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Hindsight modulates Delta expression during *Drosophila* cone cell induction

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The induction of cone cells in the *Drosophila* larval eye disc by the determined R1/R6 photoreceptor precursor cells requires integration of the Delta-Notch and EGF receptor signaling pathways with the activity of the Lozenge transcription factor. Here, we demonstrate that the zinc-finger transcription factor Hindsight (HNT) is required for normal cone-cell induction. R-cells in which *hindsight* levels are knocked down using RNAi show normal subtype specification, but these cells have lower levels of the Notch ligand Delta. We show that HNT functions in the determined R1/R6 precursor cells to allow *Delta* transcription to reach high enough levels at the right time to induce the cone-cell determinants Prospero and D-Pax2 in neighboring cells. The Delta signal emanating from the R1/R6 precursor cells is also required to specify the R7 precursor cell by repressing *seven-up*. As *hindsight* mutants have normal R7 cell-fate determination, we infer that there is a lower threshold of Delta required for R7 specification than for cone-cell induction.

KEY WORDS: Delta, *Drosophila*, Hindsight (Pebbled), Notch, Cone cell, Eye

INTRODUCTION

Notch signaling is a conserved pathway that is required to regulate cell specification and behavior at many different venues throughout metazoan development and adult life. The first step of canonical Notch signaling depends on the binding of the membrane-bound ligands, Delta or Serrate (Jagged in mammals), to Notch receptor presented by an adjacent cell (Ilagan and Kopan, 2007; Kadesch, 2004). Typically, one cell expresses higher or more sustained levels of ligand. Thus, inductive signaling occurs via unidirectional activation of Notch. The expression patterns of Notch ligands through development are complex and contribute to differential activation of the pathway during signaling. Although regulating the cell-specific transcription of Notch ligands is conceptually the simplest mode for control of the polarity and intensity of Notch signaling, we are just beginning to understand some of the signals and transcription factors involved (Bash et al., 1999; Ciechanska et al., 2007; Sasaki et al., 2002). Additional ways of modulating ligand activity include post-translational modification via ubiquitylation to regulate endocytosis and degradation (Le Borgne et al., 2005; Nichols et al., 2007), proteolysis to affect Notch-binding affinity (Nichols et al., 2007) and regulation of intracellular localization by other proteins (Bray, 2006). Controlling the level or the activity of the Notch ligand is crucial for determining cell fate in both the responding and, possibly, signaling cells. For example, studies in the *Drosophila* ovary have established that modulated levels and durations of Delta signaling are responsible for inducing different follicle cell fates (Assa-Kunik et al., 2007).

The *Drosophila* eye disc provides a tractable system to study how the coordination of juxtacrine signaling pathways with cell-specific intrinsic factors leads to differential cell-fate determination (Doroquez and Rebay, 2006). During *Drosophila* eye development, a stereotypical sequence of cell recruitments and inductions pattern the cells comprising an ommatidial cluster (Freeman, 1997). The first five photoreceptor (R) precursor cells to be born – R8, -2, -5, -3 and -4 – are patterned immediately posterior to the morphogenetic furrow. A second wave of division gives rise to the rest of the cells that will form an ommatidial unit. These include precursor cells for the R1, -6 and -7 neurons as well as for the non-neuronal cone and pigment accessory cells (Ready et al., 1976; Wolff and Ready, 1991). In mutants where the cone cells are not correctly specified, apical lens secretion is defective and the structural integrity of the ommatidium is compromised (Batterham et al., 1996; Fu and Noll, 1997). Determination of R1, -6, -7 and the cone cells occurs in the larval eye disc. Various cell-subtype-specific transcription factors for these cells are regulated by the AML-1 like transcription factor, Lozenge (LZ) (Daga et al., 1996). Differential EGF receptor (EGFR) and Notch-Delta (N-DL) signaling among these cells act in concert with LZ to evoke cell-specific readouts: the R1 and R6 precursor pair express the Bar and Seven up (SVP) transcription factors (Daga et al., 1996; Higashijima et al., 1992; Mlodzik et al., 1990); the R7 precursor cell expresses Prospero (PROS) (Chu-Lagraff et al., 1991; Tomlinson and Struhl, 2001; Xu et al., 2000) and represses SVP (Daga et al., 1996); and the cone cells express D-PAX2 (Shaven – FlyBase), CUT and PROS (Blochlinger et al., 1988; Flores et al., 2000; Fu and Noll, 1997; Xu et al., 2000) (see Fig. 1).

Differential cell fate in the eye disc depends on the precise timing of *Dl* transcription. The initiation of *Dl* transcription in the eye disc requires the activity of two secreted factors: Hedgehog (HH) and Decapentaplegic (DPP) at the furrow (Greenwood and Struhl, 1999). After neuronal determination, *Dl* transcription in R-precursor cells is elevated by the EGFR pathway in the developing ommatidial clusters. Clones lacking the EGFR ligand, Spitz (SPI), show reduced DL expression posterior to the furrow (Tsuda et al., 2002). Activation of EGFR relieves repression of the *Dl* gene by the transcription factor Charlatan (CHN) (Fig. 1) (Tsuda et al., 2006).

The zinc-finger transcription factor, Hindsight (HNT) (Pebbled – FlyBase), regulates several aspects of eye development (Pickup et al., 2002; Yip et al., 1997). It is required for the assembly of the five-cell preclusters and the timing of their neuronal determination, as well as their subsequent rotation. Later in eye development, HNT is necessary for photoreceptor rhabdomere morphogenesis and ommatidial integrity. Genetic screens have uncovered several

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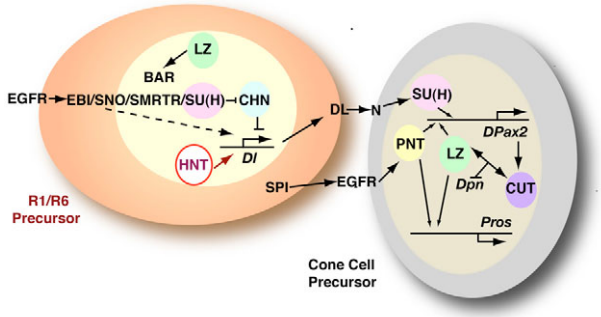

Fig. 1. Model for cone-cell induction. A schematic of the cone-cell induction pathway, based on published data (Canon and Banerjee, 2003; Flores et al., 2000; Tsuda et al., 2006; Tsuda et al., 2002; Xu et al., 2000), showing how the combination of the Notch (N) ligand Delta (DL) and the EGFR ligand Spitz (SPI), emanating from the R1/R6 precursor cell, leads to the activation of the cone-cell determinant *D-Pax2* in the adjacent cone-cell precursor. *Delta* transcription is derepressed in a precise window of time by the activation of the EGFR in the R1/R6 photoreceptor precursor cells. The Lozenge (LZ) transcription factor is required in both cells to activate *Bar* and to co-activate *D-Pax2* and *Prospero* (*Pros*) in the cone-cell precursor. From the results in this paper, we hypothesize that Hindsight (HNT) is required in the R1/R6 precursors to regulate *Dl* transcription in a pathway independent of Charlatan (CHN).

potential transcriptional targets for HNT. Loss-of-function alleles of both *charlatan* and *Delta* are dominant modifiers of a temperature-sensitive *hnt* allele, hnt^{peb} (Wilk et al., 2004).

Here we report that HNT is necessary for cone-cell induction. In *hnt* mutants, cone-cell determination fails as a result of reduced *Delta* transcription in the R1 and R6 photoreceptor precursor cells. We infer that the wild-type function of HNT is to elevate DL ligand concentration to a high-enough level in the photoreceptor precursor cells to activate the N receptor in the adjacent cone-cell-precursor cell. In conjunction with EGFR receptor signaling, this N activation regulates both *D-Pax2* and *Pros* expression, leading to timely cone-cell induction.

MATERIALS AND METHODS

Drosophila mutants and lines

The following fly stocks were used in this study: hnt^{1142} FRT (Duffy et al., 1998), *spa*-Gal4 (Jiao et al., 2001), *lz*-Gal4 (Batterham et al., 1996) and Dl^{05151} (Weber et al., 2000). The *pGMR*-Gal4 (on the second chromosome), UAS-*Dl*::GFP and FLP recombinase stock w^{1118}; *MKRS*, *P[ry+t7.2=hsFLP]86E/TM6B,Tb* were obtained from the Bloomington *Drosophila* Stock Center. The *hnt* mRNAi lines 2A and 2B (on the second and third chromosomes, respectively) were generated as follows: the *hnt* RNAi construct was targeted to a 0.7 kb region of the third exon and made by fusion of *hnt* genomic and cDNA fragments (Kalidas and Smith, 2002). The 1 kb genomic fragment, including 0.3 kb of the second intron and a 0.7 kb third exon portion downstream of the splice donor sequence, was PCR-isolated, ligated to a 0.7 kb inverted *hnt* cDNA fragment corresponding to the genomic region, and cloned into pUAST. The ligated RNAi construct was injected into fly embryos to produce the transgenic *hnt* RNAi fly lines. Primer sequences used in construction of the *hnt* RNAi transgenes are available upon request.

To generate the UAS-*hnt* transgenic line full-length *hnt* PCR products containing the *hnt* coding region and second intron, but not the 5′ and 3′ UTRs, were synthesized by PCR from w^{1118} genomic DNA. PCR products were first cloned into pCR2.1-TOPO vector (Invitrogen), then into a pUAST-*egfp* vector. Transgenic lines were generated by germline transformation in a w^{1118} background.

FLP-induced clones in the eye disc

Clones were induced in the eye as described previously (Pickup et al., 2002).

Immunohistochemistry

Antibody staining was carried out using standard protocols (Wolff, 2000). Primary antibodies obtained from the Developmental Studies Hybridoma Bank were mouse anti-HNT monoclonal (27B8 1G9, 1:10 dilution) (Yip et al., 1997), rat anti-ELAV (7E8A10-s, 1:20) (O'Neill et al., 1994), mouse anti-CUT (2B10-s, 1:30) (Blochlinger et al., 1993), mouse anti-ROUGH (62C2AB, 1:100) and mouse anti-PROS (MR1A-s, 1:5) (Kauffmann et al., 1996). Other primary antibodies were rabbit anti-D-PAX2 (1:50) (Fu and Noll, 1997), rat anti-BAR-H1 (1:1000) (Higashijima et al., 1992), rabbit anti-CHN (1:100) (Tsuda et al., 2006), chicken anti-GFP (Abcam, 1:500) and rabbit anti-β-galactosidase (Cappel, 1:1000). All secondary antibodies were used at a dilution of 1 in 250 and were obtained from Molecular Probes or Invitrogen. They were Alexa fluor 555-conjugated goat anti-mouse antibody, Alexa fluor 488-conjugated goat anti-rat antibody, Alexa fluor 555-conjugated goat anti-rabbit antibody, Alexa fluor 488-conjugated goat anti-rabbit antibody, Alexa fluor 488-conjugated goat anti-chicken antibody and Alexa fluor 647-conjugated goat anti-rat antibody.

To visualize two different mouse primaries simultaneously (Fig. 3C″), we used a double-labeling technique described in the Jackson ImmunoResearch catalog. Eye discs were incubated in the mouse anti-CUT antibody first, followed by an unconjugated rabbit anti-mouse Fab antibody (Jackson, dilution of 1:250), which was then visualized by a standard Alexa fluor 488-conjugated anti-rabbit tertiary antibody. The discs were then placed into the mouse anti-HNT antibody, which was detected by an Alexa fluor 555-conjugated anti-mouse secondary antibody (1:500 dilution). Although this technique was optimized, we have not achieved perfect separation of the two different primary signals and, as a result, some cross-reactivity is seen in the cone cells.

Microscopy, image capture and processing

A Zeiss Axiovert 100 microscope with LSM510 software was used for laser confocal microscopy. Images were reconstructed using the Volocity software (Improvision) and processed and displayed using Photoshop (Adobe). Adult eyes were photographed using a Canon G5 camera mounted on a Leica MZ75 microscope. Images were processed using Photoshop software (Adobe).

RESULTS

Reduction of HNT in developing R cells results in a glossy eye phenotype

Our previous studies demonstrated that HNT is required in photoreceptor (R) precursor cells to maintain epithelial integrity (Pickup et al., 2002). To assess the causes of this phenotype we produced transgenic flies in which *hnt* expression was knocked down by RNAi (Fig. 2). In wild-type eye discs, HNT is expressed in all R-cell precursors posterior to the furrow but is not found in cone-cell precursors (Fig. 2A,A′; see Fig. S1A,B in the supplementary material) (Pickup et al., 2002). When UAS-*hnt*RNAi constructs were expressed using the p*GMR*-Gal4 driver in all cells posterior to the morphogenetic furrow, HNT protein levels were partially reduced in the R3 and R4 cells and strongly reduced in the R1, R6 and R7 cells (Fig. 2C-D′; see Fig. S2A-B″ in the supplementary material). Anti-ELAV antibody staining demonstrated that neuronally determined R cells formed, even though they lacked HNT protein (Fig. 2B′ and inset). This result is consistent with those from a previous clonal analysis, which showed that HNT function is not absolutely required for R cell development but, rather, affects the timing of differentiation and the morphology of the developing R cell clusters (Pickup et al., 2002).

The majority of *hnt* RNAi flies die during late pupal stages; however, with only one dose of the *hnt* RNAi transgene, adult escapers hatched. The eyes of these escapers were glossy with

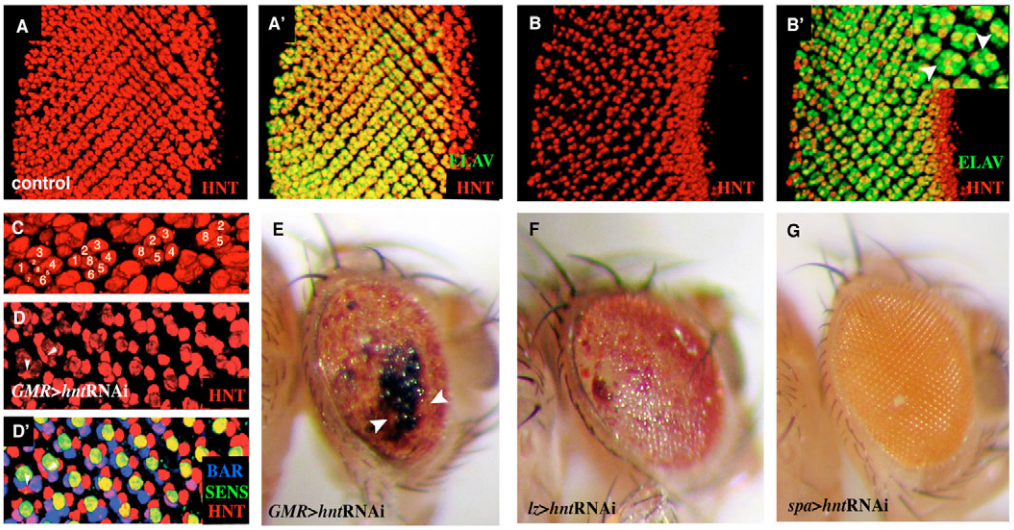

Fig. 2. *hnt* mRNAi knockdown eye phenotypes. (**A-D′**) Apical views of third-instar larval eye discs stained with anti-HNT antibody (red) and (A′,B′ and inset) anti-ELAV antibody (green) and (D′) anti-SENS (green) and anti-BAR (blue). Anterior is to the right. (A,A′) A no-driver control disc: UAS-*hnt* mRNAi2A/+; UAS-*hnt* mRNAi2B/+. (A) Developing ommatidial clusters express HNT posterior to the furrow. (A′) All of the cells expressing HNT also express ELAV, demonstrating that they are photoreceptor (R) precursor cells. No other apical cells stain for HNT. (B,B′ and inset) An *hnt* RNAi mutant disc (UAS-*hnt* mRNAi2A/p*GMR*-Gal4; *hnt* mRNAi2B/+). (B) HNT is expressed in the R8, R2 and R5 cells, at low levels initially in, but then absent from, the R3 and R4 cells. HNT is very reduced, or absent from, the more-posterior R1, R6 and R7 cells. (B′ and inset) Cells that lack HNT expression still express ELAV (arrowheads in B′ inset), demonstrating that these cells are neuronally determined. (C) Wild-type eye disc stained with anti-HNT antibody to show the normal configuration of the R precursor cells in a developing cluster. (D,D′) An *hnt* mRNAi mutant disc (UAS-*hnt* mRNAi2A/p*GMR*-Gal4; *hnt* mRNAi2B/+). (D) HNT expression is reduced after knockdown. (D′) Specifically, HNT expression is retained in three cell nuclei: the SENS-positive R8 nucleus and the neighboring R2 and R5 nuclei. HNT is reduced/absent in the BAR-positive R1/R6 pair and later absent from the R3/R4 pair and the R7 nucleus. In more-posterior rows HNT expression is occasionally seen late in the R7 nuclei (arrowheads). (**E-G**) Light-microscope pictures of the external adult eye. Anterior is to the right. (E) A UAS-*hnt* mRNAi2A/p*GMR*-Gal4 fly eye. The eye of this escaper fly has several defects. The surface of the eye is glossy and most facets lack intact lenses. The red pigmentation is patchy and there is a large necrotic scar (arrowheads) and smaller black pocks, which may indicate ruptured lenses. (F) A *lz*-Gal4/Y; UAS-*hnt* mRNAi2A/+ fly eye. The eye of this fly has a gradient of pigmentation lessening towards the posterior side. The eye has facets, but is somewhat smoothened compared with a wild-type eye. (G) The eye of this *spa*-Gal4/+; UAS-*hnt* mRNAi2A/+ fly has a wild-type appearance. The pigmentation is normal and the facets all have intact lenses.

mottled pigmentation and no well-defined facets (Fig. 2E). The same phenotype was observed for another line with a different insertion site of the *hnt* RNAi transgene (data not shown). Smoothened eyes are a sign of faulty lens secretion by the underlying cone and pigment cells (Cagan and Ready, 1989b), whereas altered patterns of pigmentation imply defective secondary and/or tertiary pigment cells. The eyes of *hnt* RNAi knockdown flies also have melanized necrotic patches on their surface, indicative of considerable cell death (Fig. 2E, arrowheads). All aspects of this *hnt* knockdown phenotype are reminiscent of the eyes of *lozenge* (*lz*) mutants where there are demonstrable defects in the accessory cone and pigment cells that shape the ommatidial lattice (Batterham et al., 1996).

A similar but milder external eye phenotype was observed when the same *hnt* RNAi transgene was expressed in only the R1/R6/R7 and cone-cell precursors using a *lz*-Gal4 driver (Crew et al., 1997). *hnt* knockdown in these cells gave a gradient of loss of pigmentation (most severe at the posterior) (Fig. 2F) in every fly, again indicating that at least a part of the phenotype was caused by defects in the accessory cells. Although *lz*-Gal4 drives expression in the cone-cell precursors, this phenotype was unlikely to have been caused by *hnt* knockdown in these cells as HNT is not expressed in cone cells. Furthermore, when the *hnt* RNAi transgene was expressed in the cone cells in the larval eye disc but not in the R cells, using the *sparkling*-Gal4 driver (Jiao et al., 2001), there was no smoothening, loss of pigmentation or necrosis (Fig. 2G). This driver promotes expression in at least some of the cone-cell precursors at the right time in the larval eye disc, although it is not clear whether it is as strong a driver as the p*GMR* and *lz*-drivers (see Fig. S3A,A′ in the supplementary material).

In summary, knockdown of *hnt* in a subset of R-cell precursors causes defects in lens secretion and pigmentation that appear to result from a non-autonomous effect on accessory cells in the developing eye disc.

HNT function in developing R cells is necessary for cone-cell induction

The R1, R6 and R7 precursor cells arise from a basal pool of undifferentiated cells in a second wave of division posterior to the morphogenetic furrow. They are the last R cells to be determined in the third-instar eye disc (Ready et al., 1976) and play a pivotal role in cone-cell induction (Lai, 2002). This induction requires R-cell-precursor specification by LZ (Daga et al., 1996), as well as a precisely timed window of activation of the N-DL and EGFR signaling pathways (Flores et al., 2000) (Fig. 1).

As reduction of HNT function in just the inducing R-precursor cells produced a glossy eye phenotype (Fig. 2F), we reasoned that part or all of this phenotype might be caused by a cone-cell defect. To test this hypothesis we examined expression of the cone-cell determination marker, CUT (Blochlinger et al., 1993), in third-instar

eye discs of pGMR-Gal4; *hnt* RNAi flies. In control discs, a pair of cone-cell precursor nuclei expressed CUT in a 'bow-tie' configuration by column 7. By column 10, CUT was found in two more nuclei per ommatidium, making up a cone-cell 'quartet' at the posterior of the disc (Fig. 3A). In eye discs in which HNT expression had been knocked down, there was a depletion of CUT expression: CUT was found later (column 10) and at lower levels than in the control discs at a similar stage of development (compare Fig. 3A and 3B). No cone-cell precursor quartets were found at the posterior of the mutant discs; at most two cells per ommatidium expressed CUT. When pupal discs of this genotype were examined, CUT expression was initially lower than that in a no-driver control, but later increased to control levels (data not shown). This result suggests that the phenotype we observe in discs where HNT expression is knocked down may be due to a delay, rather than an outright block, in cone-cell induction. A weaker phenotype was observed in *lz*-Gal4; *hnt* RNAi larval eye discs in which knockdown occurred in R1, 6 and 7: CUT protein levels were close to wild type; but again, CUT was most often expressed in only two of the four cone-cell precursors (data not shown).

To determine whether depletion of CUT was a bona fide hypomorphic phenotype of *hnt*, we tested for expression of the same marker within clones mutant for hnt^{1142}, an antibody-null allele (Wilk et al., 2000) (Fig. 3C-C″). Within these clones we found that CUT protein was absent from ommatidia lacking HNT expression (n=11 clones). Occasionally, a few isolated HNT-negative, CUT-positive cells were found along the borders of clones, indicating possible local non-autonomy. In order to establish which R cells are required to induce CUT expression in the cone-cell precursor, we scored border ommatidia along the edges of hnt^{1142} clones (Fig. 3D,D′). For technical reasons we are only able to examine clusters with a full complement of R cells in a wild-type configuration. This allowed us to unequivocally assign cone cells at the two-cell stage to a particular cluster. We examined 38 clones and found four examples of clusters where only R1/R6 (as marked by BAR) and R7 were mutant for *hnt* (Fig. 3D,D′). In all four cases, there was no, or significantly reduced, CUT expression. We never saw any cases of ommatidia in which all the R cells (including R1 and R6) expressed HNT and CUT was not expressed (n=68). We were not able to do the reciprocal experiment (where R1/R6 express HNT and all the other R cells are mutant) as this almost always gave defective ommatidia with incorrect R-cell specification or altered polarity (Pickup et al., 2002). In ommatidia in which one cell out of the R1-R6 pair was mutant for *hnt,* these clusters sometimes had normal cone-cell induction (67%, n=24), indicating that one signaling cell may sometimes be enough to induce some CUT expression (scored at the two-cone-cell stage). A similar scenario has been shown for R7 induction by these cells (Tomlinson and Struhl, 2001).

We also examined hnt^{1142}-mutant clones for expression of a second-cone-cell-determination marker, D-PAX2 (Fu and Noll, 1997). In wild-type third-instar eye discs, this transcription factor is expressed in a similar pattern to CUT and is part of the same pathway required for cone-cell differentiation (Flores et al., 2000; Fu and Noll, 1997) (see Fig. 1). In the interior of the *hnt*-mutant patches, D-PAX2 expression was absent (n=10 clones) (Fig. 3E-E″). Early D-PAX2 expression is required for timely initiation of CUT expression in wild-type larval and early pupal eye discs (Fu and Noll, 1997). Later pupal eye discs from strong *D-Pax2* mutants did express CUT, indicating that this late phase of CUT expression is D-PAX2-independent. Thus the effects we observed on CUT expression in the *hnt* RNAi mutants are likely to have been caused by depletion of D-PAX2.

Taken together, the results shown in Fig. 3 and the knockdown phenotype generated by the *lz*-Gal4 driver (Fig. 2F) suggest that HNT function is required in the R1/R6-cell precursor cells during larval development for proper cone-cell induction. As HNT is not expressed in pigment cells, the effect of *hnt* RNAi on adult eye pigmentation is likely to be a secondary effect of this cone-cell

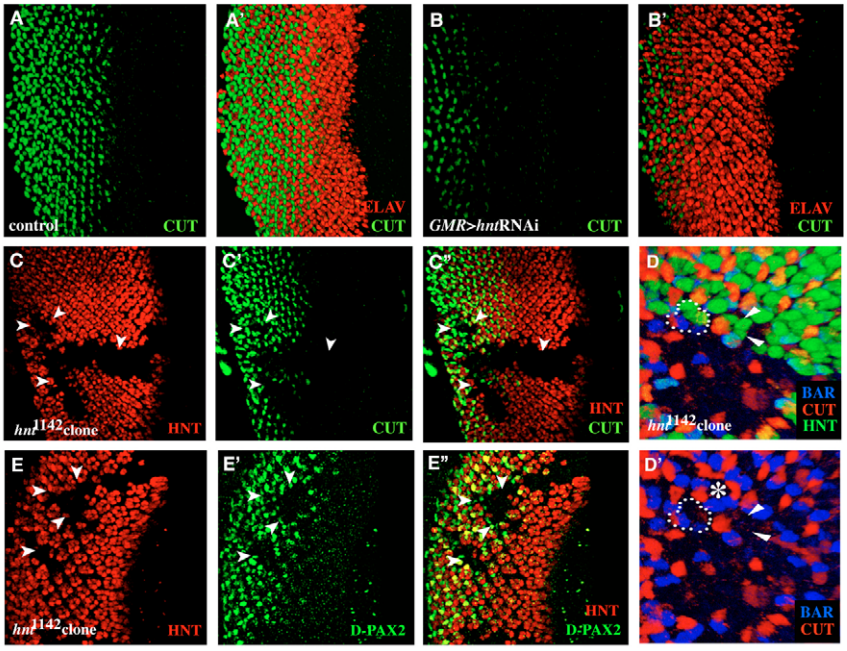

Fig. 3. Cone precursor cell phenotypes in *hnt* mutant eye discs. Third-instar larval eye discs. Anterior is to the right. (**A-B′**) Antibody staining with anti-CUT (green) and anti-ELAV (red). (A,A′) A no-driver control disc: UAS-*hnt* mRNAi2A/+;UAS-*hnt* mRNAi2B/+ shows a wild-type pattern of CUT expression in the cone precursor cells beginning at about row 7 of ELAV expression. (B,B′) In an *hnt* mRNAi mutant disc (UAS-*hnt* mRNAi2A/p*GMR*-Gal4;*hnt* mRNAi2B/+) of the same age, there is delayed and reduced expression of the cone-cell precursor marker CUT. (**C-C″**) Antibody staining with anti-CUT (green) and anti-HNT (red). A clone of the allele hnt^{1142} (bounded by arrowheads) is marked by a lack of HNT and CUT expression (green). (**D**,**D′**) Antibody staining with anti-CUT (red) and anti-BAR (blue), and in D also with anti-HNT (green). In this hnt^{1142} clone, within the ommatidium where only R1/R6 and R7 cells are mutant for *hnt* (dotted line), the levels of CUT staining are significantly reduced relative to the cone-cell staining in adjacent wild-type ommatidia (asterisk in D′). The disc has been rotated to clearly show the R1/R6 cells, but has been serially reconstructed to score the remaining HNT^+ R cells. An adjacent mosaic ommatidium (arrowheads) has only one CUT-positive cone cell, but is not counted because it has only one BAR-positive cell. (**E-E″**) Antibody staining with anti-D-PAX2 (green) and anti-HNT (red). Larval eye discs with several clones of hnt^{1142} (bounded by arrowheads and identified by their lack of HNT expression) have no D-PAX2 expression (green) compared with the adjacent wild-type tissue.

defect. Cone cells have been shown to be necessary for proper pigment-cell determination during pupal stages (Nagaraj and Banerjee, 2007).

In order to test whether HNT is sufficient to induce cone-cell fate we used a UAS-*hnt* line to over- and misexpress HNT early in all (p*GMR*-Gal4 driver) or a subset (*lz*-Gal4 driver) of the undifferentiated cells behind the furrow (see Fig. S4A-C′ in the supplementary material). In this context, HNT expression is sufficient to induce ectopic anti-CUT expression in many of the basal undifferentiated cells (see Fig. S4B′,C′ in the supplementary material). These cells have thus taken on this aspect of cone-cell determination.

HNT is not required autonomously for R1/R6/R7 subtype specification

There are at least two possible explanations for the failure of cone-cell precursors to adopt a normal fate in the larval eye disc. One is that the R-cell precursor cells that usually induce their neighboring cells to initiate the cone-cell determination pathway are not able to signal normally – the signal is reduced or not timed correctly. A second possibility is that the signaling cells themselves are not specified normally. We tested this latter hypothesis by staining for the R1/R6 subtype marker, BarH1 (BAR) (Higashijima et al., 1992), as well as for the R7/cone precursor cell marker, Prospero (PROS) (Kauffmann et al., 1996) (Fig. 4). Both of these transcription factors are necessary to determine their respective cell subtypes and they also serve as readouts for the LZ pathway. In *lz* mutants these proteins are not expressed and normal R cells fail to develop (Daga et al., 1996).

When p*GMR*-Gal4; *hnt* RNAi knockdown flies are compared with a non-driver control that has normal levels of HNT, expression of BAR in the R1 and R6 precursor cells was found to be normal; BAR expression began at the same time and was at comparable levels to BAR-positive cells in the control eye discs (Fig. 4A,B). Thus, the mutant phenotype we observed in *hnt* knockdown discs was not a result of delayed R1/R6 determination. The R7 precursor cell was also specified correctly. In *hnt* RNAi mutant discs, a single ELAV-positive cell also strongly expressed PROS (Fig. 4D, inset). As PROS expression in the R7 precursor is in part activated by EGFR effectors (Xu et al., 2000), this result also indirectly indicates that there is functional SPI ligand secreted by the R1/R6 cells (see Fig. 1). The PROS expression normally found in the cone-cell precursors in control eye discs (distinguishable by their lack of ELAV in Fig. 4C, inset) was much lower in the cone-cell precursors in 'mutant' discs (arrowheads in Fig. 4D, inset). This observation is consistent with the results described previously and reflects a lack of proper cone-cell determination.

In summary, the results presented above demonstrate that wild-type HNT function is not directly required to determine R1/R6 cell identity. The R-cell precursor cells that are necessary to induce the cone cells are specified correctly after *hnt* knockdown. As the markers tested are induced by LZ signaling, this result also indicates that these cells have a normal LZ pathway operating in them in the knockdown scenario.

HNT is necessary for high-level DL expression and signaling

The membrane-bound N ligand, DL, is required in determined R cells for cone-cell induction (Flores et al., 2000). The timing of *Delta* expression in the R1 and R6 precursor cells is regulated by the activation of the SU(H)-EBI-SMRTR complex, which removes the transcriptional repressor CHN from the *Dl* promoter (Tsuda et al., 2006) (see Fig. 1). We have previously demonstrated that clones of *hnt* antibody-null alleles have lowered levels of *Dl-lacZ* expression in their R cells (Wilk et al., 2004). To test whether the R1 and R6 cells in particular have less *Dl* expression in a *hnt* RNAi mutant eye, we examined the expression of the Dl^{05151} enhancer trap in a *lz*-Gal4; *hnt* RNAi background. Control larval eye discs showed expression of this *Dl* reporter gene in R cells that mimics endogenous *Dl* expression in the late-developing clusters (Weber et al., 2000): β-galactosidase was found in the R1 and R6 precursor cells but was low in the R7 precursor cell (Fig. 5C,C′). This asymmetrical ligand expression is thought to account for the role of R1/R6 in inducing R7 cell fate (Cooper and Bray, 2000; Parks et al., 1995). In eye discs from *lz*-Gal4; *hnt* RNAi larvae, the levels of *Dl* reporter expression in the R1 and R6 cells (identified by Bar expression) were reduced (Fig. 5D,D′). Cells that lacked HNT expression had lowered *Dl* expression 37% of the time (n=165 R1/R6) (Fig. 5B,D,D′). Apparently, this reduced level of DL is sufficient for normal R7 induction, as the R7 precursor cell still expressed PROS and ELAV (Fig. 4D and inset). No extra cells expressed the R1/R6 determination marker, BAR, in the mutant eye discs (Fig. 4B), indicating there had been no R7 to R1/R6 fate transformation. Notably, *Dl* expression was also reduced in the other

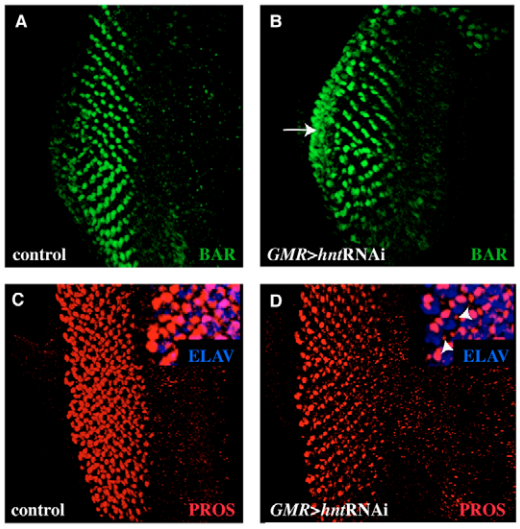

Fig. 4. R1/R6- and R7-precursor-cell specification in *hnt* mutant eye discs. Third-instar larval eye discs stained with antibodies for (A,B) BARH1 (green) and (C,D) for Prospero (PROS, red). The insets also show staining for the pan-neural marker ELAV (blue). (**A**) A no-driver control disc: UAS-*hnt* mRNAi2A/+;UAS-*hnt* mRNAi2B/+ shows wild-type anti-BAR antibody staining in the determined R1/R6 precursor nuclei. (**B**) In a *hnt* mRNAi mutant disc (UAS-*hnt* mRNAi2A/p*GMR*-Gal4;*hnt* mRNAi2B/+) the anti-BAR antibody staining looks very similar to the control. There is slightly elevated staining in the peripodial membrane compared with the control (arrow), only some of which is shown in this image. (**C**) A no-driver control disc: UAS-*hnt* mRNAi2A/+;UAS-*hnt* mRNAi2B/+ shows wild-type anti-PROS antibody staining in determined R7 precursor cell nuclei and in the cone-cell precursor nuclei. (**D**) In a *hnt* mRNAi disc (UAS-*hnt* mRNAi2A/p*GMR*-Gal4;*hnt* mRNAi2B/+), the anti-PROS antibody staining in the R7 cells looks like that in the control. In the inset we confirm that these PROS-positive nuclei are the R7 precursor cell nuclei because they express the neural marker for ELAV (blue), which does not stain cone-cell precursor nuclei. In this knockdown disc, there is reduced anti-PROS staining in the cone-cell nuclei compared with the control disc shown in C and C inset. These nuclei do not stain with anti-ELAV (blue) in the D inset.

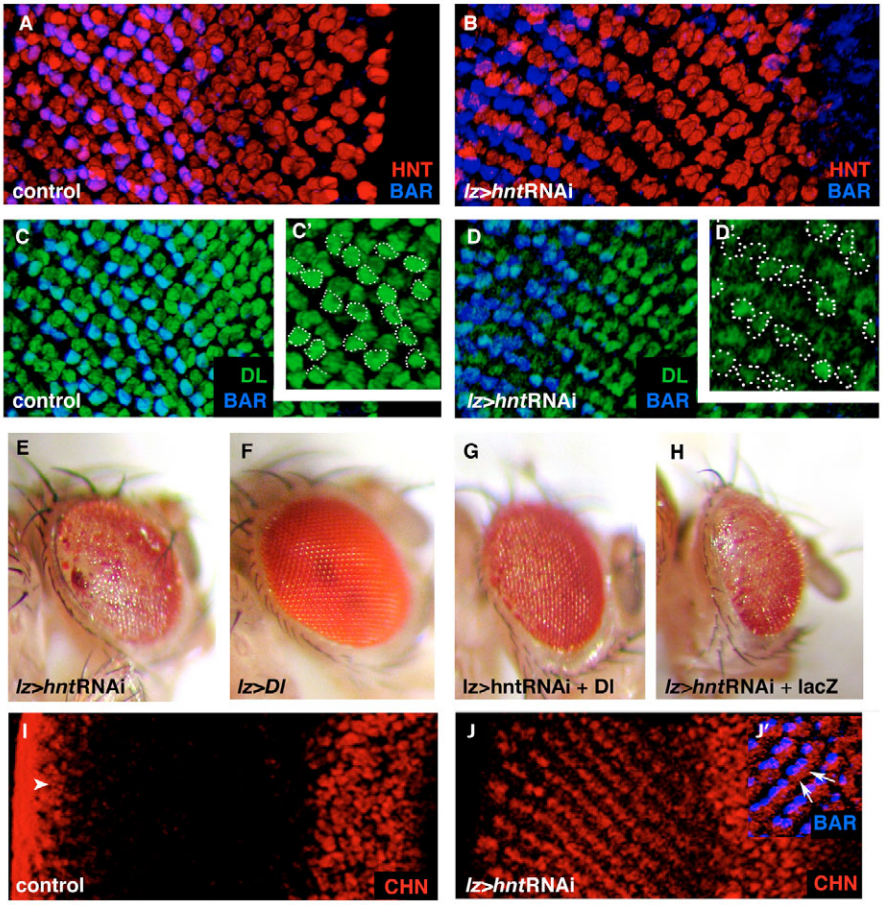

Fig. 5. *hnt* RNAi affects *Delta* expression. Basal views of third-instar larval eye discs stained with (A,B) anti-HNT antibody (red) and anti-BARH1 antibody (blue) to mark the R1/R6 precursor nuclei or (C-D′) anti-β-galactosidase antibody (green) to detect Dl^{05151} enhancer trap expression and anti-BARH1 antibody. (**A**) A no-driver control disc: UAS-*hnt* mRNAi2A/+; UAS-*hnt* mRNAi2B/*Dl-lacZ* shows HNT expression (red) in all the R1/R6 precursor cells (blue). (**C**,**C′**) These same cells (blue and dotted lines in C′) express high levels of *Dl* (green) all the way to the posterior of the disc (left). (**B**) A *lz*-Gal4/*Y*; UAS-*hnt* mRNAi2A/+; UAS-*hnt* mRNAi2B/*Dl-lacZ* eye disc lacks HNT expression (red) in almost all of the R1/R6 precursor cells (blue). (**D**,**D′**) There is reduced or absent *Dl* expression (green) in 37% of the R1 and R6 precursor nuclei (blue and dotted lines in D′). (**E-H**) Light-microscope pictures of the external adult eye. Anterior is to the right. (E) A *lz*-Gal4/+; UAS-*hnt* mRNAi2A/+ fly eye, as described in detail in Fig. 2F. (F) A control *lz*-Gal4/UAS-*Dl*::GFP fly eye looks like a wild-type eye externally. (G) A *lz*-Gal4/+; UAS-*hnt* mRNAi2A/UAS-*Dl*::GFP fly eye is partially rescued relative to the *hnt* knockdown phenotype shown in E. The eye has almost normal pigmentation, no glossy surface, but is still slightly rough compared with a wild-type eye. (H) A control *lz*-Gal4/+; UAS-*hnt* mRNAi2A/UAS-*lacZ* fly eye is not rescued relative to the HNT knockdown phenotype shown in E. (**I-J′**) Basal view of third-instar eye discs stained with anti-CHN antibody (red) and anti-BAR antibody (blue in inset). (I) A no-driver control disc: UAS-*hnt* mRNAi2A/+; UAS-*hnt* mRNAi2B/+ shows CHN expression in a band of basal nuclei around the furrow and in a very few late R precursor cell clusters at the posterior edge of the disc (arrowhead). (J,J′) In UAS-*hnt* mRNAi2A/p*GMR*-Gal4; *hnt* mRNAi2B/+ eye discs, the early CHN expression is unaffected. In the late-developing R-cell clusters, where HNT expression is knocked down, there is novel CHN expression in some of the R-cell precursors (arrows in J′), but not in the BAR-positive R1 and R6 precursor cells (blue in J′) that emit the DL signal required for cone-cell induction.

R cells (where HNT was still expressed). One possible explanation for this is that HNT-regulated signals from R1-6/7 are necessary to maintain later *Dl* levels in other cells.

To test whether the adult eye phenotype in *hnt* RNAi flies is caused by lowered *Dl* we performed a rescue experiment restoring DL levels by driving the expression of a UAS-*Dl*::GFP transgene in the R1/R6 and R7 cells. Both complementation and overexpression analyses led to the conclusion that the DL-GFP protein was active in signaling to N (De Joussineau et al., 2003). When we drove DL-GFP in the *lz*-Gal4; *hnt* RNAi background, it partially suppressed the knockdown phenotype (compare Fig. 5E and 5G). The eyes of these rescued flies were less rough and had normal pigmentation, implying that adding DL back just to the R1, R6 and R7 cells compensated for their lack of HNT. This effect was not caused by Gal-4 titration, as a UAS-nuclear *lacZ* transgene is not able to suppress the *lz*-Gal4; *hnt* RNAi eye phenotype (Fig. 5H).

Taken together, these results suggest that, although not involved in R1 and R6 subtype specification, HNT is required in these cells for efficient DL expression in, and signaling by, these cells. The fact that DL expression is sufficient to almost completely suppress the *hnt* RNAi phenotype is consistent with a role for HNT protein upstream of *Delta* transcription and shows that HNT does not regulate any DL-independent pathways that are necessary for cone-cell induction.

HNT does not regulate DL expression in the R1-6 precursor cells via CHN

The current understanding of *Dl* regulation in the R1/R6 signaling cells entails an EGFR-induced derepression of the block by CHN on *Dl* transcription (Tsuda et al., 2006) (see Fig. 1). To determine whether HNT modulates DL levels via this pathway, we assayed CHN expression in eye discs with lowered HNT activity. In no-driver control discs, CHN is expressed at high levels in a band of basal nuclei around the furrow (Tsuda et al., 2006). CHN expression then drops and only resumes in the most posterior column of developing clusters (Fig. 5I, arrowhead). In eye discs where *hnt* expression had been knocked down in late-developing clusters (Fig. 5J,J′), CHN expression at the furrow was unaffected. In the posterior parts of these discs, there was still no CHN expression in the R1- and R6-cell precursors (marked with BAR in Fig. 5J′). This implies that HNT does not act upstream of *Chn* to affect *Dl* expression in these cells (Fig. 1). Notably CHN was derepressed in some of the other determined R-cell precursors in the *hnt* mutant (arrows in Fig. 5J′). This is consistent with the non-autonomous effect on *Dl* expression shown in Fig. 5D and D′, and supports the notion that there may be a HNT-dependent signal required for late *Chn* repression in these precursor cells. Taken together these results suggest that HNT affects cone-cell induction by the R1/R6 precursor cells in a CHN-independent pathway (Fig. 1).

DISCUSSION

Here we have shown that HNT function is necessary to elevate the DL ligand in the R1/R6 precursor cells to a level high enough to achieve cone-cell induction. Notably, HNT is not an on/off switch for *Dl* expression; rather it potentiates the level of *Dl* transcription in the R1/R6 precursor cells. Our data suggest that this modulation is likely to be independent of CHN, which is itself a transcriptional repressor of *Dl* (see Fig. 1). Although we have not shown in this paper that this HNT effect is due to direct action, we have found the exact sequence for two HNT binding sites in the upstream and intronic sequences of the *Delta* transcription unit (R. Wilk, L.M. and H.D.L., unpublished).

Earlier reports describing HNT function in the ovary show that HNT expression is regulated by the Notch signaling pathway and controls follicle cell proliferation and differentiation (Sun and Deng, 2007). In this paper we report that HNT acts upstream of Notch activation by regulating DL ligand expression levels. These two modes of regulation are not necessarily mutually exclusive, but we do not think that Notch activates the *hnt* gene in the eye. First, HNT is expressed in all the R-cell precursors in the eye, whereas the Notch pathway is activated at high levels only in a subset of these precursors, as well as in the accessory cone and pigment cell precursors, where HNT is not expressed at all (Cooper and Bray, 2000). Second, when Notch activity is attenuated by using the N^{ts} mutant (Cagan and Ready, 1989a), HNT expression in the furrow expands to all cells that now acquire a neuronal fate (Pickup et al., 2002). This result cannot be interpreted as a simple repression of HNT expression by Notch activation in non-neuronal cells, as HNT expression is not complementary to Notch activation in the eye disc (Pickup et al., 2002). Third, Notch activation cannot be sufficient to induce HNT expression in the eye disc, as we do not see any expansion of HNT expression into adjacent, non-determined cells when we ectopically express *Dl* early in the cone-cell precursors (with the *lz*-Gal4 driver). Fourth, we have shown here that the expression of *Dl* in the R-cell precursors is partly dependent on HNT function. Others have clearly demonstrated that this late *Dl* expression does not require Notch activity, as it is unaffected in a N^{ts1} mutant (Tsuda et al., 2002).

The two-signal model for R7-fate determination

The two-signal model of R7 fate hypothesizes that R7 determination requires a strong RTK signal (achieved by the additive effects of Sevenless and EGFR activation) together with Notch activation (Tomlinson and Struhl, 2001). These signals are necessary to activate *pros* and repress *svp* expression, respectively (Kauffmann et al., 1996; Miller et al., 2008; Xu et al., 2000). As the cone-cell precursor cells do not contact the determined R8 cell at the appropriate time, they will not 'see' the SEV ligand BOSS (Van Vactor et al., 1991). Cone cell precursors, then, will not ordinarily activate their SEV receptors. In this model, different fates have been reinforced in the R7/cone equivalence group by adding a second, activating ligand for EGFR (Tomlinson and Struhl, 2001).

In this paper we suggest a further level of complexity. We have shown, by manipulating the level of *Dl* in the R1/R6 signaling cells, that activation of the key players in cone-cell determination requires high levels of the Notch activation in the cone-cell precursor cell. Several lines of evidence support the idea that the level of the DL ligand is translated into cell-fate differences in a responding R precursor cell. As there is low DL expression in the R7 precursor cell and only late expression of DL in the cone-cell precursor cell (Flores et al., 2000; Nagaraj and Banerjee, 2007), the adjacent R1/R6 precursor cells never activate their Notch receptors (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). Both the R7 precursor and the cone-cell precursor cells receive their ligand signal from the R1/R6 precursor cells (Tomlinson and Struhl, 2001; Tsuda et al., 2002). In our hypothesis, the R7 precursor cell requires only a low level of ligand signal to activate the R7-like program: turning on *pros* and off *svp*.

We suggest that the cone-cell precursor requires a high level of ligand signal to activate the cone-cell program. Expressing a dominant-negative form of DL in the R1/R6 signaling cells prevents cone-cell, but not R7-cell, determination (Tsuda et al., 2002). As both the cone and R7 precursor cells receive their DL input from the same R1/R6 cells (Flores et al., 2000; Tomlinson and Struhl, 2001), it is possible that an intrinsic feature of the R7 precursor cell – possibly the high RTK activation – antagonizes N signaling, so that *D-Pax2* transcription does not occur in that cell (Rohrbaugh et al., 2002). The transcriptional repressor, Lola, may also be involved in this distinction, as it is known to bias precursor cells towards R7- over cone-cell fate (Zheng and Carthew, 2008).

A coordinated program for cone-cell induction

Although a role for Notch signaling in cone-cell induction has been shown to be necessary for *D-Pax2* expression (Flores et al., 2000), it has not been directly demonstrated as necessary for *pros* regulation in cone cells (Xu et al., 2000). The experiments presented here suggest that high levels of Notch signaling may indirectly or directly be required for *Pros* expression in the cone-precursor cells. This requirement is independent of the role of SU(H) in inducing *D-Pax2*, as there are normal levels of PROS in the cone-cell precursors of a *D-Pax2* null mutant (A.T.P., unpublished observation). Ectopically activating the Notch pathway in the R1/R6 precursor cells occasionally induces ectopic PROS (but eliminates ELAV) in these cells (Miller et al., 2008). Although this effect on PROS expression may be a secondary result of a cell-fate transformation, it could also be interpreted as a more direct effect of Notch signaling on *pros* transcription. In a different context, PROS expression has been shown to be affected by DL-activated Notch signaling in a subset of glial cells in the embryonic CNS (Thomas and van Meyel, 2007).

Why would there be two DL thresholds for different cell fates? There is some preliminary work that suggests different mechanisms for Notch-activated transcriptional readout in the responding cell, depending on the level of signal received. In the cone-cell equivalence group, the cone-cell determination pathway requires that D-PAX2 and PROS be expressed. It is hypothesized that *D-Pax2* may require a higher level of Notch activation than *Pros*, which is also required for R7 determination [Hayashi et al., unpublished data referenced by Zheng and Carthew (Zheng and Carthew, 2008)]. Our experiments indicate that there may be coordinated regulation of both *D-Pax2* and *Pros* expression in the cone cells. Based on the experiment shown in Fig. 4D, we postulate that the mechanism of *Pros*-gene induction in the cone cells is different from *pros* regulation in R7. By potentiating the level of *Dl* gene expression in the R1/R6 signaling cells, it is possible to overlay the cone-cell fate over the transcriptional module necessary for R7-cell fate. This simple change has, thus, allowed for the elaboration of very different cell fates from the same equivalence group.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/6/975/DC1

References

Assa-Kunik, E., Torres, I. L., Schejter, E. D., Johnston, D. S. and Shilo, B. Z. (2007). Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. *Development* **134**, 1161-1169.

Bash, J., Zong, W. X., Banga, S., Rivera, A., Ballard, D. W., Ron, Y. and Gelinas, C. (1999). Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. *EMBO J.* **18**, 2803-2811.

Batterham, P., Crew, J. R., Sokac, A. M., Andrews, J. R., Pasquini, G. M., Davies, A. G., Stocker, R. F. and Pollock, J. A. (1996). Genetic analysis of the lozenge gene complex in Drosophila melanogaster: adult visual system phenotypes. *J. Neurogenet.* **10**, 193-220.

Blochlinger, K., Bodmer, R., Jack, J., Jan, L. Y. and Jan, Y. N. (1988). Primary structure and expression of a product from cut, a locus involved in specifying sensory organ identity in Drosophila. *Nature* **333**, 629-635.

Blochlinger, K., Jan, L. Y. and Jan, Y. N. (1993). Postembryonic patterns of expression of cut, a locus regulating sensory organ identity in Drosophila. *Development* **117**, 441-450.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* **7**, 678-689.

Cagan, R. L. and Ready, D. F. (1989a). Notch is required for successive cell decisions in the developing Drosophila retina. *Genes Dev.* **3**, 1099-1112.

Cagan, R. L. and Ready, D. F. (1989b). The emergence of order in the Drosophila pupal retina. *Dev. Biol.* **136**, 346-362.

Canon, J. and Banerjee, U. (2003). *In vivo* analysis of a developmental circuit for direct transcriptional activation and repression in the same cell by a Runx protein. *Genes Dev.* **17**, 838-843.

Chu-Lagraff, Q., Wright, D. M., McNeil, L. K. and Doe, C. Q. (1991). The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila. *Development* **Suppl. 2**, 79-85.

Ciechanska, E., Dansereau, D. A., Svendsen, P. C., Heslip, T. R. and Brook, W. J. (2007). dAP-2 and defective proventriculus regulate Serrate and Delta expression in the tarsus of Drosophila melanogaster. *Genome* **50**, 693-705.

Cooper, M. T. and Bray, S. J. (2000). R7 photoreceptor specification requires Notch activity. *Curr. Biol.* **10**, 1507-1510.

Crew, J. R., Batterham, P. and Pollock, J. A. (1997). Developing compound eye in lozenge mutants of Drosophila: lozenge expression in the R7 equivalence group. *Dev. Genes Evol.* **206**, 481-493.

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.

De Joussineau, C., Soule, J., Martin, M., Anguille, C., Montcourrier, P. and Alexandre, D. (2003). Delta-promoted filopodia mediate long-range lateral inhibition in Drosophila. *Nature* **426**, 555-559.

Doroquez, D. B. and Rebay, I. (2006). Signal integration during development: mechanisms of EGFR and Notch pathway function and cross-talk. *Crit. Rev. Biochem. Mol. Biol.* **41**, 339-385.

Duffy, J. B., Harrison, D. A. and Perrimon, N. (1998). Identifying loci required for follicular patterning using directed mosaics. *Development* **125**, 2263-2271.

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.

Freeman, M. (1997). Cell determination strategies in the Drosophila eye. *Development* **124**, 261-270.

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.

Greenwood, S. and Struhl, G. (1999). Progression of the morphogenetic furrow in the Drosophila eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway. *Development* **126**, 5795-5808.

Higashijima, S., Kojima, T., Michiue, T., Ishimaru, S., Emori, Y. and Saigo, K. (1992). Dual Bar homeo box genes of Drosophila required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. *Genes Dev.* **6**, 50-60.

Ilagan, M. X. and Kopan, R. (2007). SnapShot: notch signaling pathway. *Cell* **128**, 1246.

Jiao, R., Daube, M., Duan, H., Zou, Y., Frei, E. and Noll, M. (2001). Headless flies generated by developmental pathway interference. *Development* **128**, 3307-3319.

Kadesch, T. (2004). Notch signaling: the demise of elegant simplicity. *Curr. Opin. Genet. Dev.* **14**, 506-512.

Kalidas, S. and Smith, D. P. (2002). Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. *Neuron* **33**, 177-184.

Kauffmann, R. C., Li, S., Gallagher, P. A., Zhang, J. and Carthew, R. W. (1996). Ras1 signaling and transcriptional competence in the R7 cell of Drosophila. *Genes Dev.* **10**, 2167-2178.

Lai, E. C. (2002). Developmental signaling: shrimp and strawberries help flies make cones. *Curr. Biol.* **12**, R722-R724.

Le Borgne, R., Bardin, A. and Schweisguth, F. (2005). The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development* **132**, 1751-1762.

Miller, A. C., Seymour, H., King, C. and Herman, T. G. (2008). Loss of seven-up from Drosophila R1/R6 photoreceptors reveals a stochastic fate choice that is normally biased by Notch. *Development* **135**, 707-715.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M. (1990). The Drosophila seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**, 211-224.

Nagaraj, R. and Banerjee, U. (2007). Combinatorial signaling in the specification of primary pigment cells in the Drosophila eye. *Development* **134**, 825-831.

Nichols, J. T., Miyamoto, A. and Weinmaster, G. (2007). Notch signaling-constantly on the move. *Traffic* **8**, 959-969.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.

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.

Pickup, A. T., Lamka, M. L., Sun, Q., Yip, M. L. and Lipshitz, H. D. (2002). Control of photoreceptor cell morphology, planar polarity and epithelial integrity during Drosophila eye development. *Development* **129**, 2247-2258.

Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the Drosophila retina, a neurocrystalline lattice. *Dev. Biol.* **53**, 217-240.

Rohrbaugh, M., Ramos, E., Nguyen, D., Price, M., Wen, Y. and Lai, Z. C. (2002). Notch activation of yan expression is antagonized by RTK/pointed signaling in the Drosophila eye. *Curr. Biol.* **12**, 576-581.

Sasaki, Y., Ishida, S., Morimoto, I., Yamashita, T., Kojima, T., Kihara, C., Tanaka, T., Imai, K., Nakamura, Y. and Tokino, T. (2002). The p53 family member genes are involved in the Notch signal pathway. *J. Biol. Chem.* **277**, 719-724.

Sun, J. and Deng, W. M. (2007). Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. *Dev. Cell* **12**, 431-442.

Thomas, G. B. and van Meyel, D. J. (2007). The glycosyltransferase Fringe promotes Delta-Notch signaling between neurons and glia, and is required for subtype-specific glial gene expression. *Development* **134**, 591-600.

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.

Tsuda, L., Kaido, M., Lim, Y. M., Kato, K., Aigaki, T. and Hayashi, S. (2006). An NRSF/REST-like repressor downstream of Ebi/SMRTER/Su(H) regulates eye development in Drosophila. *EMBO J.* **25**, 3191-3202.

Van Vactor, D. L., Jr, Cagan, R. L., Kramer, H. and Zipursky, S. L. (1991). Induction in the developing compound eye of Drosophila: multiple mechanisms restrict R7 induction to a single retinal precursor cell. *Cell* **67**, 1145-1155.

Weber, U., Paricio, N. and Mlodzik, M. (2000). Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development* **127**, 3619-3629.

Wilk, R., Reed, B. H., Tepass, U. and Lipshitz, H. D. (2000). The hindsight gene is required for epithelial maintenance and differentiation of the tracheal system in Drosophila. *Dev. Biol.* **219**, 183-196.

Wilk, R., Pickup, A. T., Hamilton, J. K., Reed, B. H. and Lipshitz, H. D. (2004). Dose-sensitive autosomal modifiers identify candidate genes for tissue autonomous and tissue nonautonomous regulation by the Drosophila nuclear zinc-finger protein, hindsight. *Genetics* **168**, 281-300.

Wolff, T. (2000). Histological techniques for the Drosophila eye. Part 1, larva and pupa. In *Drosophila Protocols* (ed. W. Sullivan, M. Ashburner and R. S. Hawley), pp. 201-227. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Wolff, T. and Ready, D. F. (1991). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. *Development* **113**, 841-850.

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.

Yip, M. L., Lamka, M. L. and Lipshitz, H. D. (1997). Control of germ-band retraction in Drosophila by the zinc-finger protein HINDSIGHT. *Development* **124**, 2129-2141.

Zheng, L. and Carthew, R. W. (2008). Lola regulates cell fate by antagonizing Notch induction in the Drosophila eye. *Mech. Dev.* **125**, 18-29.