophila*. It has been shown that Spitz and EGFR act in the development of the *Drosophila* central nervous system and compound eye. Here we show that *spitz* function is required in developing ommatidia for the first cell recruitment step, and that Spitz pro-protein is expressed in the retinal neurons as they begin to differentiate. We propose a ‘two-key’ model for additive signal transduction from EGFR and other receptor tyrosine kinases, via the Ras pathway, in the developing eye.

**Key words:** *Drosophila*, spitz, EGF-receptor, retina, patterning

**INTRODUCTION**

The EGFR family are transmembrane receptor tyrosine kinases that transduce signals from outside of cells, into the cytoplasm, where they activate a cascade of cytoplasmic and nuclear functions collectively known as the Ras pathway (reviewed by McCormick, 1993; Perrimon, 1994; Schlessinger and Bar-Sagi, 1994) and play a role in early vertebrate development (reviewed by Wiley et al., 1995). Activating ligands for EGFR family receptors include small polypeptides of the Epidermal Growth Factor (EGF) family, as well as those of Transforming Growth Factor-\(\alpha\) (TGF-\(\alpha\)) family (reviewed by Massagué, 1990; Derynck, 1992). TGF-\(\alpha\) is translated as a pro-protein with an N-terminal signal sequence, an extracellular domain, a transmembrane domain and a C-terminal domain. Four cleavage sites exist and the smallest diffusible form is a 52 residue peptide. Both cleaved (diffusible) and membrane tethered forms are expressed and can act as ligands (reviewed by Massagué, 1990; Derynck, 1992). The *Drosophila* Argos protein has been proposed to act as a negative regulatory ligand of the EGFR (Schweitzer et al., 1995a). Activating mutations of the EGFR are among the most common causes of human cancer and EGFR gain-of-function mutations have been isolated as oncogenes (reviewed by Aaronson, 1991). In mice, loss-of-function mutations of the EGFR (\textit{waved-2}) produce epithelial, nervous system and other phenotypes including defects in eye development, and overexpression of the EGFR can cause changes in retinal cell fate (Luetke et al., 1994; Lillien, 1995). Similarly, loss-of-function mutations for TGF-\(\alpha\) affect epithelial and eye development (\textit{waved-1}; Luetke et al., 1993; Mann et al., 1993), and TGF-\(\alpha\) has been shown to act as a chemo-attractant in developing mouse eyes (Reneker et al., 1995). A second TGF-\(\alpha\) family member, Glial Growth Factor (GGF), also plays a role in mammalian retinal development (Bermingham-McDonogh et al., 1996).

The *Drosophila* EGFR homolog acts at several points in development, and mutations have been identified (and named) for three different phenotypes. \textit{torpedo} (\textit{Egfr}\textsuperscript{\textit{bop}}) alleles are maternal effect mutations that affect the dorso/ventral (d/v) patterning of the oocyte (Clifford and Schüpbach, 1992), \textit{faint little ball} (\textit{Egfr}\textsuperscript{\textit{blb}}) alleles are zygotic mutations that affect the development of the ventral midline and central nervous system (CNS; Mayer and Nüsslein-Volhard, 1988; Price et al., 1989) and \textit{Ellipse} (\textit{Egfr}\textsuperscript{\textit{Elp}}) alleles are dominant mutations that affect the eye (see below and Baker and Rubin, 1989) and wing (Sturtevant and Bier, 1995). Two TGF-\(\alpha\)-like activating ligands are known in *Drosophila*: Gurken, which is secreted by the oocyte and functions in d/v patterning (reviewed by Lehman, 1995) and Spitz, which acts in patterning the embryonic ventral midline and CNS (Rutledge et al., 1992), and later in imaginal development (Freeman, 1994; Tio et al., 1994; Sturtevant and Bier, 1995). The Spitz protein is roughly equally similar to mammalian TGF-\(\alpha\) and GGF, with strong sequence similarities in the diffusible factor domain, as well as in the overall domain structure of the pro-protein. While the cleavage sites have not been conserved, a diffusible form of Spitz can function in vivo (Schweitzer et al., 1995b). There are several alternatively spliced *spitz* mRNAs (Rutledge et al., 1992; Tio et al., 1994), and the regulation of ligand transcription and posttranslational maturation are likely to be complex. In addition to *Egfr* and *spitz*, mutations at several other loci have similar effects on embryonic CNS and midline development, and these have been dubbed ‘the *spitz* group’ (Mayer and Nüsslein-Volhard, 1988). These are thought to be members of the EGFR signal transduction pathway, and include downstream functions, such as the transcription factors Pointed and Single-minded (Crews et al.,...
1988, Klämbt, 1993), as well as proteins that may act upstream in ligand maturation such as Star and Rhomboid (Bier et al., 1990; Sturtevant et al., 1993; Kolodkin et al., 1994).

The Drosophila compound eye consists of about 800 facets or ommatidia, each containing 20 cells: eight photoreceptor neurons (conventionally numbered R1 through R8) and twelve accessory cells (Ready et al., 1976). The adult retina develops from the eye field of the larval eye-antennal imaginal disc (Weismann, 1864). Early in larval development the presumptive eye field is an unpatterned monolayer epithelium. In the last larval instar a wave of progressive development, known as the morphogenetic furrow, sweeps across this field from posterior to anterior (Ready et al., 1976; Tomlinson and Ready, 1987). This furrow is associated with changes in cell shape, cell-cycle, gene expression and developmental pattern formation and is driven by a molecular system that includes segment polarity and basic-HLH genes (reviewed by Heberlein and Moses, 1995). In the furrow all of the cells are held in G1 arrest and evenly spaced ommatidial preclusters are established, first as a shell of cells around a rosette, which later resolve to five cell preclusters. These five cells differentiate as neurons (by antigenic and morphological criteria) in a precise order: R8, then R2 and R5 as a pair, then R3 and R4 (Tomlinson and Ready, 1987; Wolff and Ready, 1991). The cells surrounding the five cell preclusters undergo a final round of the cell-cycle, and then constitute an equivalence group from which the remaining three photoreceptors, and all twelve accessory cells are selected by local positional signaling (reviewed by Banerjee and Zipsursky, 1990; Cagan, 1993).

Notch and Delta are clearly involved in ommatidial spacing (Cagan and Ready, 1989; Baker and Zitron, 1995), and it has also been suggested that the EGFR mediates lateral inhibition between nascent ommatidia in the morphogenetic furrow, based on the fact that Egrf/E trim (gain-of-function) homoyzgoeotes appear to have increased inter-ommatidial spacing (Baker and Rubin, 1989). However, the retinal phenotype of Egrf loss-of-function mosaic clones is not decreased spacing, but a failure of neural differentiation (Xu and Rubin, 1993) and it may be that the Egrf+E trim phenotype can be better interpreted as a progressive disorientation of the furrow. We and others have examined adult retinal mosaic clones and shown that the Drosophila TGFα/EGF homolog, spitz is required for the normal development of ommatidia (Freeman, 1994; Tio et al., 1994). Furthermore, partial loss-of-function spitz mutations do not decrease spacing between the developing ommatidia but result in a less precise array of developing clusters, and reduced numbers of cells in each ommatidium in the adult (Tio et al., 1994).

We have developed a new system to negatively mark mosaic clones in the developing retina and we present data here (using this system) to show that the primary spitz defect in the developing retina is a failure of the first photoreceptor recruitment step: the R8 cell appears to develop normally but no further cells differentiate as neurons. We have developed antisera against the Spitz pro-protein and find that consistent with the mosaic data, Spitz is expressed in the photoreceptor neurons as they begin to differentiate. The simplest interpretation of these new data is that the founding R8 cell signals to the future R2 and R5 cells by means of Spitz and the EGFR. However, our previous data show that in rare cases spitz wild-type R2 and R5 cells can rescue an ommatidium with a spitz mutant R8 cell, and that would imply reverse signaling. A second difficulty with this simple model is that of signal specificity: the EGFR is not the only receptor-tyrosine kinase to signal in these cells via the Ras cascade (Simon et al., 1991; Perrimon et al., 1995). How can a signal from the EGFR via the Ras cascade instruct one cell to differentiate as an R2/5 cell, and a later signal from Sevenless via the same Ras cascade instruct another cell to differentiate as an R7 cell? We now suggest a ‘two-key’ model for the function of receptor tyrosine kinase signaling in the developing retina: that the quantity of Ras activation is critical (and must reach a threshold level), that the Ras pathway signal is permissive for retinal cell differentiation but is not cell-type specific, that different receptors act in an additive manner to achieve full Ras activation and that other factors (such as the pattern of pre-existing transcription factors) control retinal cell-type.

**MATERIALS AND METHODS**

**Drosophila stocks and mosaic clones**

The wild-type stock used was Canton-S. Nineteen new insertions of the β-galactosidase expression element ‘construct D’ (Moses and Rubin, 1991) were recovered and mapped by chromosome in situ hybridization (as described by Langer-Safer et al., 1982). Four were chosen to generate recombinants with FRT elements (as described by Xu and Rubin, 1993), as appropriate for mosaic analysis experiments for each of the four major autosomal chromosome arms: P[construct D]25A P[rs-hsp70:neoFRT]40A, P[rs-hsp70:neoFRT]24D P[construct D]56A, P[construct D]69E P[rs-hsp70:neoFRT]80E and P[rs-hsp70:neoFRT]82B P[construct D]96A. For the mosaic experiments, males of the genotype P[rs-hsp70:FLP]1 w1118; P[w+;ry+]/SC1 spitz[P[rs-hsp70:neoFRT]40A/TSTL+/+ were crossed to virgin females of the genotype P[rs-hsp70:FLP]1 w1118; P[construct D]25A P[rs-hsp70:neoFRT]40A/TSTL+. TSTL is a T(2;3) translocation double balancer between In(2LR)O Cy on and TM6B Tb Hu c. The progeny were then heat-shocked once at late embryogenesis and once again in the first early instar (37°C, 2 hours). Eye discs were prepared from Tb+ third instar larvae and stained as described below. The spitz alleles used were: spitz1, spitz1cl and spitz2cl, which have been shown to behave genetically as nulls (Tio et al., 1994). The kinesin-β-galactosidase stock was a gift from Ed. Ginger (described by Clark et al., 1994).

**Histology**

Eye discs were prepared (as described by Tomlinson and Ready, 1987) and in some cases sectioned in a freezing microtome. Samples were mounted in Vectashield (Vector Labs, H-1000). Embryos were prepared as described by Dequin et al. (1984). Samples were examined with DIC illumination or by laser-scanning confocal microscopy as appropriate. Primary antibodies were: rabbit anti-β-galactosidase (Cortex Biochem, CR7001P21), mouse anti-β-galactosidase (Promega, Z378A), mouse mAb 22C10 (gift from Larry Zipursky and Seymour Benzer), rat anti-Elav (from University of Iowa, Developmental Studies Hybridoma Bank), rabbit anti-SalM (gift from Ulrike Gaul), rabbit anti-Beh1 (gift from Kwang-Wook Choi), mouse monoclonal anti-Boss (gift from Larry Zipursky), rabbit anti-Ato (gift from Yuh-Nung Jan and Andrew Jarman) and mouse anti-Sca (gift from Nick Baker). Secondary antibodies used were: Cy5-conjugated goat anti-mouse (Jackson Labs, 115-176-003), or goat anti-rabbit (Jackson Labs, 111-176-003), FITC-conjugated goat anti-mouse (Jackson Labs, 115-095-003), or goat anti-rat (Jackson Labs, 112-096-003), or goat anti-rabbit (Jackson Labs, 116-096-003), HRP-conjugated goat anti-mouse (Biorad, 170-6516), LRSC-conjugated donkey anti-rat (Jackson Labs, 170-6516) and TRITC-conjugated donkey anti-mouse (Jackson Labs, 170-6516) primary Abs, and mouse Abs 22C10 (1:1000) and 22C5 (1:1000) (gift from Larry Zipursky). Secondary Abs were goat anti-mouse (Jackson Labs, 115-176-003), or goat anti-rabbit (Jackson Labs, 116-096-003), or goat anti-rat (Jackson Labs, 112-096-003), or goat anti-rabbit (Jackson Labs, 116-096-003), HRP-conjugated goat anti-mouse (Biorad, 170-6516), LRSC-conjugated donkey anti-rat (Jackson Labs, 170-6516) and TRITC-conjugated donkey anti-mouse (Jackson Labs, 170-6516). S-phase cells were detected by 5-bromo-2′-deoxyuridine incorporation, BrdU, as described by Wolff and Ready, 1991, using Sigma BrdU (catalog no. B-0631) and detected with mouse anti-BrdU (Becton Dickinson, 347580). Cytoplasmic actin was
detected with rhodamine-conjugated phalloidin (Molecular Probes, R-415). Whole-mount in situ hybridization to eye imaginal discs was as described by Tautz and Pfeifle, (1989) and modified by Thomas et al. (1994).Probe was RNA from a spitz cDNA (clone BA3; Tio et al., 1994) labeled with digoxigenin-dUTP using a labeling kit (Boehringer, 1175-025). Signal was detected with rhodamine-conjugated anti-digoxigenin Fab-fragment (Boehringer, 1207-750).

**Spitz antiserum**

DNA encoding amino acids 28-70 from the amino-terminal of the spitz protein was amplified by PCR with restriction enzyme linked primers (EcoRI and BamHI) and ligated in frame into pGEX-2T (Amrad Corp.) and verified by sequencing. Fusion protein was isolated essentially as described by Smith and Johnson (1988). Five Swiss Webster mice were immunized five times with 10-20 μg of fusion protein per injection by the CIT hybridoma facility. Sera were screened on embryos and eye imaginal discs. Polyclonal ascites were purified by ammonium sulfate precipitation and dialyzed against PBS before use (Harlow and Lane, 1988).

**RESULTS**

**spitz is required for the initial recruitment step in ommatidial assembly**

We and others have previously shown that spitz is required in the initial five precluster cells (R8, 2, 5, 3 and 4) of the developing ommatidium (Freeman, 1994; Tio et al., 1994), and that the strength of this requirement follows the order in which these cells differentiate as neurons (almost absolutely required

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**Fig. 1. spitz phenotypes in the developing retina.** All panels show mosaic clones negatively marked with β-galactosidase and double stained to show other phenotypes (see Materials and Methods). β-galactosidase is shown in green (except for H, in which it is brown) note that the homozygous spitz areas show no β-galactosidase expression. Anterior is to the right, arrowheads mark the furrow, and the scale is the same in all panels. A and B show developing neurons in red, stained with mAb 22C10 (A) and anti-Elav (B). Note that the clusters in mutant areas contain only one neuron. C and D show the expressions of proteins in cells that are recruited after the R8 cell. C shows anti-SalM in red (normally expressed in R3 and R4). D shows anti-BarH1 in red (normally expressed generally in the furrow, and later in R1 and R6). Note that there are no R3, R4, 1 or 6 cells in the spitz mutant clones. E,F and G show the expression of proteins that act upstream of R8 cell specification or in the R8 cell itself. E shows anti-Boss in red, F shows anti-Atonal in red and G shows anti-Sca in red. Note that the remaining neuron in each cluster in the spitz mutant clones is an R8 cell by these antigenic criteria, and that spitz mutations do not affect the specification, pattern or differentiation of the R8 photoreceptor cells. H shows a marker for cell-cycle: the incorporation of BrdU (in black). Note that the ‘second wave’ of S-phase cells are not affected by spitz. Bar in A is 25 μm.
in R8, strongly required in R2 and 5 and weakly required in R3 and 4). However, these results were based on an analysis of the phenotypes of adult retinal mosaic clones, and thus could not resolve the primary developmental defect in ommatidial development. We thus chose to examine such mutant clones earlier in development, at the time when the furrow is still moving in the third larval instar, using the FLP recombinase system (Xu and Rubin, 1993). To overcome technical difficulties with the MYC epitope marking system, we made use of a β-galactosidase based marker. Construct ‘D’ (Moses and Rubin, 1991) has been shown to direct the uniform expression of β-galactosidase in the larval eye imaginal disc, and we reinjected this element, selected insertions on the four major autosomal arms and placed them in cis to appropriate FRT elements to negatively mark mosaic clones (see Materials and Methods).

We induced spitz mutant mosaic clones with three different spitz alleles (see Materials and Methods), and obtained indistinguishable results. In each case, we visualized the clones by staining with an anti-β-galactosidase antibody. We are able to distinguish clearly both the homozygous mutant and wild-type twin-spots from the heterozygous background tissue. We double-stained to examine several developmental markers within the mutant tissue. Markers of neural differentiation (mAb 22C10 and Elav, Fujita et al., 1982; Robinow and White, 1991) normally show sequential expression in devel-

**Fig. 2.** spitz expression in the developing eye. Third larval instar eye imaginal discs stained to show spitz mRNA and protein expression. Anterior is to the right and arrowheads mark the furrow.

(A) shows anti-SpiN. Note staining in the distal tips of cells in the furrow, and later punctate, perinuclear staining. B shows spitz mRNA. Note the general similarity to the pattern of the SpiN antigen. C-F show surface views of one developing retina, stained for cytoplasmic actin (C and green in F); Elav (D and blue in F); and SpiN (E and red in F). Note that the early furrow expression of SpiN correlates to the ‘rosettes’ seen with the actin stain, and that the later perinuclear SpiN staining correlates with neural differentiation as seen with Elav. G-J show a section of one developing retina, stained for cytoplasmic actin (G) and green in J); Elav (H and blue in J); and SpiN (I and red in J). Note that the early furrow expression of SpiN is largely apical, the later perinuclear SpiN staining decorates those nuclei that stain with Elav, and there is additional SpiN antigen in the apical tips of the developing neurons and in their efferent axons. K-N show surface views of one developing retina, containing a spitz homozygous mutant clone to serve as a control for the specificity of the SpiN antiserum. The three stains are: β-galactosidase (K) and green in N); Elav (L and blue in N); and SpiN (M and red in N). Note that the early furrow expression of SpiN is not affected by the spitz mutant clone, and that the later perinuclear SpiN staining is eliminated in the spitz mutation. The following groups of panels are to the same scale: (A,B,G-J), (C-F), (K-N) and all scale bars are 25 μm.
oping ommatidia: first in the R8 cell, then in R2 and R5 (to give a three cell stage), then in R3 and R4 (a five cell stage), then in R1 and R6 and finally in R7 (Tomlinson and Ready, 1987). In the spitz mutant clones the initial single neuron stage ommatidia appear normal, as do their spacing. However, no further progression occurs, and the clusters appear to be arrested at the one neuron stage (Fig. 1A,B). We tested two markers that are specific for later cell types: SalM (for R3 and R4; Fig. 1C; Reuter et al., 1996) and BarH1 (for R1 and R6; Fig. 1D; Higashijima et al., 1992) and found that these markers are not expressed in the spitz mutant clones. We examined a marker that is expressed in the founding R8 photoreceptor cell (Boss; Fig. 1E; Krämer et al., 1991) and its expression is unaffected by spitz, as are the products of two genes that act upstream of R8 cell specification: Atonal (Ato; Fig. 1F; Jarman et al., 1994) and Scabrous (Sca; Fig. 1G; Mlodzik et al., 1990; Baker and Zitron, 1995). We observed that the wave of DNA synthesis immediately following the furrow (Ready et al., 1976; Wolff and Ready, 1991) is unaffected by spitz (Fig. 1H). We also examined the developing rosettes and preclusters in the furrow using a stain against cytoplasmic actin (phalloidin; Wolff and Ready, 1991) and found that the early development of the preclusters is normal up to the five-cell stage (data not shown). We conclude that in the spitz mutant clones, development proceeds normally anterior to and in the furrow (up to the specification and early differentiation of the founding R8 photoreceptor cells) but that while cells do occupy the correct adjacent positions, the first recruitment step fails (specification of the R2 and R5), as do all subsequent steps. Thus the primary spitz defect in the developing retina is a failure of the first recruitment step.

It is important to note that the homozygous spitz mutant clones are apparently equal in size to their homozygous wild-type twin-spots. This suggests that there is no prior spitz function between the time that the clones are induced (first instar), and the arrival of the morphogenetic furrow. This is very different from the results reported for homozygous mutant clones for null alleles of the Egfr (Xu and Rubin, 1993), which are much smaller than their twin-spots. This suggests that the EGFR must have other, earlier functions that are linked to other ligands, or are ligand independent.

**spitz mRNA and protein are expressed in the developing eye, and embryonic brain**

The mosaic data presented above suggest a simple model: that the R8 cell secretes Spitz to induce the two neighboring cells to be specified as R2 and R5. However, it has been previously shown that spitz function is partially required in R2 and R5 as well as in R8 (and in rare cases wild-type R2 and R5 cells can rescue the development of an ommatidium with a mutant R8 cell; Tio et al., 1994). This must eliminate the simple model, and taken together, all these mosaic data suggest that Spitz may be secreted by each cell-type as it differentiates as a non-specific and general positive inductive factor. We generated an antiserum against the Spitz pro-protein to determine if the expression pattern follows this developmental series.

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**Fig. 3.** SpiN expression in the stage 13 embryo. Embryos are stained to show Elav (green) and SpiN (red). Anterior is to the left. A-C are parasagittal optical sections and D-F are horizontal optical sections. Note that Elav and high level perinuclear SpiN staining do not overlap, and that the SpiN-positive cells are in the position reported for the optic lobe neuroblasts attached to the basal surfaces of the brain hemispheres. Scale bar for A,B,D, 50 μm; C,E, F, 50 μm.
expression collocates with the first column of repeated clusters in the furrow (the ‘rosette’ stage; Wolff and Ready, 1991) as seen with cytoplasmic actin. The later, perinuclear expression closely follows the expression of Elav in the developing neurons. Star is required in the early pre-cluster cells (Heberlein et al., 1993) and encodes a probable transmembrane protein (Kolodkin et al., 1994). Star mutations affect the gain-of-function phenotype of ectopically expressed full length Spitz, but not of a truncated diffusible form, and this suggests that Star may act in Spitz protein maturation (Schweitzer et al., 1995b). Taken together these data suggest that Star may act upstream of Spitz, and regulate its translation or maturation. The localization of both spitz mRNA andSpiN antigen in perinuclear granules suggests that these may represent a regulated and limiting step in the secretory/maturation pathway for Spitz that might involve Star.

The Boss and Sevenless signaling molecules are concentrated in apical microvilli in the developing ommatidial clusters (Tomlinson et al., 1987; Cagan et al., 1992) and therefore we expected to find Spitz to be apically localized also. We examined the position of the SpiN antigen in sections of developing eye-imaginal discs (Fig. 2G-J). We found that the later expression is not only located in perinuclear granules, but also in the apical tips of the cluster cells, and in the efferent basal axons. This axonal expression is interesting as it suggests that Spitz may be subject to anterograde transport and signal to cells in the developing brain, as is Hedgehog protein (Huang and Kunes, 1996). We examined the expression of the SpiN antigen in the developing embryo. There is a low level of staining in the developing ventral nerve cord, but the highest level of expression seen is perinuclear and is localized in the position of the presumptive optic lobe neuroblasts, which are just ventral to the brain hemispheres (Hartenstein, 1993) at stage 13 (Fig. 3).

To control for the specificity of the SpiN antiserum, we stained homozygous spitz mutant mosaic clones in the developing retina (with three different EMS induced alleles, see Materials and Methods and Fig. 2K-N). The results were indistinguishable with all three alleles: the later perinuclear SpiN staining is undetectable in the spitz mutant tissue, and thus the later staining represents SpiN antigen in vivo. However, in the control clones there was some residual early signal in the furrow and thus this early furrow stain is likely to represent a cross-reacting epitope (not a spitz gene product).

**Spitz function correlates with the establishment of apical/basal nerve cell polarity**

The morphogenetic furrow is associated with changes in cell shape and nuclear position (Tomlinson, 1985). Anterior to the furrow, cells span the epithelium from the basal to the apical side, and the nuclei lie at random levels. Cells undergoing mitosis transiently lose their basal attachment. In the furrow the apical actin cytoskeleton constricts, and the epithelium bows inwards and nuclei are forced to the basal side. Following the furrow, nuclei rise only as the cells are specified as neurons. Similar epithelial dynamics are seen in other developing systems, such as the vertebrate central nervous system (reviewed by Walsh, 1996). In the Drosophila oocyte, changes in cell cytoskeletal polarity and nuclear movements are associated with localization of gurken mRNA (which, like Spitz, is a TGFβ homolog; Lehman, 1995).

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**Fig. 4.** Epithelial cell polarity follows the furrow. A section of one developing retina, stained for cytoplasmic actin (A and red in C) and kinesin-β-galactosidase (B and green in C). Note that following the furrow a subset of cells can be seen to develop a polarized expression pattern of kinesin-β-galactosidase, coincident with their nuclei rising. Later kinesin-β-galactosidase is localized only in the basal axons. Anterior is to the right, arrowheads mark the furrow. Scale bar 25 μm.
To see if similar phenomena are associated with *spitz* expression in the developing eye, we examined sections of eye imaginal discs that carried a transgene that directed the expression of a kinesin-β-galactosidase fusion protein (Clark et al., 1994). This fusion protein localizes to the positive ends of cytoplasmic microtubules. We found that anterior to the furrow, there is no localization of the fusion protein, but that beginning in the furrow, individual cells begin to localize the fusion protein, as their nuclei rise. Later (more posteriorly) the kinesin-β-galactosidase fusion protein is localized to the basal efferent axons (Fig. 4). While the time of nuclear rising corresponds to the onset of spitz transcription, unlike gurken, the *spitz* mRNA is evenly distributed around the nuclei (Fig. 2B), and is not limited to the apical side, as the gurken analogy might suggest.

**DISCUSSION**

Previously published mosaic analyses have shown that *spitz* function is almost essential in the founding R8 photoreceptor cell (99.3%), but that it is also required to a lesser degree in the next two cells (R2 and R5, ~50%), and weakly in the last two cells of the five-cell precluster (R3 and R4, Freeman, 1994; Tio et al., 1994). Here we have shown that the primary defect for loss of *spitz* function in the developing eye is an arrest of ommatidial neural induction at the first recruitment step: the founding R8 cells are specified in their normal pattern, and begin to differentiate (they express neural and R8 cell-type specific antigens), but they fail to recruit the next two cells (R2 and R5), and the clusters progress no further. Taken together, these results suggest to us a model for the function of Spitz in eye development as follows: Spitz protein is a permissive, but non-specific positive signal for neural specification. The first neuron (R8) is specified by mechanisms in the morphogenetic furrow that are independent of Spitz, involving Notch, Delta, Scabrous and basic-HLH proteins including Extra-macrochaetae, Daughterless and Atonal (reviewed by Heberlein and Moses, 1995). Once the R8 cell has begun to differentiate, it secretes Spitz, which can diffuse a short distance (perhaps only a single cell diameter), and this is a necessary signal for the specification of R2 and R5 (hence the strong *spitz* requirement in the R8 cell, and the primary defect in mosaic clones). Once R2 and R5 begin to differentiate they also secrete Spitz protein, which increases the local Spitz concentration, and may help with the recruitment of R3 and R4 (hence the partial *spitz* requirement in R2 and R5). As R3 and R4 differentiate they add yet more Spitz to the local domain of the developing cluster, and this may be an inductive signal for the development of the remaining photoreceptors. Thus, as the cluster develops, a local domain of Spitz protein is established and increases in concentration (consistent with the SpiN antigen expression series that we observe), and this signals to the nearby uncommitted cells (via the EGFR) at each step to push them towards cell-type specification (see Fig. 5A). Furthermore, Spitz is not specific to any one retinal cell type, but is a general stimulus towards such specification. The positive inductive effects of Spitz secreted by the developing ommatidia may be balanced and antagonized by Argos secreted by the surrounding cells (Schweitzer et al., 1995a).

One other receptor tyrosine kinase (RTK), Sevenless, is known to act in ommatidial assembly, and there may be more. It is also clear that both Sevenless and the EGFR signal through the Ras pathway (reviewed by McCormick, 1993; Perrimon, 1994). How can these two (and maybe more) signals both pass through the same signal transduction cascade, and what is their purpose? We propose a ‘two key’ model for this dual function (Fig. 5B): just as no single (crazy) man can turn both keys and launch an intercontinental ballistic missile, perhaps no single

![Fig. 5. A ‘two key’ model for signal transduction in the developing eye. (A) Diagram representing part of the developing eye imaginal disc. Anterior is to the right, and the small circles represent the field of cells. The furrow is shown as a blue line on the right. In the first column of developing ommatidia, a single cell (the future R8) is expressing Spitz (pink), which diffuses out to the adjacent cells (yellow circles). As more cells join the cluster, more cells express Spitz, and the concentration of the Spitz protein rises. This creates zones of partially activated cells around each developing cluster. More distant cells are not activated at all. (B) is a diagram illustrating the ‘two key’ model for RTK signal transduction. A single target cell is shown. It receives two signals: one mediated by Spitz and the EGFR (red), and a second signal mediated by Boss and Sevenless (green). Each contributes partially to the activation of those components of the Ras cascade that are common to both. Each signal is necessary, neither is sufficient to trigger the differentiation of the target cell. The EGFR signal is common to all recruitment steps, and specifies no particular photoreceptor cell type (it is general and permissive). Other RTKs may substitute for Sevenless in other target cells.](image-url)
REFERENCES


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