

# Combinatorial signaling in the specification of primary pigment cells in the *Drosophila* eye

Raghavendra Nagaraj and Utpal Banerjee\*

In the developing eye of *Drosophila*, the EGFR and Notch pathways integrate in a sequential, followed by a combinatorial, manner in the specification of cone-cell fate. Here, we demonstrate that the specification of primary pigment cells requires the reiterative use of the sequential integration between the EGFR and Notch pathways to regulate the spatiotemporal expression of Delta in pupal cone cells. The Notch signal from the cone cells then functions in the direct specification of primary pigment-cell fate. EGFR requirement in this process occurs indirectly through the regulation of Delta expression. Combined with previous work, these data show that unique combinations of only two pathways – Notch and EGFR – can specify at least five different cell types within the *Drosophila* eye.

**KEY WORDS:** *Drosophila*, Notch, Pigment cells, EGFR, Lz, Combinatorial signaling

## INTRODUCTION

A majority of cell-fate-specification events during metazoan development depend upon short-range signaling between adjacent cells. Given the extensive array of cell types in most multicellular organisms and the rather limited number of conserved signaling pathways, one must postulate that these pathways will function reiteratively during development. However, it is unclear how temporal specificity is generated such that, in a given tissue, the same signaling components used at different times mediate unique fate-specification events. In this study, we use the *Drosophila* eye as a model to demonstrate that two multifunctional signaling pathways can function reiteratively to generate several different cell fates from a set of pluripotent precursors.

The *Drosophila* eye has long been used to study cell fate specification mediated by short-range intercellular signals between adjacent cells (Freeman, 1997; Ready et al., 1976). Studies from several laboratories have shown that EGFR and Notch function in multiple events in the developing *Drosophila* eye (reviewed in Baker, 2001; Freeman, 2002; Voas and Rebay, 2004). Activation of EGFR by its ligand initiates the canonical Ras-Raf-MAPK pathway, culminating in the phosphorylation of the ETS-domain protein Pointed (Pnt), which binds to upstream enhancer elements and promotes transcriptional activation (reviewed in Shilo, 2005). Likewise, activation of the Notch receptor in the eye by its ligand Delta causes a cascade of proteolytic events resulting in the generation of a cleaved intracellular domain, Notch<sup>intra</sup>, which migrates to the nucleus, binds to Su(H) and activates the transcription of target genes (reviewed in Artavanis-Tsakonas et al., 1999). Both EGFR and Notch pathways are regulated at the level of ligand expression, as the receptors and the components downstream of them are ubiquitously expressed.

The developing *Drosophila* eye disc exhibits two phases of patterning as cells exit from the morphogenetic furrow, which is a dynamic zone for cell-fate-specification events. In the first phase,

groups of five cells (R8, R2/R5, R3/R4) attain photoreceptor fate and constitute the ‘precluster’. The rest of the cells undergo a terminal round of cell division and form a pool of multipotent undifferentiated cells that contribute to the formation of both neuronal (R1, R6 and R7) as well as non-neuronal (cone and pigment) cell types. The differentiation of all of these cell types is dependent on the function of EGFR, Notch and the Runt-domain-containing protein Lozenge (Lz) (reviewed in Nagaraj et al., 2001). Depending upon the context, the EGFR and Notch pathways function either in a synergistic or antagonistic fashion in the specification of unique cell fates (Flores et al., 2000; Yang and Baker, 2006). In photoreceptor cells (R cells), EGFR activation promotes the transcription of *Delta* by a proteasome-mediated mechanism involving the nuclear proteins Sno and Ebi (Tsuda et al., 2002). This is a derepression mechanism in which EGFR/Sno/Ebi promote the nuclear export of SMRTER, disrupting the Su(H)-SMRTER repressor complex that keeps *Delta* repressed. Thus, EGFR activation provides a localized source of Notch signal that leads to a sequential integration of the Notch and EGFR pathways during development (Fig. 1A). Delta that is expressed in the R cells then signals the adjacent undifferentiated cells and promotes their differentiation into cone cells. In addition to Notch, the specification of cone-cell fate also requires EGFR activation and the transcription factor Lz. The earliest marker for this cell fate is the expression of the *Drosophila* Pax2 (D-Pax2) protein (Fu and Noll, 1997), the expression of which is controlled in cone cells by the direct binding of Pnt, Su(H) and Lz (Flores et al., 2000). This highlights the importance of direct integration of signaling pathways in a combinatorial fashion to activate key cell-fate regulators.

Genetic studies have suggested that later in the pupal eye discs, specification of the primary pigment-cell fate involves Notch and EGFR (Cagan and Ready, 1989a; Cagan and Ready, 1989b; Freeman, 1996; Miller and Cagan, 1998), but their precise role has not been defined. In this paper we show that EGFR activation in the pupal cone cells causes the transcriptional upregulation of Delta. The activation of the Notch signal in the adjacent undifferentiated cells in combination with Lz expression promotes their specification into primary pigment cells. Thus, the entire logic of sequential integration of EGFR and Notch pathways is used reiteratively to regulate the spatiotemporal expression of Delta in R cells and then again later in cone cells. This Notch signal then integrates with the

Department of Molecular, Cell and Developmental Biology, Department of Biological Chemistry, Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA.

\*Author for correspondence (e-mail: banerjee@mbi.ucla.edu)

cell-type-specific transcription factor Lz in a cell autonomous manner, independently of the EGFR pathway, in the specification of primary pigment-cell fate.

## MATERIALS AND METHODS

### *Drosophila* stocks

*UAS-EGFR<sup>DN</sup>*, *UAS-N<sup>DN</sup>*, *UAS-ebi<sup>DN</sup>* and *UAS-Delta* (*UAS-Dl*) were obtained from the Bloomington stock center. *UAS-EGFR<sup>act</sup>* on the third chromosome was obtained from T. Suchpbach (Queenan et al., 1997). *EGFR<sup>To1</sup>* and *EGFR<sup>ts1</sup>* were obtained from K. Moses (Kumar et al., 1998). *FRT42D EGFR<sup>ts1</sup>* was obtained from G. Campbell (Wang et al., 2000). *spa-Gal4* was obtained from M. Noll (Kronhamm et al., 2002). *54C-Gal4* driver line was obtained from R. Cagan. *UAS-Su(H)<sup>DN</sup>* was generated using *Xenopus* Su(H) DNA binding mutant cDNA [obtained from C. Kintner (Wettstein et al., 1997)], cloned in a pUAST vector and transgenic flies generated using standard procedures.

### Heat-shock protocols

For the temperature-shift experiments involving *N<sup>ts</sup>* and *EGFR<sup>ts</sup>*, *lz<sup>ts114</sup>* white pupae were collected and heat pulsed for 10 hours during the mid-pupal stages (10–20 hours after pupal formation). Pupal eye disc were dissected and stained with the appropriate antibodies.

For heat-shock experiments involving the expression of *EGFR<sup>DN</sup>* and *N<sup>DN</sup>*, white pupae were collected and heat pulsed at 30°C for 12 hours during the mid-pupal stages (10–25 hours after pupal formation). Pupal eye disc were dissected and stained using anti-Bar antibody (Hayashi et al., 1998).

### EGFR<sup>ts</sup> clones

*EGFR<sup>ts1</sup>* clones were generated using the *ey-flp/FRT* system (Newsome et al., 2000). The cross was maintained at 18°C. White pupae from the cross were collected and subjected to a non-permissive temperature (29°C) for 10 hours during the mid-pupal stages. Pupal eye discs were dissected following heat shock and stained with anti-Delta or anti-Cut antibody.

### Immunohistochemistry

Larval or pupal eye discs were dissected in PBS and fixed in 4% formaldehyde in PBS for 40 minutes. The fixed tissue was permeabilized in PBST (0.4% Triton) and incubated in primary antibody [anti-Delta, 1/100; anti-Bar 1/50; anti-Cut, 1/20; anti-Dlg, 1/20; and anti-β-Gal, 1/50 (mouse; Promega) and 1/200 (rabbit; Capel)] overnight, washed in PBST three times and incubated in an appropriate secondary fluorescent-labeled antibody. Images were captured using a BioRad Confocal microscope.

## RESULTS AND DISCUSSION

The events leading to the activation of Delta in the photoreceptor cells of the third instar eye disc are summarized in Fig. 1A (see also Flores et al., 2000; Tsuda et al., 2002). Unlike R cells, cone cells do not express Delta at this stage of development (Parks et al., 1995). However, we found that these same cone cells express Delta at the pupal stage (Fig. 1B,C). In addition, correlated with this Delta expression, we observed the upregulation of phosphorylated MAPK in these cells (Fig. 1D–F). This is very similar to the earlier events seen in R cells during larval development, in which the activation of MAPK causes the expression of Delta (Tsuda et al., 2002). Also, as in the larval R cells, the pupal upregulation of Delta in cone cells is transcriptional. The *Delta-lacZ* reporter construct, off in the larval cone cell (Fig. 1G–I), is detected in the corresponding pupal cone cells (Fig. 1J–L). To determine whether EGFR is required for the activation of Delta in the pupal cone cells, we used the temperature-sensitive allele *EGFR<sup>ts1</sup>* (Kumar et al., 1998). We generated marked clones of this allele in the eye disc using *ey-flp* at permissive conditions and later, in the mid-pupal stages, shifted the larvae to a non-permissive temperature. Cells mutant for EGFR, but not their adjacent wild-type cells, showed a loss of Delta expression (Fig. 2A–C). However, both mutant and wild-type tissues showed normal cone-cell development, as judged by Cut (a cone-cell marker) expression (Fig. 2D–F). As

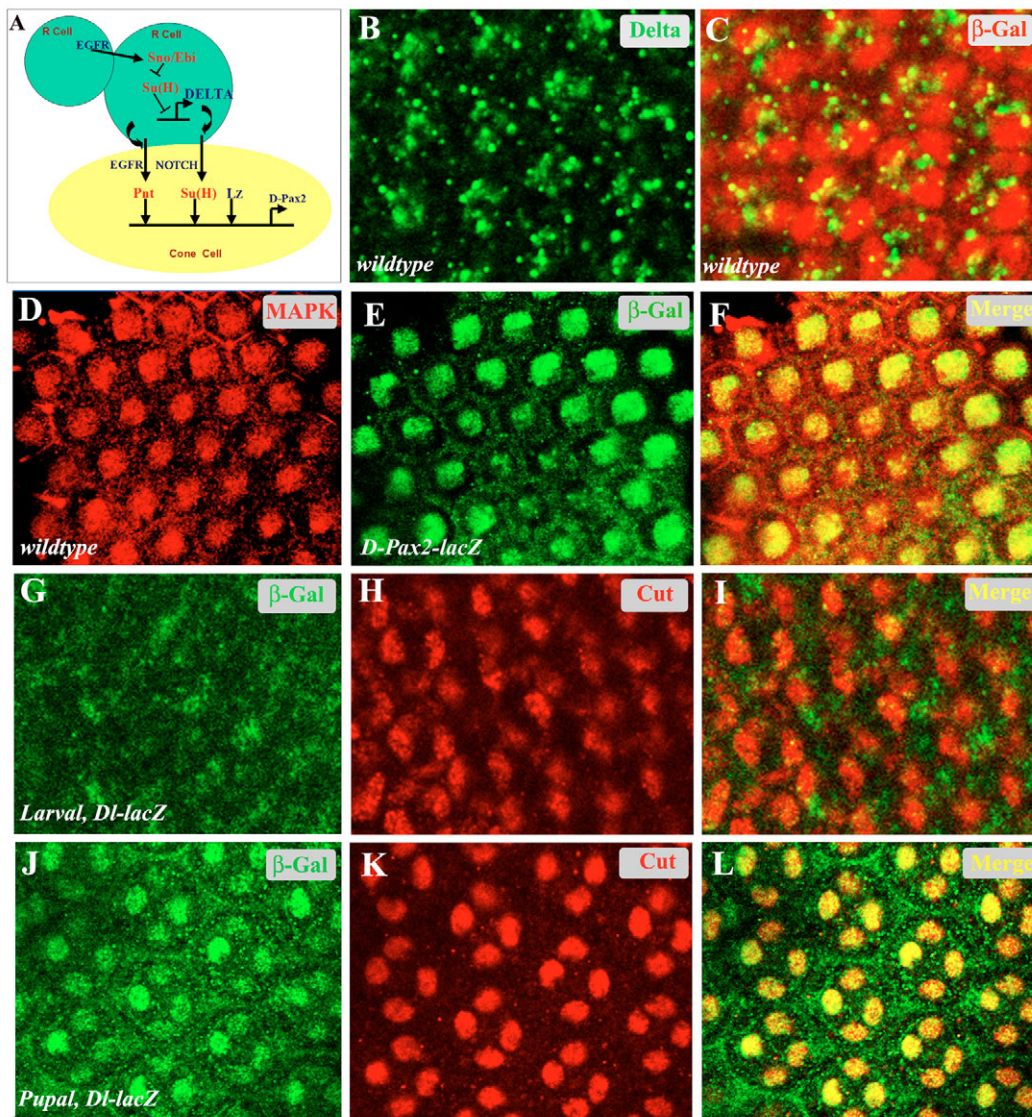
supporting evidence, ectopic expression of a dominant-negative version of *EGFR* (*EGFR<sup>DN</sup>*) in cone cells using *spa-Gal4* after the cells have already undergone initial fate specification also causes a complete loss of Delta expression without compromising the expression of the cone-cell-fate-specification marker (Fig. 2G–J).

Gain-of-function studies further support the role of EGFR signaling in the regulation of Delta expression in cone cells. Although weak EGFR activation is required for cone-cell fate (Flores et al., 2000), activated MAPK is not detectable in cone-cell precursors of the third instar larval eye disc (Fig. 2K). When *spa-Gal4* is used to express an activated version of EGFR in larval cone cells, detectable levels of MAPK activation in these cells were found (Fig. 2L) and the consequent ectopic activation of Delta in the larval cone cells occurred (Fig. 2M,N). Taken together, these gain- and loss-of-function studies show that, during pupal stages, EGFR is required for the activation of Delta. However, this Delta expression is not essential for the maintenance of cone-cell fate.

In larval R cells, the activation of Delta transcription in response to EGFR signaling is mediated by two novel nuclear proteins, Ebi and Sno (Tsuda et al., 2002). To determine the role of these genes in wild-type pupal-cone-cell Delta expression (Fig. 3A–C), we selectively blocked *sno* and *ebi* function in the pupal eye disc. A heteroallelic combination of the temperature-sensitive allele *sno<sup>E1</sup>* and the null allele *sno<sup>93i</sup>* exposed to a non-permissive temperature for 12 hours caused a significant reduction in Delta expression (Fig. 3D–F). Similarly, a dominant-negative version of *ebi* (Dong et al., 1999) also caused the loss of Delta expression (Fig. 3G–I). Importantly, pupal eye discs of neither *spa-Gal4*, *UAS-ebi<sup>DN</sup>* nor *sno<sup>E1</sup>/sno<sup>93i</sup>* showed any perturbation in cone-cell fate, as judged by the expression of Cut (Fig. 3F,I). Thus, as in the case of larval R cells (Tsuda et al., 2002), the loss of *ebi* and *sno* in the pupal cone cells causes the loss of Delta expression without causing a change in cone-cell fate.

To test whether the expression of Delta in pupal cone cells is required for the specification of primary pigment cells, we incubated *N<sup>ts</sup>* pupae at a non-permissive temperature for 10 hours during pupal development and monitored pigment-cell differentiation using BarH1 (also known as Bar) expression (Fu and Noll, 1997; Hayashi et al., 1998) as a marker (Fig. 4A). Loss of Notch signaling during the mid-pupal stages caused a loss of Bar, further demonstrating the requirement of Notch signaling in the specification of primary pigment-cell fate (Fig. 4B). Similarly, when the *54CGal4* driver line, which is activated in pigment cells, was used to drive the expression of a dominant-negative version of Notch (Go et al., 1998), pupal eye discs lost primary pigment-cell differentiation, again suggesting an autonomous role for Notch in pigment-cell precursors (Fig. 4C). In neither the *N<sup>ts</sup>* nor the *54C-Gal4*, *UAS-N<sup>DN</sup>* genetic background, no perturbation was observed in cone-cell fate specification (Fig. 4D–F). We conclude that Delta activation mediated by EGFR-Sno-Ebi in pupal cone cells is essential for neighboring pigment-cell fate specification.

Delta-protein expression in pupal cone cells is initiated at 12 hours and is downregulated by 24 hours of pupal development (Parks et al., 1995). To determine the functional significance of this downregulation, we used the genetic combination of *spa-Gal4/UAS-Delta*, in which Delta is expressed in the same cells as in wild type, but is not temporally downregulated (Fig. 4G). Whereas, in wild type, a single hexagonal array of pigment cells surrounded the ommatidium (Fig. 4H), in the pupal eye disc of *spa-Gal4*, *UAS-Delta* flies, multiple rows of pigment cells were observed surrounding each cluster (Fig. 4I). Furthermore, in wild type, only two primary pigment cells were positive for Bar expression in each

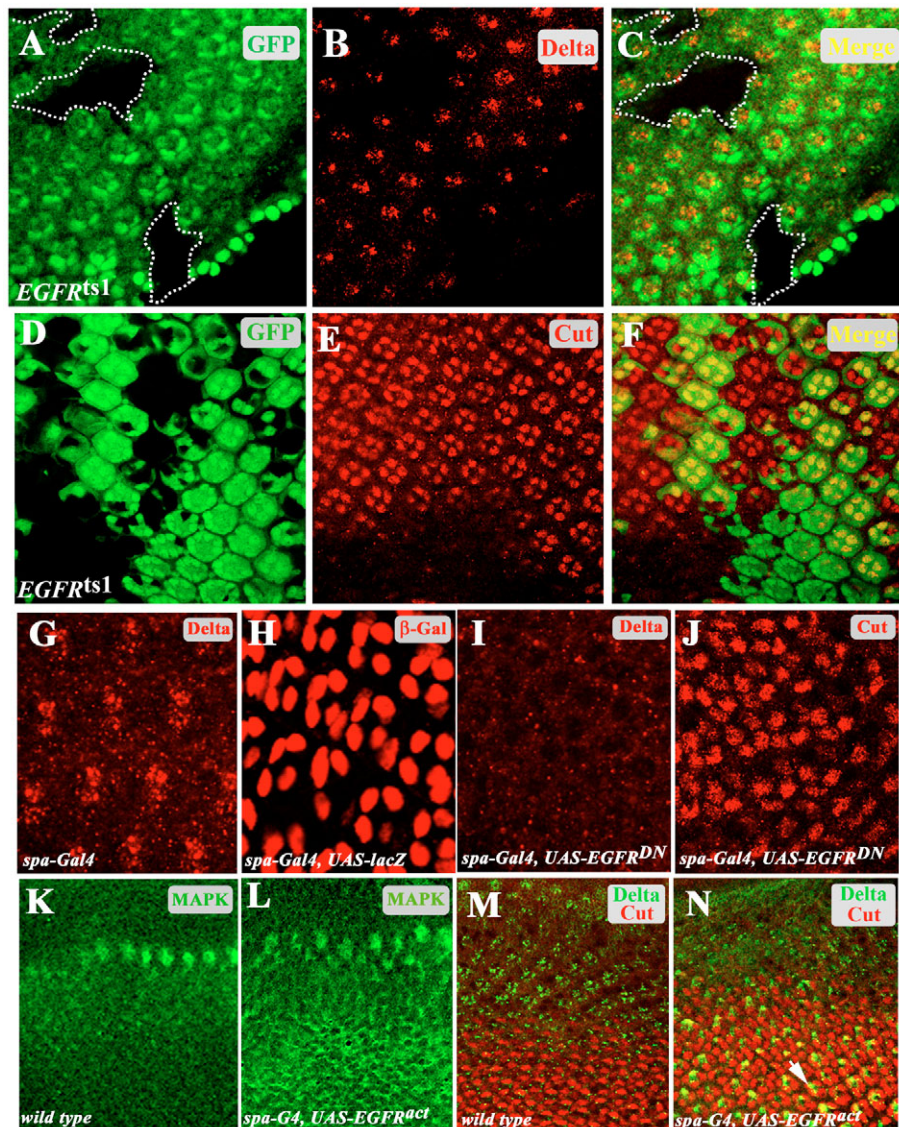


**Fig. 1. EGFR activation and Delta expression in pupal cone cells.** (A) Summary of events in the larval third instar eye disc based on Flores et al. (Flores et al., 2000) and Tsuda et al. (Tsuda et al., 2002). Activation of EGFR in R cells (green) causes the derepression of *Delta*. This process also requires the function of two novel nuclear proteins (Ebi and Sno) and the proteasome complex (Tsuda et al., 2002). Sequential and combinatorial integration of EGFR, Notch and Lz in cone-cell precursors (yellow) causes the expression of *D-Pax2* and other genes involved in the specification of cone-cell fate. (B,C) Wild-type mid-pupal eye disc. Delta protein (green) is expressed in apical tips of pupal cone cells (B). The corresponding nuclei are marked by the expression of *D-Pax2 lacZ* (red, C). (D-F) MAPK activation in cone cells of a mid-pupal eye discs. Eye discs were stained for activated MAPK (red, D) and also for *D-Pax2 lacZ* (green, E). These two signals co-localize in the pupal cone-cell nuclei (yellow, F). (G-L) Delta is transcribed in pupal but not in larval cone cells. (G-I) In third instar eye discs, *Delta-lacZ* (*DI-lacZ*; green, G) is not expressed in cone cells, which are marked with Cut (red, H). Residual *lacZ* expression is in R cells (notice the lack of overlap in I). (J-L) In pupal eye discs, *DI-lacZ* (green, J) is expressed in cone cells, which are marked with Cut (red, K). The overlap is evident in the merged panel (yellow, L).

cluster (Fig. 4A), whereas, in *spa-Gal4, UAS-Delta* pupal eye discs, ectopic expression of Bar was evident in the interommatidial cells (Fig. 4J). Therefore, the temporal regulation of Notch signaling and its activation, as well as its precise downregulation, are essential for the proper specification of primary pigment-cell fate.

By contrast to the autonomous requirement for Notch signaling in primary pigment cells, the function of the EGFR signal appears to be required only indirectly in the establishment of primary pigment-cell fate through the regulation of Delta expression in the pupal cone cells. When a dominant-negative version of EGFR was expressed using *hsp70-Gal4* at 10-20 hours after pupation, we observed no perturbation in the specification of primary pigment cells, as

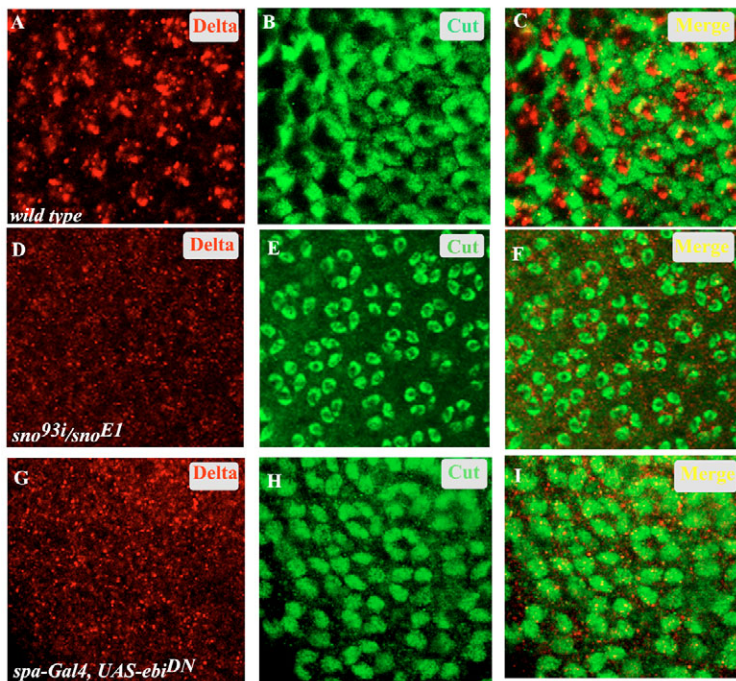
monitored by the expression of the homeodomain protein Bar (Fig. 5A,B). By contrast, the expression of dominant-negative Notch under the same condition resulted in the loss of Bar-expressing cells (Fig. 5C). Thus, in contrast to Notch, blocking EGFR function at the time of primary pigment-cell specification does not block the differentiation of these cells. Importantly, blocking EGFR function in earlier pupal stages caused the loss of Delta expression in cone cells and the consequent loss of pigment cells (Fig. 2A-C). Based on these observations, we conclude that, in the specification of primary pigment-cell fate, the Notch signal is required directly in primary pigment cells, whereas EGFR function is required only indirectly (through the regulation of Delta) in cone cells.



**Fig. 2. EGFR is required for *Delta* expression in pupal cone cells.** (A-C) *Delta* expression in *EGFR<sup>ts1</sup>*-mutant clones subjected to a non-permissive temperature during pupal development. (A) GFP expression marks wild-type cells (green); non-GFP cells are mutant for *EGFR<sup>ts1</sup>*. (B) The same disc as in A was co-stained for *Delta* expression (red). (C) The merged panel shows that the *Delta* protein is restricted to wild-type cells. (D-F) Expression of the cone-cell marker, *Cut*, in *EGFR<sup>ts1</sup>* clones transferred to non-permissive conditions during the mid-pupal stages. (D) GFP marks wild-type tissue; non-GFP cells are mutant for *EGFR*. (E) The same disc as in D was co-stained for *Cut* (red). (F) The merged panel shows that the loss of *EGFR* function in pupal stages does not compromise cone-cell fate. (G-J) Expression of a dominant-negative version of *EGFR* (*EGFR<sup>DN</sup>*) in cone cells blocks *Delta* expression. (G,H) Wild-type (control) pupal eye discs stained for *Delta* showed its expression in cone cells (red, G); the *spa-Gal4* driver was also expressed in cone cells (red, H). *spa-Gal4, UAS-EGFR<sup>DN</sup>* pupal eye discs stained for *Delta* showed loss of *Delta* expression in the cone cells (I), whereas *spa-Gal4, UAS-EGFR<sup>DN</sup>* pupal eye discs stained for *Cut* showed that a loss of *EGFR* function during pupal stages does not disrupt cone-cell fate specification (J). (K-N) Ectopic activation of *EGFR* in cone cells promotes *Delta* expression. MAPK activation in wild-type third instar eye disc (K) is seen in cells at the furrow followed by low levels of activation in the differentiated R cells behind the furrow (K). (L) In *spa-Gal4, UAS-EGFR<sup>act</sup>* third instar eye disc, MAPK is activated at high levels at later stages in the developing cone cells, which express *spa-Gal4*. (M) Wild-type expression of *Delta* (green) in the third instar eye disc is limited to R cells and is not expressed in cone cells (red). (N) In *spa-Gal4, UAS-EGFR<sup>act</sup>* third instar eye disc, ectopic activation of *Delta* (green) is seen in cone cells (red, arrow).

The Runt-domain protein *Lz* functions in the fate specification of all cells in the developing eye disc arising from the second wave of morphogenesis (Daga et al., 1996). At a permissive temperature (25°C), *lz<sup>TS114</sup>* (Gupta and Rodrigues, 1995) pupal eye discs showed normal differentiation of primary pigment cells (Fig. 5D). *lz<sup>TS114</sup>* is a sensitized background in which the *Lz* protein is functional at a threshold level. When combined with a single-copy loss of *Delta*, a

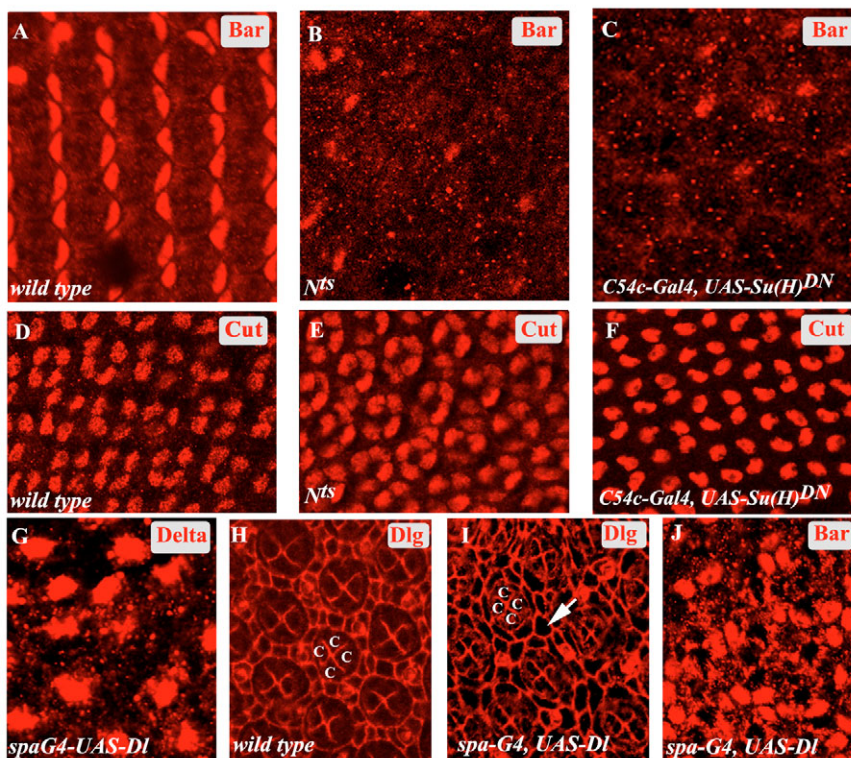
dosage sensitive interaction caused the loss of primary pigment cells (Fig. 5E). By contrast, under identical conditions, a single-copy loss of *EGFR* function had no effect on the proper specification of primary pigment-cell fate (Fig. 5F). This once again supports the notion that the specification of primary pigment cells directly requires *Lz* and *Notch*, whereas *EGFR* is required only indirectly to activate *Delta* expression in cone cells.



**Fig. 3. Delta expression in the pupal cone cells requires the function of *ebi* and *sno*.** (A-C) Delta protein expression is observed in pupal cone cells in wild type (red, A) and is reduced in pupal cone cells of *sno<sup>E1</sup>/sno<sup>93i</sup>* (D-F) and *spa-Gal4, UAS-ebi<sup>DN</sup>* (G-I) genotypes. Cone cells are marked with Cut (green; B,E,H) and the merged panels are shown in C,F and I.

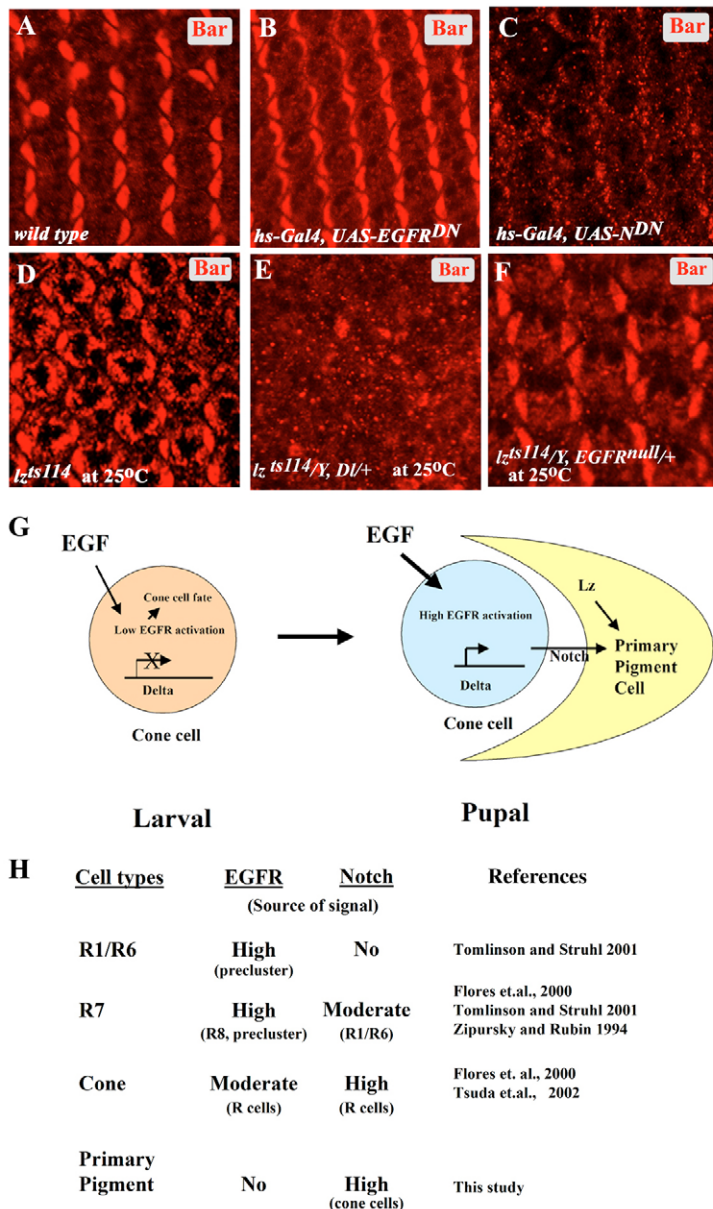
This study highlights two temporally distinct aspects of EGFR function in cone cells. First, this pathway is required for the specification of cone-cell fate at the larval stage, and EGFR is then required later in the pupal cone cell for the transcriptional activation of Delta, converting the cone cell into a Notch-signaling cell. Delta that was expressed in the cone cell through the activation of the Notch pathway functioned in combination with Lz in a cell-autonomous fashion and promoted the specification of the primary pigment-cell fate (Fig. 5G).

Studies using overexpressed secreted Spitz have shown that ectopic activation of the EGFR signal in all cells of the pupal eye disc results in excess primary pigment cells (Freeman, 1996). Here, we show that EGFR activation in the pupal eye disc is required for the transcriptional activation of Delta in cone cells, but that the loss of EGFR function at the time when primary pigment cells are specified does not perturb their differentiation. We conclude that the ectopic primary pigment cells seen in an activated-EGFR background result from the ectopic activation of Delta, which then



**Fig. 4. Requirement of Notch signaling for primary pigment-cell specification.**

(A-C) Pupal eye discs stained for Bar. Bar expression is seen in the primary pigment cells (A) and is lost in *N<sup>ts</sup>* pupal eye discs shifted to a non-permissive temperature 10 hours after pupation (B) or when *UAS-Su(H)<sup>DN</sup>* is expressed in the pigment cells using the pigment-cell-specific driver *54C-Gal4* in pupal eye discs (C). (D-F) Wild-type cut expression in pupal cone cells (D) remains unchanged in *N<sup>ts</sup>* when shifted to a non-permissive temperature 10 hours after pupation (E) or in an *54C-Gal4 UAS-Su(H)<sup>DN</sup>* background (F). (G-J) Overexpression of Delta in pupal cone cells using the *spa-Gal4, UAS-Delta (UAS-Dl)* combination causes ectopic primary pigment-cell specification; a high level of expression of the Delta protein is shown (G, compare with Fig. 1B). (H) Wild-type pupal eye disc stained for Dlg to mark the membranes shows four cone cells and a single row of pigment cells between ommatidia. (I) *spa-Gal4, UAS-Dl* pupal eye disc stained for Dlg show multiple rows of cells between ommatidia (arrow). (J) *spa-Gal4, UAS-Dl* pupal eye disc stained for Bar show the over specification of Bar-positive primary pigment cells.



**Fig. 5. Requirement for Lz and Notch, but not EGFR, in primary pigment-cell specification.** (A-F) Pupal discs stained for Bar (red). (A) Wild-type mid-pupal eye disc showed Bar expression in two primary pigment cells per cluster. (B) *hsp70-Gal4-UAS-EGFR<sup>DN</sup>* pupal eye disc subjected to heat shock at 29°C at the mid-pupal stage showed no defects in Bar expression or primary pigment-cell specification. (C) *hsp70-Gal4, UAS-N<sup>DN</sup>* pupal eye discs subjected to heat shock at 29°C at the mid-pupal stage showed a loss of Bar expression. (D) Primary pigment-cell specification in *Lz<sup>ts114</sup>* at a permissive (25°C) temperature showed a wild-type pattern of primary pigment-cell specification. (E,F) Dosage-sensitive interaction between *Notch* and *Lz* during primary pigment-cell fate specification. (E) *Lz<sup>ts114/Y</sup>, Delta/+ (Dl/+)* combination incubated at 25°C showed dosage-sensitive interactions causing a loss of Bar-expressing primary pigment cells. (F) *Lz<sup>ts114</sup>, EGFR<sup>null/+</sup>* pupal eye disc from flies incubated at 25°C showed no disruption in the specification of primary pigment-cell fate. (G) Signal integration during primary pigment-cell specification. In the larval cone cells, a low level of EGFR activation is required for the establishment of their fate but is insufficient to promote transcriptional activation of Delta. Once cone-cell fate is established, EGFR activation continued to rise and, in the pupal stages, caused the transcription of Delta. Activation of the Notch pathway in the adjacent undifferentiated cells in combination with Lz promoted the specification of primary pigment-cell fate. This process did not require input from the EGFR pathway. (H) Summary of cell-autonomous combinations of Notch and EGFR inputs in the specification of neuronal (R1, R6 and R7) and non-neuronal cell types from undifferentiated cells behind the furrow. The fate specification of these cells also requires a transcriptional input from Lz.

signals adjacent cells and promotes their differentiation into primary pigment cells. Indeed, we show here that excessive Delta activity results in the over specification of primary pigment cells. Our results are also consistent with the previous observation that the EGFR target gene *Argos* is not expressed in primary pigment cells in pupal eye discs (Wildonger et al., 2005). Additionally, Frankfort and Mardon have shown that the loss of EGFR function in pupal eye discs does not perturb the normal patterning of interommatidial bristle development, which develop even later than the primary pigment cells (Frankfort and Mardon, 2004).

The elucidation of the Sevenless pathway for the specification of R7 led to the suggestion that different cell types within the developing eye in *Drosophila* will require combinations of dedicated signaling pathways for their specification (Tomlinson, 1988). However, studies from several laboratories have suggested that the Sevenless pathway seems to be an exception, in that cell-fate-specification events usually require reiterative combinations of a very small number of non-specific signals (Voas and Rebay, 2004). Cone-cell fate is determined by the sequential integration of the

EGFR and Notch pathways in R cells followed by the parallel integration of the EGFR and Notch pathways in cone-cell precursors (Flores et al., 2000; Tsuda et al., 2002; Xu et al., 2000). Here, we show that the most important function of EGFR in the specification of primary pigment cells is to promote the transcriptional activation of Delta in cone cells through the EGFR-Ebi-Sno-dependent pathway. The sequential integration of the EGFR and Notch pathways, first used in the larval stage for Delta activation in R cells, is then reused a second time in cone cells to regulate the spatiotemporal expression of Delta, converting the cone cells at this late developmental stage to Notch-signaling cells. Delta present in the cone cell then signals the adjacent undifferentiated cells for the specification of primary pigment cells. For this process, the Notch pathway functions directly with Lz but indirectly with EGFR. Through extensive studies of this system (Cagan and Ready, 1989a; Cagan and Ready, 1989b; Flores et al., 2000; Freeman, 1996; Tomlinson and Struhl, 2001; Xu et al., 2000) it now seems conclusive that different spatial and temporal combinations of Notch and EGFR applied at different levels can generate all the signaling

combinations needed to specify the neuronal (R1, R6, R7) and non-neuronal (cone, pigment) cells in the second wave of morphogenesis in the developing eye disc (Fig. 5H).

The EGFR and Notch pathways are sequentially integrated, in a manner similar to that described here, in multiple locations during *Drosophila* development. In the development of wing veins, EGFR that is activated in the pro-vein cells causes the expression of Delta, which then promotes the specification of inter-vein cells (Shilo, 2005). Similarly, these two pathways are sequentially integrated in the patterning of embryonic and larval PNS, and during muscle development (Artero et al., 2003; Modolell, 1997). Indeed, there are striking similarities between the manner in which the EGFR and Notch pathways are integrated in the developmental program in the *C. elegans* vulva and the *Drosophila* eye (Sundaram, 2005). During vulval fate specification in the *C. elegans* hermaphrodite gonad, anchor cells are a source of EGFR signal (Lin3), which induces the specification of the nearest (P6) cell to the primary cell fate from within a group of six equipotent vulval precursor cells (VPC) (Sternberg, 2005). This high level of EGFR activation induces the transcriptional activation of Notch ligands in the primary cells in what can be considered sequential integration of the two pathways (Chen and Greenwald, 2004) – the Notch signal from the primary cell both inhibits EGFR activity in the VPCS on either side of P6.p and also promotes the secondary cell fate (Yoo et al., 2004). Thus, the reiterative integration of these two signals, in series and in parallel, can be used successfully to specify multiple cell fates in different animal species. Given that the RTK and Notch pathways function together in many vertebrate developmental systems, it is likely that similar networks will be used to generate diverse cell fates using only a small repertoire of signaling pathways.

We thank G. Campbell, R. Cagan, K. Moses, M. Noll and T. Schupbach for fly stocks. We are grateful to T. Kojima for providing the anti-Bar antibody. Monoclonal anti-Delta, anti-Cut and anti-Dlg antibodies, developed by S. Artavanis Tsakonas, G. Rubin and C. Goodman, respectively, were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by NIH grant EY08152 to U.B.

## References

- Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.
- Artero, R., Furlong, E. E., Beckett, K., Scott, M. P. and Baylies, M. (2003). Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during *Drosophila* myogenesis. *Development* **130**, 6257-6272.
- Baker, N. E. (2001). Cell proliferation, survival, and death in the *Drosophila* eye. *Semin. Cell Dev. Biol.* **12**, 499-507.
- Cagan, R. L. and Ready, D. F. (1989a). The emergence of order in the *Drosophila* pupal retina. *Dev. Biol.* **136**, 346-362.
- Cagan, R. L. and Ready, D. F. (1989b). Notch is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3**, 1099-1112.
- Chen, N. and Greenwald, I. (2004). The lateral signal for LIN-12/Notch in *C. elegans* vulval development comprises redundant secreted and transmembrane DSL proteins. *Dev. Cell* **6**, 183-192.
- Daga, A., Karlovich, C. A., Dumstrei, K. and Banerjee, U. (1996). Patterning of cells in the *Drosophila* eye by Lozenge, which shares homologous domains with AML1. *Genes Dev.* **10**, 1194-1205.
- Dong, X., Tsuda, L., Zavitz, K. H., Lin, M., Li, S., Carthew, R. W. and Zipursky, S. L. (1999). ebi regulates epidermal growth factor receptor signaling pathways in *Drosophila*. *Genes Dev.* **13**, 954-965.
- Flores, G. V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M. and Banerjee, U. (2000). Combinatorial signaling in the specification of unique cell fates. *Cell* **103**, 75-85.
- Frankfort, B. J. and Mardon, G. (2004). Senseless represses nuclear transduction of Egrf pathway activation. *Development* **131**, 563-570.
- Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87**, 651-660.
- Freeman, M. (1997). Cell determination strategies in the *Drosophila* eye. *Development* **124**, 261-270.
- Freeman, M. (2002). A fly's eye view of EGF receptor signalling. *EMBO J.* **21**, 6635-6642.
- Fu, W. and Noll, M. (1997). The Pax2 homolog sparkling is required for development of cone and pigment cells in the *Drosophila* eye. *Genes Dev.* **11**, 2066-2078.
- Go, M. J., Eastman, D. S. and Artavanis-Tsakonas, S. (1998). Cell proliferation control by Notch signaling in *Drosophila* development. *Development* **125**, 2031-2040.
- Gupta, B. P. and Rodrigues, V. (1995). Distinct mechanisms of action of the Lozenge locus in *Drosophila* eye and antennal development are suggested by the analysis of dominant enhancers. *J. Neurogenet.* **10**, 137-151.
- Hayashi, T., Kojima, T. and Saigo, K. (1998). Specification of primary pigment cell and outer photoreceptor fates by BarH1 homeobox gene in the developing *Drosophila* eye. *Dev. Biol.* **200**, 131-145.
- Kronhamn, J., Frei, E., Daube, M., Jiao, R., Shi, Y., Noll, M. and Rasmuson-Lestander, A. (2002). Headless flies produced by mutations in the paralogous Pax6 genes *eyeless* and *twin of eyeless*. *Development* **129**, 1015-1026.
- Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B. Z. and Moses, K. (1998). Dissecting the roles of the *Drosophila* EGF receptor in eye development and MAP kinase activation. *Development* **125**, 3875-3885.
- Miller, D. T. and Cagan, R. L. (1998). Local induction of patterning and programmed cell death in the developing *Drosophila* retina. *Development* **125**, 2327-2335.
- Modolell, J. (1997). Patterning of the adult peripheral nervous system of *Drosophila*. *Perspect. Dev. Neurobiol.* **4**, 285-296.
- Nagaraj, R., Canon, J. and Banerjee, U. (2001). *Cell fate specification in the Drosophila eye*. In *Drosophila Eye Development* (ed. K. Moses), pp. 73-88. Heidelberg: Springer-Verlag.
- Newsome, T. P., Asling, B. and Dickson, B. J. (2000). Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-860.
- Parks, A. L., Turner, F. R. and Muskavitch, M. A. (1995). Relationships between complex Delta expression and the specification of retinal cell fates during *Drosophila* eye development. *Mech. Dev.* **50**, 201-216.
- Queenan, A. M., Ghabrial, A. and Schupbach, T. (1997). Ectopic activation of *torpedo/Egfr*, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. *Development* **124**, 3871-3880.
- Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53**, 217-240.
- Shilo, B. Z. (2005). Regulating the dynamics of EGF receptor signaling in space and time. *Development* **132**, 4017-4027.
- Sternberg, P. S. (2005). Vulval development. In *Worm Book* (ed. The *C. elegans* Research Community), doi/10.1895/wormbook.1.6.1, <http://www.wormbook.org>.
- Sundaram, M. V. (2005). The love-hate relationship between Ras and Notch. *Genes Dev.* **19**, 1825-1839.
- Tomlinson, A. (1988). Cellular interactions in the developing *Drosophila* eye. *Development* **104**, 183-193.
- Tomlinson, A. and Struhl, G. (2001). Delta/Notch and Boss/Sevenless signals act combinatorially to specify the *Drosophila* R7 photoreceptor. *Mol. Cell* **7**, 487-495.
- Tsuda, L., Nagaraj, R., Zipursky, S. L. and Banerjee, U. (2002). An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. *Cell* **110**, 625-637.
- Voas, M. G. and Rebay, I. (2004). Signal integration during development: insights from the *Drosophila* eye. *Dev. Dyn.* **229**, 162-175.
- Wang, S. H., Simcox, A. and Campbell, G. (2000). Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271-2276.
- Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The *Xenopus* homolog of *Drosophila* Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.
- Wildonger, J., Sosinsky, A., Honig, B. and Mann, R. S. (2005). Lozenge directly activates argos and klumpfuss to regulate programmed cell death. *Genes Dev.* **19**, 1034-1039.
- Xu, C., Kauffmann, R. C., Zhang, J., Kladny, S. and Carthew, R. W. (2000). Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the *Drosophila* eye. *Cell* **103**, 87-97.
- Yang, L. and Baker, N. E. (2006). Notch activity opposes Ras-induced differentiation during the Second Mitotic Wave of the developing *Drosophila* eye. *BMC Dev. Biol.* **6**, 8.
- Yoo, A. S., Bais, C. and Greenwald, I. (2004). Crosstalk between the EGFR and LIN-12/Notch pathways in *C. elegans* vulval development. *Science* **303**, 663-666.
- Zipursky, S. L. and Rubin, G. M. (1994). Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. *Annu. Rev. Neurosci.* **17**, 373-397.