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

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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\textsuperscript{intracellular}, 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...
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**

US-EGFRΔN, UAS-ΔN, UAS-ebiΔN and UAS-Delta (UAS-DI) were obtained from the Bloomington stock center. UAS-EGFRΔN on the third chromosome was obtained from T. Suchpbach (Queenan et al., 1997). EGFRΔN and EGFRΔE were obtained from K. Moses (Kumar et al., 1998). FRT42D EGFRΔE was obtained from G. Campbell (Wang et al., 2000). spa-Gal4 was obtained from M. Noll (Kronhamm et al., 2002). 54CGal4 driver line was obtained from R. Cagan. UAS-Sa(H)ΔN was generated using Xenopus Sa(H) DNA binding mutant CDNA obtained from C. Kintner (Weinstein et al., 1997), cloned in a pUAST vector and transgenic flies generated using standard procedures.

**Heat-shock protocols**

For the temperature-shift experiments involving N6 and EGFRΔE, Pcu114 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 EGFRΔN and N6, 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).

**EGFRΔN clones**

EGFRΔN 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), 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 EGFRΔE (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 (EGFRΔE) 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 snoΔE1 and the null allele snoΔE3 showed no perturbation in cone-cell fate, as judged by the expression of Cut (Fig. 3D-F). 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 N6 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 N6 nor the 54CGal4, UAS-ΔN, UAS-ΔN, UAS-DN, UAS-DN 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.
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.
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), lzTS114 (Gupta and Rodrigues, 1995) pupal eye discs showed normal differentiation of primary pigment cells (Fig. 5D). lzTS114 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. 2. EGFR is required for Delta expression in pupal cone cells.** (A-C) Delta expression in EGFRts1-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 EGFRts1. (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 EGFRts1 clones transferred to non-permissive conditions during the mid-pupal stages. (D) GFP marks wild-type tissue; non-GFP cells are mutant for EGFRts1. 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-I) Expression of a dominant-negative version of EGFR (EGFRDN) 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-EGFRDN pupal eye discs stained for Delta showed loss of Delta expression in the cone cells (I), whereas spa-Gal4, UAS-EGFRDN 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-EGFRact 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). In spa-Gal4, UAS-EGFRact third instar eye disc, ectopic activation of Delta (green) is seen in cone cells (red, arrow).
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

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

**Fig. 4.** Requirement of Notch signaling for primary pigment-cell specification. (A-C) Pupal eye discs stained for Bar. Bar expression in seen in the primary pigment cells (A) and is lost in N^0 pupal eye discs shifted to a non-permissive temperature 10 hours after pupation (B) or when UAS-Su(H)^DN 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^0 when shifted to a non-permissive temperature 10 hours after pupation (E) or in an 54C-Gal4 UAS-Su(H)^DN 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.
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 equivalent 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 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.

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