Three Distinct Roles for Notch in *Drosophila R7* Photoreceptor Specification

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**Abstract**

Receptor tyrosine kinases (RTKs) and Notch (N) proteins are different types of transmembrane receptors that transduce extracellular signals and control cell fate. Here we examine cell fate specification in the *Drosophila* retina and ask how N acts together with the RTKs Sevenless (Sev) and the EGF receptor (DER) to specify the R7 photoreceptor. The retina is composed of many hundred ommatidia, each of which grows by recruiting surrounding, undifferentiated cells and directing them to particular fates. The R7 photoreceptor derives from a cohort of three cells that are incorporated together following specification of the R2-R5 and R8 photoreceptors. Two cells of the cohort are specified as the R1/6 photoreceptor type by DER activation. These cells then activate N in the third cell (the R7 precursor). By manipulation of N and RTK signaling in diverse combinations we establish three roles for N in specifying the R7 fate. The first role is to impose a block to photoreceptor differentiation; a block that DER activation cannot overcome. The second role, paradoxically, is to negate the first; Notch activation up-regulates Sev expression, enabling the presumptive R7 cell to receive an RTK signal from R8 that can override the block. The third role is to specify the cell as an R7 rather than an R1/6 once RTK signaling has specified the cells as a photoreceptor. We speculate why N acts both to block and to facilitate photoreceptor differentiation, and provide a model for how N and RTK signaling act combinatorially to specify the R1/6 and R7 photoreceptors as well as the surrounding non-neuronal cone cells.

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**Introduction**

During development, cells receive signals from local or distant sources and respond by sending other such signals. As a result, complex systems of spatial and temporal information emerge and individual cells make decisions about their fates based on this information. Remarkably, only a few ligands and receptors mediate these signaling events. Hence, the question arises as to how a small number of signaling pathways can generate elaborate cell patterns such as the array of photoreceptors, lens and pigment cells that make up each facet of the insect compound eye.

Two concepts have emerged. The first is the iterative use of signals. In this case, each signaling event alters the state of a cell and determines how it responds to subsequent signals. Accordingly, a cell can receive a sequence of signals over time and progress in a stepwise manner towards its ultimate fate. The second concept is the combinatorial use of the signals. In this case, the informative value of a signal depends on whether it is received alone or together with one or more additional signals. Both mechanisms appear to apply in most developmental contexts, but how they are used, either separately or in conjunction to dictate any given cell fate is poorly understood. Here we investigate how receptor tyrosine kinase (RTK) and Notch (N) signaling are used to specify a unique cell type: the R7 photoreceptor of the *Drosophila* eye.

The *Drosophila* ommatidium is a complex assembly of 20 cells arrayed in a stereotypical pattern in which each cell can be uniquely identified by both its type and its position in the structure (Figure 1A–1C). Each ommatidium is composed of a core assembly of eight photoreceptors and four cone cells (non-neural lens elements) surrounded by an array of primary, secondary and tertiary pigment cells, and mechanosensory bristle complexes (Figure 1B) [1]. The core assembly arises in two distinct phases. In the first phase, a “precluster” of five cells withdraws from the cell cycle and differentiates into five photoreceptors in a stepwise fashion: first R8, then the pair R2/R5 and then the R3/R4 pair. In the second phase, the assembly grows by successive rounds of accretion of surrounding cells (Figure 1F). Cells are recruited to specific positions (niches; white cells in Figure 1F) on the surface of the growing cluster, and as they begin to differentiate they then contribute to the formation of new niches into which the next round of cells will be recruited [2,3]. During the first round of accretion, a group of three undifferentiated cells is added to the R2/8/5 face of the precluster (Figure 1Fii). Upon joining, the two end cells, which abut the R2 and R3 cells, rapidly differentiate as the R1 and R6 cells (Figure 1Fiii). Several hours later, the middle
**Author Summary**

Cells are often directed to their developmental fates by the signals they receive from other cells. The Drosophila eye has become a classic paradigm for studying such signaling, and in this system direct neighbor-to-neighbor signaling plays a large role. The R7 photoreceptor is directed to its fate by signals derived from two different neighboring cell types. One sends a signal that activates tyrosine kinase signaling in the R7 precursor, whereas the other activates the Notch signaling pathway. Here we examine Notch signaling and find that it induces three responses in the R7 precursor. We show that one role acts to inhibit the specification of the cell as a photoreceptor, while another role opposes this function, and acts to direct the cell to the photoreceptor fate. The third role specifies the cell as the specialized R7 photoreceptor rather than as the generic photoreceptor type. These results demonstrate that activation of a single signaling pathway can result in multiple cellular responses, even antagonistic ones.

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**Figure 1. Details of the developing and adult ommatidia.** (A) Scanning EM of the adult eye. Each facet corresponds to a single ommatidium. (B) A schematic adult ommatidium. The four cone cells (c, light blue) overlie the eight photoreceptors. Green labels the outer photoreceptors (R1-R6); R7 is colored purple and R8 is dark blue. R7 lies higher in the retina than R8. (C) TEM section through an ommatidium corresponding to the middle cross-section in (B). R7 is evident at this level. (D) TEM section through a developing ommatidium. At this stage the cone cells are yet to join the cluster. Presumptive photoreceptors are numbered. Note that R8 is the central cell at this stage. (E) Schematic summary of the RTK signaling events that determine whether a cell becomes a photoreceptor (Ttk degraded) or not (Ttk not degraded).

Two RTKs are required for specification of the R1/6/7 photoreceptors: the Drosophila EGF-Receptor (DER) and Sevenless (Sev). DER is ubiquitously expressed and is activated by its ligand Spitz, a diffusible peptide that is secreted by differentiating cells of the precluster and is required for the specification of all the six outer photoreceptors (R1–R6) [13,14]. Sev is expressed in a complex manner; it is found in R3/4 and the mystery cells in the precluster, but at the time around R7 specification it is expressed at high levels in the R7 and cone cell precursors, and at low levels in the R1/6 progenitors. It is activated by a membrane bound ligand, Bride of Sevenless (Boss) presented on the surface of the R8 cell [15–18]. The R1/6/7 precursors contact R8 but the cone cell progenitors do not (Figure 1Fvi).

For many years only the RTK signaling pathway was clearly defined in the developing ommatidia. DER and Sev were seen as performing the same role in the R1/6 and R7 precursors respectively [14]. That is, they activated the Ras/MAPK pathway to degrade Ttk and induce photoreceptor differentiation (Figure 1E). However, the R1/6 and R7 cells are morphologically and physiologically distinct photoreceptor types, and although the activity of DER and Sev explains how they become photoreceptors, it does not explain what makes them different kinds of photoreceptors. Three models have been invoked to explain what
differentiates the R1/6 and R7 photoreceptors. One model is that the timing or intensity of the RTK signal is the critical determinant. For example, a “clock” in the cells might meter the time of receipt of the RTK signal, and those receiving it early would become R1/6 types and those late would become R7s [14]. A second model sees Sev conferring a more potent activation of the Ras/MAPK pathway than DER, sufficient to engage additional outputs that select the R7 as opposed to the R1/6 fate. The third model posits that R1/6 cells, or perhaps the R8, express a second signal that acts in combination with Boss to direct the R7 precursors to their unique fates [3]. This third model was substantiated by the subsequent discovery that N signaling was required for specification of R7. Signaling by the N ligand Delta (Dl) on R1/6 activates N in the R7 precursor, directing it to the R7 fate [19,20]. In the absence of Sev, the R7 precursor fails to differentiate as a photoreceptor and adopts the non-neuronal cone cell fate [17], and in the absence of the N signal it differentiates as an R1/6 photoreceptor [19,20].

Thus, specification of the R7 fate requires the activation of two signaling pathways, providing a paradigm for studying how a cell “decodes” two signals to choose a particular fate. The role of the RTK signal in this process is relatively well understood but that of N has remained less clear. Here we have examined the roles played by N and find that it has (at least) three distinct roles in dictating R7 specification. First, N activity in the R7 precursor creates a barrier to RTK-induced photoreceptor differentiation, one that DER activation is not able to overcome. Second, it promotes Sev expression, providing the R7 precursor with a means to receive an additional RTK signal that overcomes the N-induced barrier to photoreceptor differentiation. Third, it provides an input that dictates the choice of R7 as opposed to R1/6 photoreceptor type. Extrapolating from these results, we propose a combinatorial model for the specification of the R1/6, R7 and cone cell fates by RTK and N signaling.

Results

We define three distinct roles for N in R7 specification. A role in activating sev transcription was uncovered first; the remaining roles emerged from experiments in which this role was bypassed. Accordingly, we begin with the evidence that sev transcription is N dependent.

Role 1—N Activity Up-Regulates sev Transcription to Specify the R7 Precursor as a Photoreceptor Rather Than a Cone Cell

(i) N activity regulates sev transcription. Sev is expressed in a complex pattern in the developing ommatidium; it is absent from R8, R2 and R3, barely detectable in R1 and R6, and accumulates highly in the remaining photoreceptors (R3, R4 and R7) as well as the cone cells [21]. Using a sev promoter fragment that reproduces the expression pattern of the native sev gene [22], we previously drove a constitutively active form of N (that reproduces the expression pattern of the native [19,20]. However, it posed the question of why one of several that established a causal relationship between N precursors as ectopic R7 photoreceptors [20]. This finding was R7) as well as the cone cells [21]. Using a accumulation highly in the remaining photoreceptors (R3, R4 and R7, barely detectable in R1 and R6, and

(ii) High levels of sev transcription are required for R7 specification. In the absence of the sev gene, the R7 precursor is inappropriately specified as a cone cell, and we next asked whether the high levels of sev transcription induced by N activity are indeed necessary for the appropriate R7 specification. To do this we varied the level of Sev expression independently of N

To investigate this possibility we examined the expression of sev.lacZ in developing ommatidia of sev.N* flies, and observed a rapid and strong accumulation of ectopic β-Galactosidase in the R1/6 precursors (Figure 2B), which in wild type (Figure 2A) show no detectable expression. Accordingly, we infer that N activity can up-regulate sev transcription in these cells, allowing the sev.N* transgene to amplify its own expression as well as that of the sev.lacZ transgene.

To determine whether the normal up-regulation of sev transcription in the R7 precursor depends on N activity, we performed the reciprocal experiment of assaying sev.lacZ expression in ommatidia in which the N transduction pathway was impaired in two different ways. In the first approach, we used the sev promoter to drive expression of a chimeric protein consisting of Suppressor of Hairless (Su(H)) fused to the Engrailed repressor domain (EnR): sev.Su(H)/EnR [20]. Su(H) can function as a transcriptional activator or repressor depending on the state of N activity [23], but Su(H)/EnR is “locked” in the repressor state and acts as a constitutive repressor of N target genes. If sev transcription is normally up-regulated by N, the sev.Su(H)/EnR transgene might be subject to a self-limiting, governor effect in which N activity leads to enhanced expression of Su(H)/EnR, which in turn impairs the expression of Notch target genes, including both sev and the sev.Su(H)/EnR transgene itself. Consistent with this reduction in N signal transduction and a requirement for N input in the R7 fate choice, R7 precursors are transformed (albeit at a low penetrance) to R1/6 fates in sev.Su(H)/EnR flies [20].

To assess the effect of the sev.Su(H)/EnR transgene on sev.lacZ expression, we generated wild type clones in a background of cells carrying a single copy of the sev.Su(H)/EnR transgene, allowing us to compare sev.lacZ expression in experimental (sev.Su(H)/EnR) versus wild type (control) cells, side-by-side. sev.lacZ activity was reduced cell-autonomously in R7 precursors as it was in the other cells that expressed sev.Su(H)/EnR (R3/4 and the cone cells) (Figure 2C). Furthermore, this reduction was even more pronounced in the “twin spot” clones in which two copies of sev.Su(H)/EnR are present (Figure 2C).

The second method we used to reduce N activity was to use the temperature sensitive allele N[ts]. We examined eye discs that were fully mutant for N[ts] or had N[ts] clones induced in them. In these manipulations the animals were held at the restrictive temperature for 24 h before dissection and analysis. We observed a strong reduction in sev.lacZ expression in all cells that normally express it, in both the whole disc experiments and in the clones (not shown). Since β-Galactosidase has a long perdurance it may give an inappropriate indication of surviving sev transcripts when N activity is severely reduced. We therefore monitored Sev protein levels. Here the whole disc experiments showed an almost complete loss of Sev expression (Figure 2E), and an autonomous loss was detected in the clones (Figure 2F). Again, the effects on Sev expression were universal; all cells that express Sev (including the presumptive R7) showed a loss of the protein expression.

Since raising or lowering activity of the N signal transduction pathway correspondingly raises or lowers sev transcription, we infer that N acts to promote high levels of sev expression in the R7 precursor.
signaling by replacing the endogenous sev gene with transgenes that express within the eye in a blanket manner. First we used the low-level, ubiquitous tubulinα1 promoter (tub.sev). In sev null (henceforth sev−) flies, all R7 precursors differentiate as cone cells and the adult eyes have no R7 cells. To the sev− mutant background we introduced two tub.sev transgenes and observed no rescue of any R7s (N = 272 ommatidia). Correspondingly, the eye discs of this genotype showed almost no Sev expression (Figure 3B). When a different set of two tub.sev transgenes was tested, again no rescue of R7s was observed (N = 283 ommatidia). However, when the four transgenes were combined, 29% of the R7s were rescued (N = 344 ommatidia), and a correspondingly higher level of Sev protein was present in the eye discs (Figure 3C). GMR is a promoter element that drives at high levels in all the developing retinal cells, and into the sev− mutant background we introduced a GMR-sev transgene (sevGMR); GMR.sev) and observed almost complete rescue (97%) of the R7s (N = 511 ommatidia) with a corresponding high level of Sev expression in the eye disc (Figure 3D). Likewise, increasing the number of the tub.sev transgene to 8 copies leads to a complete rescue (100%, N = 130 ommatidia). Collectively, these data suggest that at low levels of Sev expression R7s are not specified, at moderate levels an intermediate number of R7s are rescued, and high levels correspond with a robust specification of R7s. We therefore infer that N functions to ensure a high level of Sev in the R7 precursor for its appropriate specification.

Role 2—N Activity Specifies a Photoreceptor as the R7 Rather Than the R1/6 Type

Based on the reciprocal transformations between the R1/6 and R7 fates caused by reducing or elevating N activity, we previously proposed that N normally directs the choice of the R7 fate as an alternative to R1/6 [20]. However, we now understand that the N
pathway modulates the level of sev transcription, and the question arises as to whether N directs the R7 versus R1/6 choice through the regulation of sev or through a separate mechanism.

To address this question, we used the sev0; GMR.sev flies in which there is no endogenous sev gene for N to up-regulate, and sev transcription is supplied by a heterologous promoter element that is not responsive to N. Hereafter we refer to this as the “GMR.sev-rescued” condition.

In this background we repeated the three genetic mosaic experiments used to define the requirement for N signaling in the R7 versus R1/6 choice [20]: namely assaying the effects of Dl null (henceforth Dlnull), sev.Su(H)Ener, and sev.N* clones.

(i) Dlnull clones in GMR.sev-rescued eyes. In wild type eyes, Dl is required in either R1 or R6 for R7 specification [20]. We performed Dl mosaic analysis in GMR.sev-rescued flies and scored normally constructed ommatidia to determine which photoreceptors could be Dlnull without perturbing R7 specification. As we previously observed in wild type flies, Dl could be safely removed from any of the R1, R6, or R7 precursor cells (Figure 4G), provided that it was not removed simultaneously from both the R1 and R6 precursor. We scored 127 normally constructed yet genetically mosaic ommatidia (containing a mixture of wild type and Dlnull cells). 30% of these had Dlnull cells in the R1/6/7 group (14% had either a single R1 or R6 mutant, 15% had R1/R7 or R6/R7 mutant, and 1% had only R7 mutant). No ommatidium had both R1 and R6 mutant. Thus, correct specification of the R7 photoreceptor still requires Dl signaling from either the R1 or R6 cell, even when the requirement for N in up-regulating sev is bypassed.

Determining the fate of the cell in the R7 position when both R1 and R6 are mutant for Dl is difficult as such ommatidia are structurally aberrant, and a degree of inference is required in identification of the constituent cells [20]. Given this proviso, ommatidia were observed that had a morphologically R1/6-like cell in the R7 position when the neighboring R1 and R6 cells were both Dlnull (Figure 4B and 4C), as we previously observed for Dl mosaics in otherwise wild type eyes [20]. Hence, in GMR.sev-rescued ommatidia, as in wild type ommatidia, it appears that the cell in the R7 position must receive Dl input from either the R1 or the R6 cell to avoid being mis-specified as an R1/6 cell.

(ii) sev.Su(H)Ener clones in GMR.sev-rescued eyes. As described above, sev.Su(H)Ener acts as a mild cell-autonomous suppressor of the N transduction pathway. Here, we focus on the consequences of reducing N transduction in the R7 precursor. Such analysis is complicated by the accompanying reduction of N signaling in the cone cell precursors which, as we describe below, can cause them to be mis-specified as R1/6 photoreceptors. To avoid this complication we generated mosaic eyes with only small, rare sev.Su(H)Ener clones, and scored ommatidia containing only one or a few mutant cells and lacking supernumerary photoreceptors. In an otherwise wild type background, a low frequency of sev.Su(H)Ener cells located in the R7 position differentiate inappropriately as R1/6 cells [20] and the same was observed in the GMR.sev-rescued background (Figure 4D and 4E).

We then examined the effects of sev.Su(H)Ener activity in GMR.sev-rescued ommatidia in the larval retina during the stage of R7 specification (but before supernumerary photoreceptors are added by mis-specification of cone cells) using molecular markers
Figure 4. The effects of N manipulations in the sev<sup>+</sup>; GMR.sev background. (A–C) DI clones (labeled by lack of pigment—evident in photoreceptors by the absence of the black granular mass adjacent to the rhabdomeres) in the GMR.sev-rescued background. (A) Mosaic analysis shows that normal ommatidia still form if either R1 or R6 is mutant for Di (arrows point to R1 or R6 cells lacking Di), but not when both are mutant. (B,C) The fate of R7 precursors when both R1 and R6 are mutant. (B) The lower ommatidium labeled with black numbers shows the cell in the R7 position (asterisk) appearing as an R1/6 type when the cells in the R1/6 positions are both Di. Compare with the wild type ommatidium (top right) labeled in red. (C) At the level of the R6s, the lower ommatidium (black labels) still shows the large rhabdomere cell (asterisk) consistent with it being an R1/6 type, and R8 can be seen projecting between the inferred R1/2 cells. Compare with the wild type ommatidium (red labeling) in which the cell in the R7 position is no longer evident at this depth and R8 projects between R1 and R2. (D,E) Clones of sev.Su(H)EnR labeled by the absence of pigment. (D) When the cell in the R7 position carries sev.Su(H)EnR, it can transform into an R1/6-like cell (asterisk) with (E), a rhabdomere that projects...
of cell fate. Here, α-Runt staining was used to label R7’s, and α-
Seven-up (Svp) and α-Bar used to label the early and late phases of
R1/6 specification, respectively. In an otherwise wild type
background, sev.Su(H)EnR causes a fraction of the R7 precursors
to express R1/6 markers instead of R7 markers [20], and we observe
a similar mis-specification of R7 precursors in the
GMR.sev-rescued background (Figure 4F).

Collectively, our analysis of both the Df and sev.Su(H)EnR
mutant conditions argue that N activity in the R7 precursor is
required to select the R7 fate instead of the R1/6 fate, even in the
GMR.sev-rescued background, when it is no longer required to up-
regulate sev.

(iii) sev.N* clones in GMR.sev-rescued eyes. Boss is the ligand for Sev and is expressed by the developing R8 cell. Both R1
and R6 contact R8, so any Sev expressed in these cells should gain
access to the ligand. And yet Boss has no effect on R1/6
specification, even when Sev is supplied at high levels in the R1/6
cells, as in GMR.sev-rescued ommatidia (Figure 3D), as expected if N
input is required for R7 versus R1/6 specification. Similar results
were found when heat shock expression of the sev gene was
previously used to rescue ommatidia [24,25].

Above, we have assayed the requirement for N in promoting the
R7 fate. We next examined the effects of ectopic N activity in R1/6
precursors using sev.N*. Our earlier studies showed that the R1/6
precursors are sensitive to the presence of ectopic N activity.
Although there were some escapers, the R1/6 precursors were
largely specified as R7 types [20]. We have repeated these
experiments in the GMR.sev-rescued background and obtained the
same result. Of 73 mosaic ommatidia with normal pattern only
7% of ommatidia showed R1 or R6 cells carrying the sev.N transgene. In comparison, control clones using a neutral marker showed 70% of mosaic ommatidia with R1/6 cells labeled. Thus, in the GMR.sev-rescued background the presence of sev.N* strongly interferes with the correct specification of the R1/6 fates. Furthermore, in entirely mutant sev.N* GMR.sev-rescued eye discs, the vast majority of R1/6 precursors express the R7 marker, Runt, at the expense of the R1/6 marker, Svp (Figure 4H).

In summary, the level of N signaling in the R1/6 precursors, as
in the R7 precursor, distinguishes between alternative R1/6 and
R7 fates, even when the N-dependent requirement for sev up-
regulation is met by other means. When a cell has been specified
as a photoreceptor (Tik degradation) the presence of high N
activity dictates the R7 fate, whereas a low N signal directs the
R1/6 fate (Figure 4I).

Role 3—N Activity Imposes a Barrier to Photoreceptor
Specification by DER Signaling That Can Be Overcome by Sev Signaling

The first two roles of N (up-regulating sev to allow the R7
precursor to initiate photoreceptor differentiation and to distin-
guish between the R7 and R1/6 photoreceptor fates) were gleaned
primarily from examining the consequences of manipulating N
activity in the R7 precursor cell. Below we present three experiments that manipulate N and Sev/Ras transduction in the
R1/6 and cone cell precursors and we describe the effects on
whether these cells adopt the photoreceptor (R1/6/7) or non-
photoreceptor (cone cell) fate.

(i) In the absence of sev, ectopic N activity causes the R1/6
precursors to become cone cells. In both wild type and
GMR.sev-rescued ommatidia, sev.N* activity in R1/6 precursors
directs them to the R7 fate. However, in both these contexts, sev
is also up-regulated, posing the question of what fate the R1/6
precursor cells would adopt if the contribution of Sev signaling
was abolished. To address this, we compared sev,N*ommatidia in the
presence or absence of the endogenous sev gene.

Adult sev,N* ommatidia (Figure 5A) are variable in structure and
usually contain four large rhombomere cells (probably correspond-
ing to R2, R3, R4 and R5) and two to four small rhombomere cells
(probably R7 and R9 plus variable numbers of supernumerary R7
cells [26]) derived from the R1 and R6 precursors [20]). The
variability derives from the loss of cells from the presumptive R1/
6/7 positions in sev,N* ommatidial clusters. Why this happens is
not known, but it results in a variable number of supernumerary
R7s being generated [20]. When the sev gene is removed in this
background, there is a dramatic reduction in the number of small
rhombomere cells, in most cases to only a single R8 cell, without a
gain in the number of large rhombomere cells (Figure 5B). From
this we draw two inferences. First, the sev,N* induced transfor-
nation of R1/6 precursors to the R7 fate is sev-dependent. Second, in
the absence of sev, the R1/6 precursors do not default back to their
normal fate since there is no corresponding increase in numbers of
large rhombomere cells.

What do the sev*; sev.N* R1/6 precursors become? To address
this, we stained sev*; sev.N* eye discs with photoreceptor markers
(Svp, Elav, Runt) and the cone cell marker (Cut) and observed that
the cells in the R1/6 positions express Cut and none of the
photoreceptor (cone cell) fate.

(ii) Activated Ras restores R7s in sev*; sev.N*
ommatidia. Sev is specifically required for the R7 fate, in
contrast to the outer photoreceptors (including R1/6), which are
specified by DER signaling. It is generally assumed that the R1/6
precursors receive Spitz, the DER ligand, from the precluster cells
(most likely the abutting photoreceptors R2 and R5). Spitz binding
to DER activates the Ras/MAPK pathway leading to the
degradation of Ttk; the inhibitor of photoreceptor differentiation
(Figure 1E; see Introduction). Thus, in normal development,
avtivation of DER appears sufficient to promote photoreceptor
differentiation in the R1/6 precursors by destroying Ttk. How-
ever, when N is inappropriately activated in these cells in the
absence of sev (sev*; sev.N*) the cells no longer become photo-
receptors, but instead become cone cells. If, however, sev is present
(as in sev.N*, or sev*; GMR.sev; sev.N* ommatidia), the cells
differentiate as photoreceptors. Thus, N activity appears to
create a barrier to photoreceptor differentiation that DER alone
cannot overcome. Yet when Sev is concurrently activated the
photoreceptor fate is specified. This difference could be explained
in at least two ways. First, Sev and DER are different types of
RTKs, and hence, Sev may be able to activate downstream
transduction pathways that DER cannot. Second, Sev simply
provides more of the same activity that DER provides, namely an
increased activation of the Ras/MAPK pathway. Indeed, it may be that N up-regulates sev in the R7 precursor cells specifically to enable them to receive a more potent RTK signal than that normally mediated by DER.

To distinguish between these models, we used a sev.Ras* transgene to supply high levels of constitutive Ras activity to the R1/6 cells in sev; sev.N* ommatidia, allowing us to assess whether potent activation of the canonical Ras/MAPK pathway is sufficient to overcome the sev.N* induced barrier to photoreceptor differentiation. sev; sev.N*; sev.Ras* flies do not eclose which precluded an assessment of the adult phenotype, but in eye discs the cells in the R1/6 position (asterisks) expressing the cone cell marker Cut (green). (D) When sev.Ras* is supplied to the cells shown in (C), the R7 fate is restored to the cells in the R1/6 positions (asterisks) as evidenced by Runt expression (green). (E–H) Down-regulation of N signaling converts cone cells to R1/6 type cells. (E) shows a section through a sev.Su(H)EnR eye; many large rhabdomere cells are present in the ommatidia. (F) A sev.Su(H)EnR disc labeled for Cut (green) and Svp (red). Circles highlight early R3/4 pairs showing no evidence of incorporation of mystery cells. (G) Image from the posterior of a sev.Su(H)EnR disc labeled as in (F). Cells in cone cell positions expressing Cut can also express Svp (asterisks). (H) Image of a sev.Su(H)EnR 36 h pupal disc showing supernumerary Svp-expressing photoreceptors (red). (H') The same disc as (H) with the level of Cut expression (green) flattened onto the Svp layer. There are a reduced number of Cut expressing cone cells.

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(iii) Down-regulation of the N transduction pathway causes cone cell precursor cells to adopt the R1/6 fate. As described above, down-regulation of N signal transduction by the sev.Su(H)EnR transgene has at least two distinct consequences in the developing retina: first, it can cause the R7 precursor cell to adopt the R1/6 fate, and second, it can produce supernumerary large rhabdomere photoreceptors (Figure 5E). There are two likely sources of these ectopic photoreceptors: the “mystery” cells and the cone cells. Mystery cells are early companions of the R2–5 and R8 precluster cells that are subsequently lost [3], but various manipulations can induce them to differentiate as photoreceptors (e.g., [27]). To determine the source of these ectopic photoreceptors, we examined molecular markers of photoreceptors and cone cells in developing larval and pupal sev.Su(H)EnR discs. Nascent ommatidia in such discs show no evidence of mystery cell incorporation (Figure 5F), but at the posterior of such discs we detect defects in the cone cell array. Cell identification in the posterior tissue is difficult because sev.Su(H)EnR also affects the chirality of the ommatidia and they rotate in a disorganized manner compromising a simple reading of which cells belong to which ommatidium. The cone cells’ nuclei lie above the photoreceptor nuclei and express the transcription factor Cut, and these features make it easier to identify the cone cell cluster of each ommatidium. We observe a reduction in the...
number of nuclei in the cone cell positions, and a concomitant reduction in the number of nuclei expressing Cut. Indeed, we observe nuclei in cone cell positions expressing both Cut and Svp, suggesting that such cells have a confused identity (Figure 5G). In the 36 h pupal eyes individual cells belonging to each ommatidium are easy to identify, and here we observe an increase in the number of Svp-positive photoreceptors and a decrease in the number of cone cells (Figure 5H). At this stage there is no co-expression of Svp and Cut and any previous ambiguities appear to have resolved. These data suggest that at least some presumptive cone cells transform into Svp-expressing R1/6/7-like cells in the sev.Su(H)EnR background. We note that this phenotype is only partially penetrant, likely reflecting the weak suppression of the N pathway by the sev.Su(H)EnR construct.

In principle, the RTK signal responsible for inducing cone cells to differentiate as photoreceptors in sev.Su(H)EnR mutant retinas could be Sev or by DER. A Sev signal seems unlikely as its only known ligand, Boss is restricted to R8, a cell that the cone cell precursors do not contact. To confirm this we analyzed sev; sev.Su(H)EnR ommatidia and observed supernumerary photoreceptors, as in sev.Su(H)EnR ommatidia (not shown). Hence, we infer that DER is responsible for the mis-specification of cone cell precursors as R1/6 photoreceptors in sev.Su(H)EnR ommatidia.

N activity Regulates Ttk Levels

Above we have described the evidence for a role of N in inhibiting the photoreceptor fate. We next examined the effects that N modulations have on Ttk. There are two distinct populations of cells that express Ttk in the eye disc. It is expressed at high levels in the cone cells which lie in the apical regions of the disc, and it is expressed at low levels in all the basal cells representing the cells that will subsequently be incorporated into the growing ommatidia (Figure 6A). The first three cells incorporated into the precluster are the R1/6/7 precursors, and in these cells Ttk protein is degraded. But the cone cells, which are recruited next, not only fail to degrade Ttk but also show increased levels (compared to the basal cells from which they are derived; Figure 6A). We asked whether N activity was responsible for the high levels of Ttk in the cone cells. First, we attempted to monitor ttk transcriptional activity using in situ hybridization, and although we were able to detect ttk transcripts when the gene was overexpressed (sev; UAS-ttk), we could not detect the wild type level of transcription (not shown), and could not therefore determine whether it was reduced when N activity was attenuated. Second, we examined the ttk-lacZ reporter line [7] and did not detect any down-regulation in the N(01) background (not shown).

We next asked what happens to Ttk protein levels when N activity is reduced. Figure 6B shows protein expression in a N(01) eye disc that has been held at the restrictive temperature for 24 h. Control stainings for Svp and Senseless show robust expression levels and pattern disruptions typical of loss of N function. For example, Senseless is expressed in the R8 cells, and multiple Senseless-expressing cells are induced in the anterior regions of such discs (Figure 6B"); consistent with a large depletion in N activity. In these discs Ttk accumulation is severely reduced (Figure 6B). This observation allows us to correlate the loss of Ttk expression with the loss on N activity, and we infer that when the block on photoreceptor determination is removed there is a corresponding loss of Ttk protein. We next examined the Ttk protein levels in the R1/6/7 cells of sev; sev.N(01) eye discs. These cells are normally destined to form photoreceptors, but the presence of the high-level N activity instead specifies them as cone cells. In these cells we see ectopic high levels of Ttk protein (Figure 6C), and the effect of N in blocking the photoreceptor fate correlates with ectopic high levels of Ttk protein. Thus when N signaling is raised or lowered there is a corresponding raising or lowering of Ttk levels that mirrors the effects of N in blocking photoreceptor specification.

Discussion

We examine here the signaling that specifies three Drosophila ommatidial cell types: the R1/6 and R7 photoreceptors and the non-neural cone cells. We separate the fate choice into two binary decisions. The first is RTK-dependent and determines whether a cell is specified as a photoreceptor or not. If RTK signaling is sufficiently high, Ttk is degraded and the cell becomes a photoreceptor (Figure 1E); if not it becomes a cone cell. The second binary decision occurs once the choice to become a photoreceptor is made and dictates either the R7 or R1/6 fate. Here we examine the function of N in these two binary decisions and infer that it plays at least three roles. Two of these relate to the decision to become a photoreceptor: one role promotes the photoreceptor fate and the other opposes it. The third role distinguishes between the R7 and R1/6 types. We infer that all three roles operate in the specification of R7 itself, and such a complexity of N signaling was hitherto unsuspected. Below we evaluate the evidence for these three roles.

(i) N activates sev transcription to allow R7 precursors to adopt the photoreceptor fate. Three pieces of evidence indicate that N signaling regulates sev expression in the R1/6, R7 and cone cell precursors. First, ectopic N activity cell-autonomously up-regulates sev transcription. Second, and conversely, compromising N activity reduces sev transcription. Third, both the sev,N(01) and sev.Su(H)EnR transgenes, which depend on the N-responsive sev enhancer, show the expected auto-regulatory behavior: sev,N(01) is a potent amplifier of its own expression, whereas sev.Su(H)EnR is subject to a “governor effect” which limits its own expression.

Furthermore, we provide evidence that the high levels of sev supplied by N activity are essential for R7 specification. When sev is low or absent R7 precursors differentiate as cone cells, but when sev levels are high they differentiate as photoreceptors.

(ii) N activity dictates the choice of the R7 versus the R1/6 fate independently of the requirement for N in up-regulating sev. In the GMR.sev-rescued background, sev transcription is supplied at high levels in a blanket manner. Importantly, the endogenous sev gene is absent and cannot be up-regulated by N signaling. In this background we have manipulated N signaling in three different ways, and in all cases we find that high N activity dictates the R7 fate whereas low activity dictates the R1/6 fate. Thus, independent of its role in specifying photoreceptor versus non-photoreceptor (up-regulation of sev transcription), N activity determines the type of photoreceptor that an R1/6/7 precursor becomes.

(iii) N activity creates a barrier to photoreceptor differentiation. N has long been implicated in antagonizing photoreceptor specification. For example, when N function is reduced in developing eyes in the region of the furrow, almost all cells become specified as photoreceptors [28]. Our work here has defined a specific inhibitory role for N within the R7 precursor.

In wild type ommatidia DER signaling directs the R1/6 precursor cells to adopt the R1/6 fate. However, if N is ectopically activated in these cells and sev is concomitantly removed (sev; sev.N(01)) they become cone cells. In contrast, if we leave sev function intact (sev,N(01)) the R1/6 precursors develop as R7 photoreceptors. Hence, we infer that N activity imposes a barrier to photoreceptor specification that cannot be overcome by DER signaling but can
be negated when Sev is active (Figure 7B). Notably, we can also induce these cells to differentiate as R7 photoreceptors if we supply them with constitutively active Ras rather than native Sev function.

Since high-level Ras activation overcomes the N-induced block, we infer that Sev is able to activate Ras to a higher level than DER. Although DER appears insufficient to overcome the block, we have not yet activated Sev in the absence of DER to determine whether it alone can overcome the inhibition.

The cells in which the block is active also have high levels of Ttk expression. From this we surmise that N activity works indirectly to prevent the degradation of Ttk. We expect this block to act

Figure 6. Ttk expression and its correlation with the failure to differentiate as a photoreceptor. (A) Wild type expression of Ttk. Ttk (green) is expressed at high levels in the cone cells in the apical regions. (A') But is only weakly expressed in the nuclei of the basal layer. Note, the strong staining at the back of the disc is from apical tissue curving down in the disc. (A'') shows the same disc also stained for Svp and Runt to label the R1/6/3/4 and R7/8 cells respectively, to allow clear identification of the Ttk-expressing cone cells. (B) A N\textsuperscript{ts} disc held at 30°C for 24 h and stained for Ttk (green), which is significantly reduced. (B' and B'') show the same disc, respectively, stained for Svp (red) and Sens (blue) to show the persistent expression of other proteins. (C) A sev\textsuperscript{0}; sev.N\textsuperscript{*} eye disc stained for Ttk (green) and Svp (red). (C') shows a blow-up in which the cells in the R1/6/7 positions (asterisks) express high levels of Ttk. (D) is a wild type disc for comparison with (C). Here R1/6/7 do not express high levels of Ttk, but instead express high levels of either Svp (R1/6) or Runt (R7).

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A Model of Cell Fate Specification in the Developing Drosophila Eye

N signaling in the presumptive R7 appears to activate two competing pathways: one repressing the photoreceptor fate and the other facilitating it. Why would this be? We speculate that it relates to an ancient function of N in limiting photoreceptor number. The 

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mutation was named [5] after Phyllopoda crustaceans in which the ommatidia have only five photoreceptors [29], corresponding to the R2, R3, R4, R5 and R8 precluster cells. We suggest that in the ancient condition the five photoreceptor cells express Dl (or another N ligand) to prevent additional cells from becoming photoreceptors. With this view in mind we propose the following model of cell fate assignments for the cells that join the precluster (Figure 7C).

(i) The precluster cells express low levels of Dl (black arrows). (ii) Cells are recruited into defined niches; the first three being those on the R5/8/2 face. Three cells occupy these positions and Dl from the precluster activates mild N signaling (weak shade of gray), which provides a weak block to photoreceptor specification. Spitz expressed by R2/5 (red arrows) activates DER in the cells in the R1/6 position and overcomes the N block. (C) R1/6 begin differentiation as photoreceptors and express high levels of Dl as cells join the niches of the two flanking cone cells. The cell in the R7 position and these presumptive cone cells receive Dl from R1/6 and N is activated to high levels (dark gray). This provides a potent barrier to photoreceptor specification and also activates sev transcription. Binding of Sev to Boss on R8 provides high-level RTK activity (blue arrow). (iv) The RTK signaling specifies the R7 precursor as photoreceptor and the concomitant presence of activated N directs the R7 rather than the R1/6 photoreceptor type. R7 proceeds to express high levels of Dl, as do the two cone cells and R3. As the subsequent cone cells join, they receive these Dl signals and activate both the barrier to photoreceptor differentiation and sev expression. (V) None of the cone cell precursors contact R8 so none have activated Sev, and Spitz diffusing from R2/5 is unable to trigger the photoreceptor fate because of the high N activity.

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differentiate they induce a high-level of N activation in the R7 precursor and induce a N activity level in the flanking anterior and posterior cone cells, which is too high to be overcome by the DER activation, triggered by the arriving Spitz molecules. The N signaling also turns on sev transcription, providing high levels of Sev to these cells. R7 contacts the Boss-expressing R8 cell leading to a high-level activation of the Ras/MAPK pathway that overcomes the N barrier and allows photoreceptor specification. The presence of activated N then specifies the cell as an R7 rather than an R1/6 type.

(iv) As R7 and the cone cells begin differentiating they express DI. On the other side of the cluster the R3/4 pair undergo a N/DI interaction leading to high DI expression in R3. These DI expressions then activate N in the two further cone cells that join the ommatidium. The four cone cells experience high N activity and express high levels of Sev, which remains inactive because the cells do not contact R8. With Sev signaling inactive, the high-level N activity suffices to prevent the diffusing Spitz signal from specifying these cells as photoreceptors.

The Roles of DER and the Diffusion of Its Ligand

Implicit in the model above is the idea that Spitz diffuses from the precluster and reaches more distant cells with time. This concept was introduced by Freeman [14] and it explains many of the results we observe. First, consider the R7 precursor in which N activity is reduced. This cell becomes an R1/6 type. We suggest that when diffusing Spitz reaches this R7 precursor it finds the N-induced barrier absent and DER activation suffices for photoreceptor specification. Second, consider the cone cell precursors in which N signaling is also reduced. Here again the photoreceptor inhibition is weakened, and when the diffusing Spitz reaches them they too are specified as photoreceptors. Thus, we infer that in the absence of the strong N activity (providing a barrier to photoreceptor specification), diffusing Spitz can liberally induce the formation of ectopic photoreceptors.

The RTK and N Signals Propagate by Different Means

Above we consider the case of the cone cell which receives high N activation from DI expressed by its differentiating neighbors, and DER activation from Spitz diffusing from the R2/5 cells. Here we see two antagonistic signals propagating from the precluster and reaching distant cells by two distinct mechanisms. The N signal is relayed by the sequential incorporation of cells into the growing structure; as they begin to differentiate they express DI. By this cell-to-cell propagation, the DI signal progressively reaches more distant cells. The DER activation occurs by the diffusion of Spitz; it reaches more distant cells with time.

The Niche Model of Cell Incorporation

A key concept in the model presented here is that cells are “blind” to the fate-specifying signals emanating from the precluster until they enter the ommatidium. That is, cells undergo a two-step process of cell fate specification. First they occupy a niche (a specific position into which a cell can be recruited) and then they receive their fate-specifying signals. Consider the cluster shown in Figure 1F. In image (i) the precluster is surrounded by many cells. From these, three are recruited to the R1/6/7 positions (white cells – image (ii)). We suggest that these cells now respond to the signals from the cluster, the others do not. As more cells differentiate more niches become available and more cells become incorporated. The molecular nature of this recruitment mechanism remains unknown, but it plays a critical role in preventing the ectopic and premature responses of the cells to the RTK and N signaling pathways.

R1/6/7 and the Cone Cells Form an Equivalence Group

Since sev<sup>−</sup> R7 cells became cone cells, and cone cells with activated Sev became R7s, these cells were thought to belong to an equivalence group [27]. How this R7/cone cell equivalence group was established separate from the other cells that join the precluster remained unclear. Here by manipulation of N and RTK signaling in the R1/6, R7 and cone cells, we could transform any one of these into any other (Figure 7A). We infer therefore that all these cells form an equivalence group. Before they enter the ommatidium they are equipotent and it is the signals provided by the growing cluster that directs their fate rather than any pre-pattern that may exist in these cells prior to their incorporation.

DER Signaling Independent of Photoreceptor Specification

We have highlighted here the role of RTK signaling in the specification of photoreceptor fate, but we note that it is also required for the appropriate specification of the cone cells [12,14]. These cells receive a high N signal (and the concomitant barrier to photoreceptor specification) but appear to adequately transduce DER for the role in the cone cells. This again raises the question of where in the RTK pathway the N-induced barrier acts. The RTK pathway can be separated into the canonical transduction from the membrane to the nucleus, and the specific gene targets in the nucleus. Consider the photoreceptor pathway, here phyll transcription is required for the degradation of Tik, and anything lowering phyll transcription or its downstream outputs would oppose photoreceptor specification but leave other RTK outputs unaffected. Here we view the barrier as specifically targeting the photoreceptor output of the RTK pathway. Conversely, the barrier could affect the transduction through the cytoplasm to the nucleus and affect all outputs. In this situation we envisage that cone cell specification requires only a low-level activation of the RTK pathway.

Conclusions

We have examined what initially appeared to be a relatively simple process in cell fate assignment: the mechanism by which RTK and N signaling specify the R7 fate. We uncovered a complex signaling inter-relationship that probably results from the evolutionary history of the R7 cell. What appears paradoxical from the developmental perspective may be expected from the evolutionary view. The tractability of R7 as a model for fate specification has allowed the many functions of N to be uncovered here, but in other studies where manipulations cannot be as effectively performed, such complexity may go unrecognized.

Materials and Methods

All methods were previously described [20], except for PLP fixation, which was used for the Tik antibody following [3].

Stocks

<sup>sec</sup>/d2, Dl/rev10, sec>w+>N[w], sec>W+>Su(H-EnR), sec.N[ecn] [26], sec.lacZ [33], Tik.lacZ [7], N<sup>ts</sup>. To make sec.Su(H)/EnR clones the following chromosome was constructed: hs-flp, ubi-GFP, sec.Su(H)/EnR, FRT 19A. This was crossed to FRT 19A and heat shocked for 1 h at 37°C between 24 and 48 h AEL.

Antibodies

Elav.7E8A10, Cut (DSHB), Runt (gift J. Reinitz), Svp (gift Y. Hiromi), Bar (gift K. Saigo), Tik (gift P. Badenhorst), GFP
(Molecular Probes), Senseless (gift H. Bellen), Sev (Santa Cruz Biotechnology), and Beta-Galactosidase (Cappel).

**Nts** Experiments

Nts flies were reared at 18°C and shifted to 30°C in the third instar for 24 h followed by dissection. For clones, Nts was recombined with FRT 19A and clones were induced with hs-flipase or eye-flipase.

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**Author Contributions**

The author(s) have made the following declarations about their contributions: Concepted and designed the experiments: AT YEM GS. Performed the experiments: AT YEM GS. Analyzed the data: AT YEM GS. Contributed reagents/materials/analysis tools: AT YEM GS. Wrote the paper: AT YEM GS.

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