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Switching cell fates in the developing *Drosophila* eye

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SUMMARY

The developing *Drosophila* ommatidium is characterized by two distinct waves of pattern formation. In the first wave, a precluster of five cells is formed by a complex cellular interaction mechanism. In the second wave, cells are systematically recruited to the cluster and directed to their fates by developmental cues presented by differentiating precluster cells. These developmental cues are mediated through the receptor tyrosine kinase (RTK) and Notch (N) signaling pathways and their combined activities are crucial in specifying cell type. The transcription factor Lozenge (Lz) is expressed exclusively in second wave cells. Here, we ectopically supply Lz to precluster cells and concomitantly supply the various RTK/N codes that specify each of three second wave cell fates. We thereby reproduce molecular markers of each of the second wave cell types in precluster cells and draw three inferences. First, we confirm that Lz provides key intrinsic information to second wave cells. We can now combine this with the RTK/N signaling to provide a cell fate specification code that entails both extrinsic and intrinsic information. Second, the reproduction of each second wave cell type in the precluster confirms the accuracy of the RTK/N signaling code. Third, RTK/N signaling and Lz need only be presented to the cells for a short period of time in order to specify their fate.

KEY WORDS: *Drosophila*, R7, Cell fate, Eye, Lozenge

INTRODUCTION

When cells make developmental decisions, two influences can play crucial roles: the external information that is presented to cells, and the internal information that determines how the cells respond to it. The external information can be of diverse molecular nature but is usually in the form of secreted peptides that diffuse to target cells, or integral membrane proteins presented by immediate neighbors. The internal information is usually encoded by transcription factors that determine how the cells respond to these signals.

The developing *Drosophila* ommatidium provides an excellent model system with which to study how extrinsic and intrinsic information is integrated to deliver clear cellular developmental directives. Here, two classes of signaling pathway relay the external information: the receptor tyrosine kinase (RTK) and Notch (N) signaling pathways. The RTK pathway uses two distinct classes of signals. Spitz, which acts as the ligand for the *Drosophila* EGF receptor (DER; Egfr – FlyBase), is a diffusible peptide (Freeman, 1994; Freeman, 1996; Kumar et al., 1998), whereas Bride of sevenless (Boss) is an integral membrane protein that activates the Sevenless (Sev) RTK in immediately adjacent cells (Hafen et al., 1987; Krämer et al., 1991; Reinke and Zipursky, 1988). The N ligand in the developing eye is Delta (Dl), another integral membrane protein, which activates N only in direct neighbors (Artavanis-Tsakonas et al., 1995; Parks et al., 1995). The intrinsic information comes from the developmental history of the retinal tissue, in which a complex transcription factor web (Kumar, 2010) defines the tissue as 'eye', and when the cells receive a fate directive they interpret that signal as an instruction to make one of the many ommatidial cells.

The ommatidium is constructed in two distinct waves. First, a group of cells exits the cell cycle, undergoes a complex interaction and generates the precluster comprising prospective photoreceptors R2,3,4,5,8. In the second wave, cells are systematically incorporated into the cluster and the unit grows by simple accretion (Ready et al., 1976; Tomlinson and Ready, 1987). This is a reiterative process, in which cells are first recruited into specific positions within the cluster and then developmental signals from the differentiating cluster cells direct the fate of these new additions. As these cells differentiate they create new positions for cell recruitment and the process is repeated (Fig. 1A). We focus our studies on the fate specification of the first seven cells that are incorporated during the second wave in three rounds of recruitment. The first three cells that join the cluster are directed to become photoreceptors – two of the R1/6 generic class and one of the specialized R7 type. Next, two rounds of recruitment incorporate two pairs of cells, all four of which are directed to become lens-secreting cone cells. Thus, in this process three cell types are specified: the R1/6 photoreceptors, the R7 photoreceptor, and the cone cells (Fig. 1B).

A series of experiments has suggested that two binary molecular switches lie at the heart of the cell fate specifications of the second wave cells (Fig. 1C). RTK signaling determines whether Tramtrack 88 (Ttk), a transcription factor that represses photoreceptor development, is degraded (Li et al., 1997; Li et al., 2002; Tang et al., 1997). If Ttk is degraded then a second wave cell becomes a photoreceptor. If not, the photoreceptor fate is repressed and a cone cell is specified. The second binary switch relates to N activity, such that if Ttk is degraded (the photoreceptor fate) and the cell has low N activity it becomes an R1/6 photoreceptor, and if N activity is high it becomes an R7 photoreceptor (Fig. 1C) (Tomlinson et al., 2011).

Work from the Banerjee laboratory determined in a series of elegant experiments that the transcription factor Lozenge (Lz), is expressed exclusively in second wave cells (expression is absent from the precluster) (Flores et al., 1998) and that ectopic expression of Lz in precluster cells generates ectopic R7s (Daga et al., 1996), suggesting that supplying Lz to precluster cells redirects their fate responses to those of the second wave cells. Thus, Lz appears to act as the intrinsic factor that determines whether RTK and N signals are interpreted as precluster or second wave fate signals. In this paper, we expand upon this insight and demonstrate that the three

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characterized second wave fates can be systematically reproduced in the R3/4 precursors of the precluster when Lz is concomitantly supplied. Specifically, we show that when Ttk is absent and N activity is high, R7s are specified; when Ttk is absent and N activity is low the R1/6 class is formed; and when Ttk is present the cone cell fate is specified. This allows us to now incorporate Lz into the RTK/N/Ttk code of second wave fates, and thereby combine the intrinsic with the extrinsic information.

Additionally, this work provides cogent support for the signaling code of the second wave cells. Originally, the roles of RTK, N and Ttk in the specification of the second wave types were inferred from experimental manipulations of the second wave cells, and although all results were internally consistent, the complexity of the RTK and N interactions was somewhat surprising, and we sought another method for independent verification of this code. The use of precluster cells to reiterate second wave fates has now provided that verification; each of the cell types appears to be specified in the Lz-expressing R3/4 precursors in exactly the manner predicted by the cell fate codes. Thus, as we add the intrinsic information, we add it to a newly validated extrinsic signaling code.

It has been clear for some time that the signals that specify the second wave cells are presented to the cells during a short time window. In this work, we establish that the Lz information is similarly only required for a short time period, and we infer that both the intrinsic and extrinsic information need only be presented briefly to the cells for them to lock-in their final cell fates. This raises the intriguing question of the molecular mechanism by which the ephemeral specification information is converted into stable cell fate directives that guide the differentiation and maturation of the cells.

MATERIALS AND METHODS

Immunohistochemistry and histology

Protocols for adult eye sectioning and antibody staining have been described previously (Tomlinson et al., 2011). Primary antibodies: rabbit anti-β-gal (Cappel); rabbit anti-GFP, mouse anti-GFP IgG2a (Molecular Probes); guinea pig anti-Runt (gift of J. Reinitz, Stony Brook University, NY, USA); rabbit anti-Runt (gift of A. Brand, Gurdon Institute, Cambridge, UK); guinea pig anti-Sens (gift of H. Bellen, Baylor College of Medicine, Houston TX, USA); rabbit anti-Bar (gift K. Saigo, Kobe University, Japan); rat anti-Bar (gift of T. Cook, University of Cincinnati School of Medicine, USA); mouse anti-Svp (gift of Y. Hiromi, National Institute of Genetics, Japan); rabbit anti-RFP (MBL International Corporation); rat anti-Elav and mouse anti-Cut (Developmental Studies Hybridoma Bank). Alexa Fluor 488, 555 and 647 conjugated secondary antibodies were used (Molecular Probes). For analysis of adult eye sections, at least 200 individual ommatidia were scored for each genotype. A minimum of 40 individual eye imaginal discs were analyzed for each genotype.

Identification of individual cells in third larval instar eye discs

In wild type ommatidia cells join the ommatidium in a stereotypical manner, they adopt specific positions in the cluster and they express molecular markers characteristic of their fate. We use a panel of antibodies to detect these fates, including Runt, Sens, Elav, Ro, Bar and Cut. All photoreceptors express Elav; in addition, R8 expresses Runt and Sens, R3/4s express Svp, R1/6s express Bar and Svp (at lower levels than R3/4) and R7s express Runt. Cone cells express Cut (without Elav). When examining mutant ommatidia we track cells as they join the ommatidia and occupy specific positions, and then determine which molecular markers they subsequently express.

Fly stocks

The following lines were used: *sev[d2]*, *sev.N*$^{[intra]}$ (Fortini et al., 1993); *sev.Rap*^{V12} (Mavromatakis and Tomlinson, 2012); *lz*^{77a7} (Batterham et al., 1996); *sev.Su(H)enR* (Tomlinson and Struhl, 2001); *Rh3;Rh4 lacZ* (*Pan-R7 lacZ*) (Hofmeyer et al., 2006).

Generation and transformation of constructs

The coding sequences for *DsRed*, *phyl* and *lz* were each amplified using appropriate PCR primers and cloned into an attB vector containing 2× *sev* enhancer elements and the *sev* promoter element. For *ro.GFP* and *ro.Gal4* a 1.9 kb *Not*I-*Eco*RV (5204-7132) fragment of *rough* genomic DNA was cloned upstream of the minimal *hsp70* promoter and then cloned into an attB vector containing either *GFP* or *Gal4* coding sequence. Transformation of all the above attB plasmids was carried out following standard protocols (Bischof et al., 2007).

RESULTS

The ectopic expression of Lz in R3/4 precluster cells specifies them as R7s

The *sev* enhancer element (Basler et al., 1991) drives expression at high levels in R3/4, R7 and cone cell precursors (Fig. 1D), and to target Lz expression to the R3/4 precursor we engineered and transformed a *sev.lz* construct. We observed [as previously described (Daga et al., 1996)] that the cell in the R4 position frequently transformed into an R7-type cell, as evidenced by cellular morphology in adult sections (Fig. 2D, red arrowheads), and by the expression of specific molecular markers in third larval instar discs (Fig. 2E). Less frequently, both R3/4 precursors transformed into R7s (Fig. 2D, red circle), and occasionally the R3 precursor transformed into the R7 type when the R4 precursor did not (Fig. 2D, green arrowhead).

The R3/4 cells of the normal precluster undergo an N-Dl interaction that results in one cell with high N activity (R4) and the other with relatively low N activity (R3) (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Since the cell in the R4 position experiences high N activity, then the frequent specification of this cell as an R7 was consistent with the cell fate code for R7; it requires high N levels whereas the R1/6 types are specified by low N activity (Fig. 1C). R3/4 types are morphologically indistinguishable from R1/6 cells in adult sections but have a clear molecular signature in terms of the transcription factors that they display during the development of the eye disc: R3/4 express Svp whereas R1/6 cells express both Svp and Bar. The cells in the R3 positions in *sev.lz* eye discs did not express Bar, suggesting that although the high levels of N activity specified R7s, the lower level of N activity did not specify R1/6 types. We return to the specification of the R1/6 type below.

Raising N activity in the R3/4 precursors specifies both as R7s in the presence of Lz

To test whether it was indeed the high levels of N activity in R4 precursors that specified them as R7s, we concomitantly expressed activated N (N*) in both the R3 and R4 cells using a *sev.N** construct (Fortini et al., 1993; Tomlinson and Struhl, 2001) (*sev.N*; sev.lz*). *sev.N** has a number of effects on second wave cells, two of which are pertinent here. First, it directs the R1/6 cells to the R7 fate, thereby generating three R7 cells in the R1/6/7 positions (Fig. 2G-I) (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). Second, it can cause one or more of these three cells to be lost shortly thereafter, resulting in adult ommatidia with one, two or three R7 cells in this position (Tomlinson and Struhl, 2001). It should be noted that supplying N* to the R3/4 precursor using *sev.N** has no effect on their general cell fate specification (Fig. 2H). When we examined *sev.N*; sev.lz* adult eye sections the ommatidia were randomly rotated, preventing the unambiguous identification of the R1/6/7 and R3/4 sides of the ommatidia. Although there was variability in structure, ommatidia were frequently observed containing two large rhabdomere cells and four small R7-like cells

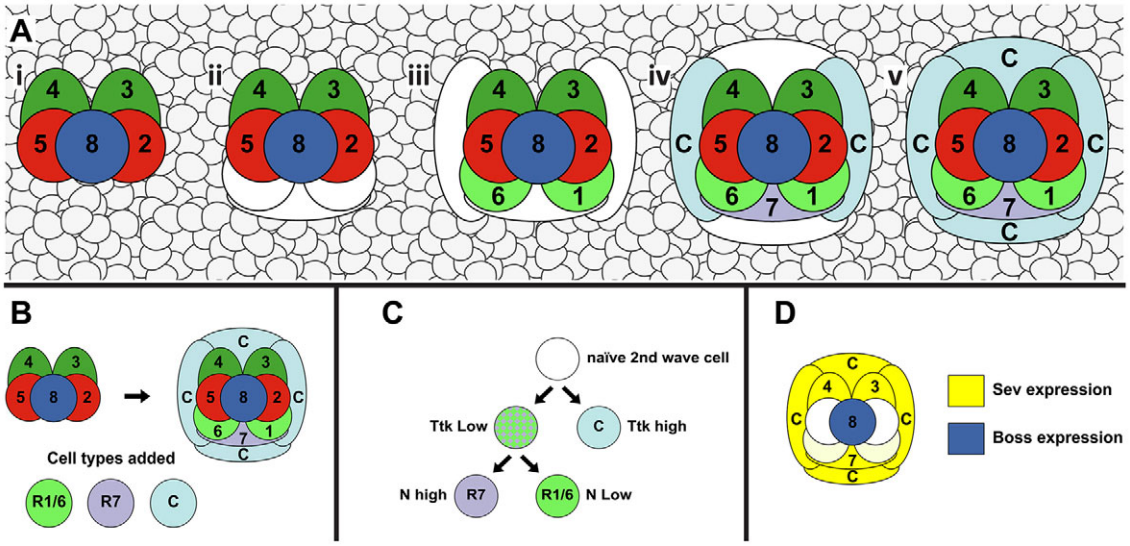

Fig. 1. Summary of the sequence of cell incorporation, cell fate specification and marker expression in the developing *Drosophila* ommatidium. (**A**) The incorporation and differentiation of the first seven cells to be added to the precluster. (i) The precluster (R2,3,4,5,8), is surrounded by a 'sea' of undifferentiated second wave cells (gray ovals). (ii) Three cells from the pool join the precluster on the R2/5/8 face. (iii) Two cells begin to differentiate as R1/6 photoreceptors while the R7 precursor between them delays differentiation and two cone cell precursors (C) join the cluster at the flanks. (iv) The R7 precursor begins to differentiate and two additional cone cell precursors join the cluster. (v) The differentiation of the cone cells ends this phase of ommatidial development with all seven of the newly added cells differentiating as specific cell types. (**B**) The three different cell types (R1/6, R7 and cone cells) that are added to the precluster. (**C**) The cell fate code for the R1/6, R7 and cone cells. If a cell degrades Ttk and has high N activity it becomes an R7, but if it has low N activity it becomes an R1/6 type. If the cell fails to degrade Ttk it becomes a cone cell. (**D**) The expression patterns of Sev and its ligand Boss.

(Fig. 2J, red arrowheads). Thus, in *sev.N*; sev.lz* eyes, in addition to the R1/6/7 cells, the R3/4 cells now appeared as R7s. This was corroborated by examination of larval eye discs in which we observed cells in the R3/4 positions now expressing R7 markers (Fig. 2K). These results suggest that, if an R3/4 precursor cell expresses Lz and has high N activity, then it is specified as an R7 cell.

In each of these experiments we identified the R7s in adult sections by their small rhabdomeres and in the eye discs by their expression of Runt. R8s share both these features, and to ensure that the cells in the eye disc and adult retina were indeed R7s we performed two controls. R8 precursors in the eye disc are uniquely identified by expressing both Runt and Sens, whereas R7 precursors express only Runt. In all experiments in which ectopic Runt-expressing cells were detected, we counterstained to ensure that they were not also expressing Sens (an example is shown in Fig. 2E′). To determine whether the supernumerary small rhabdomere cells detected in adult sections were indeed of the R7 type, we monitored the transcriptional activity of the opsin genes that are typically expressed in these cells using the combined *Rh3.lacZ* and *Rh4.lacZ* transgenes (Hofmeyer et al., 2006). In wild-type adult ommatidia all R7s were positively stained (Fig. 3A, red arrowheads), as were the supernumerary small rhabdomere cells in *sev.lz* (Fig. 3B) and *sev.lz; sev.N** (Fig. 3C) eyes. Thus, by examining molecular markers in the eye disc and by morphology and opsin expression in the adult, we corroborated that R3/4 precursors could be transformed into bona fide R7 photoreceptors by the ectopic expression of Lz.

The role of Sev in specifying precluster R7s

Above, we have discussed the role played by N in specifying the R7 versus R1/6 photoreceptor types once Ttk is degraded (Fig. 1C), but prior to this N plays two roles in determining whether Ttk is degraded or not. These two roles are, surprisingly, antagonistic. One function is to antagonize RTK signaling and thereby prevent DER activity from degrading Ttk. The other is to activate *sev* transcription and supply a high level of this RTK to the R7 precursor, following which, the combined actions of Sev and DER suffice to overcome the N-induced block to the pathway, such that Ttk is degraded and the cell is specified as a photoreceptor. Indeed, in the sev^{0} mutant, the R7 precursor becomes a cone cell because, in the absence of Sev, there is insufficient RTK activity to overcome the inhibition imposed by N, and this therefore defines a key feature of second wave cells: if they experience high N activation and are unable to activate Sev, then Ttk will persist and they will become cone cells. We therefore tested whether R7s specified in the precluster by ectopic Lz expression were Sev dependent and whether they became cone cells in its absence. Key to this experiment is the fact that the R3/4 precursors express high levels of Sev and directly contact the Boss-expressing R8 cell, so the Sev ligand is therefore directly available to them just as it is to the native R7 (Fig. 1D).

When Sev was removed from *sev.lz* eyes (sev^{0}*; sev.lz*) we inferred the constitutive loss of endogenous R7 and that any remaining R7s would arise from the R3/4 precursors. Examination of adult sections showed a frequent absence of R7-like cells from the ommatidia, and that the predominant phenotype was ommatidia containing five large rhabdomere cells (Fig. 4A, inset). However, in a minority of ommatidia, R7-like cells were clearly present. To investigate this further we examined sev^{0}*; sev.lz* eye discs. As expected, the endogenous R7 cell was specified as a cone cell, labeled with Cut, as were most of the cells in the R4 position (Fig. 3B). But, correlating with the adult investigation, in some ommatidia, cells in the R3/4 positions expressed R7-specific markers. Interestingly, some of these cells also expressed the cone cell marker Cut (not

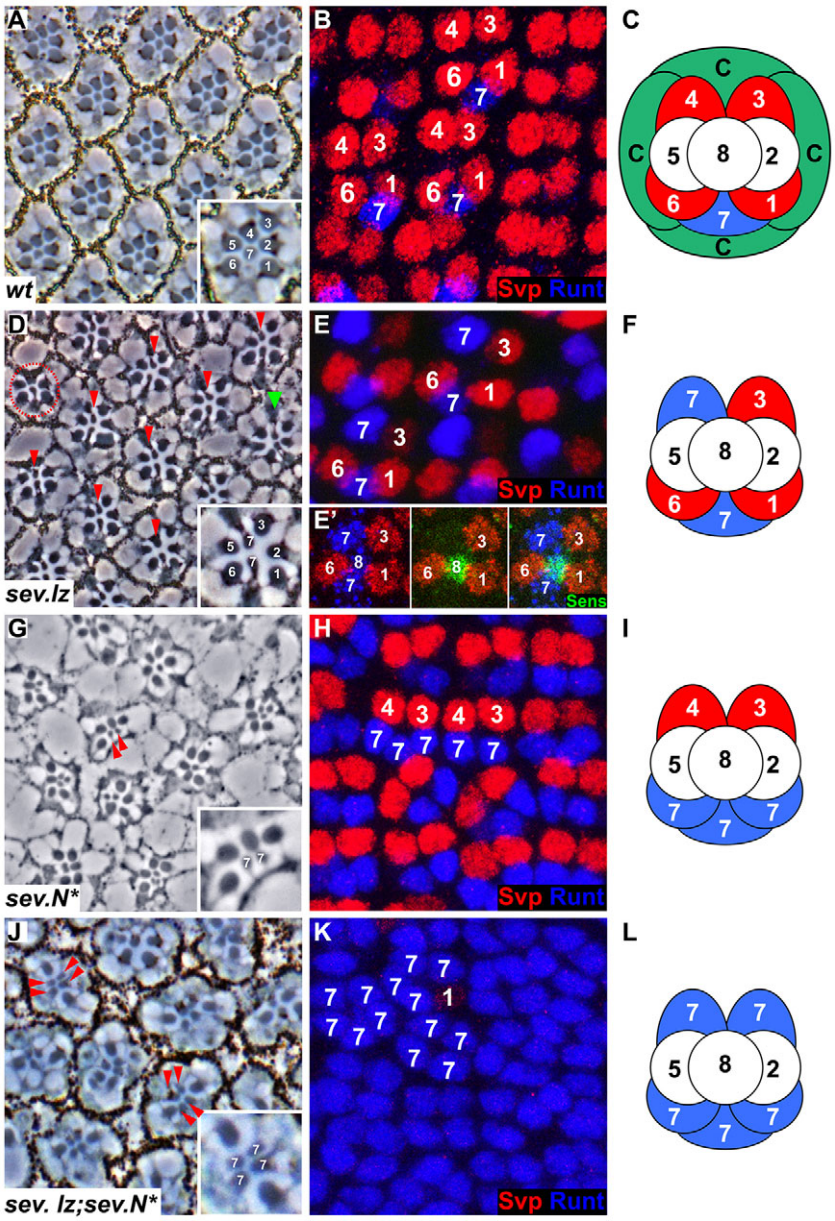

Fig. 2. The effects of expressing Lz in R3/4 precursors. (**A**) Section through an adult wild-type eye. Inset illustrates the cell arrangement in a single ommatidium. (**B**) A wild-type third instar eye disc showing expression of Svp (red) in the R3/4 and R1/6 precursors and Runt (blue) in R7. (**C**) Schematic representation of cluster cell identities. (**D**) Section through an adult *sev.lz* eye. Cells in the R4 position frequently transform into R7-like cells (red arrowheads), less frequently both R3/4 cells appear as R7s (red circle), and occasionally the cell in the R3 position appears as an R7 (green arrowhead). Inset shows a typical *sev.lz* ommatidium with the cell in the R4 position appearing as an R7. (**E**) A *sev.lz* third instar disc labeled for Svp and Runt. The cells in the R4 position frequently display the R7 marker Runt. (**E′**) A single ommatidium stained for Sens (green), Runt and Svp. Runt is expressed in both R7 and R8, whereas Sens is restricted to R8. The Runt-expressing cell in the R4 position does not express Sens. (**F**) Summary of the most frequent *sev.lz* fates. (**G**) Section through an adult *sev.N** eye. Typically, two or three R7-like cells (arrowheads) are observed in the R1/6/7 positions caused by the transformation of R1/6 precursors to R7s accompanied by the loss, or not, of one or more of the R7s. (**H**) A *sev.N** third instar eye disc showing the cells in the R1/6/7 positions differentiating as R7s, evidenced by the expression of Runt and the absence of Svp. (**I**) Summary of sev.*N** ommatidia indicating the transformation of R1/6 precursors to the R7 fate. (**J**) Section through an adult *sev.lz; sev.N** eye. Ommatidia typically contain two large rhabdomere cells and four R7-like cells (arrowheads). (**K**) A *sev.lz; sev.N** third instar eye disc showing the potent transformation of R3/4 and R1/6 into R7 cells. The cell marked 1 is an infrequent R1/6 cell that failed to transform. (**L**) Schematic representation of a typical *sev.lz; sev.N** ommatidium.

shown) suggesting that they were in an ambiguous cell state – a condition not observed in R4 precursors of *sev.lz* flies. From these results we infer that there is a partial dependence on Sev for the specification of the *sev.lz* R3/4 precursors as R7s.

We next examined the Sev dependence of R3/4-to-R7 transformations when N* was additionally supplied (*sev*^{0}*; sev.N*;* *sev.lz*), and found that Sev was now critically required. Here, adult ommatidia frequently showed only two large rhabdomere cells (inferred as R2/5), and a small rhabdomere cell that appeared morphologically to be R8, suggesting that none of the R1/6/7 or R3/4 precursors differentiated as photoreceptors (Fig. 4D). To validate this interpretation and to determine the fate of these cells

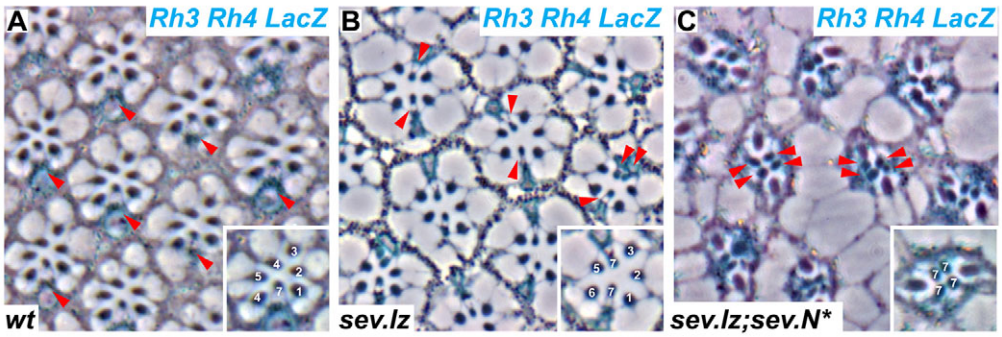

Fig. 3. *Rh3;Rh4-lacZ* reporter activity in wild-type and experimental adult eyes sections. (**A**) A wild-type eye showing *lacZ* reporter staining of R7s (blue, arrowheads). (**B**) In *sev.lz* eyes the extra R7-like cells are all labeled (arrowheads). (**C**) In *sev.lz; sev.N** eyes the four R7-like cells are all labeled (arrowheads).

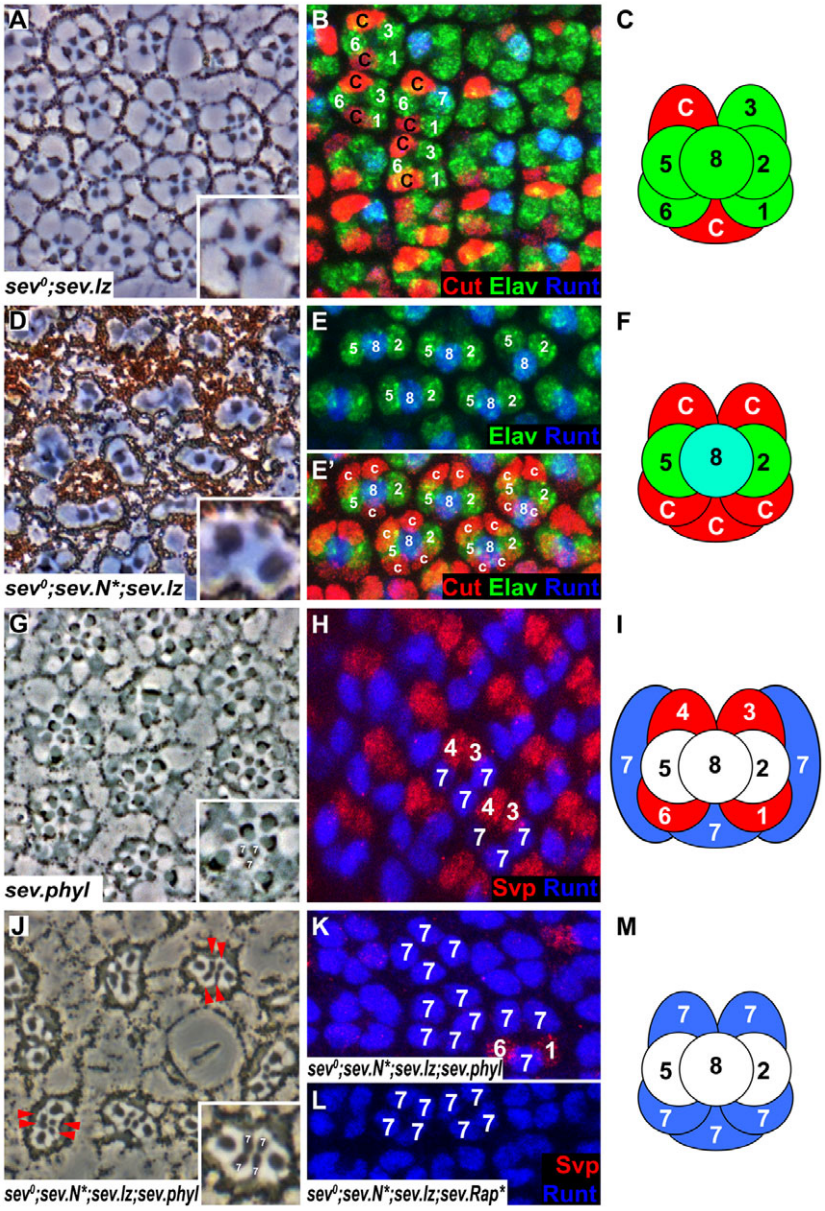

Fig. 4. The role of Sev in *sev.lz* R3/4 precursors. (**A**) Section through a *sev^{0}; sev.lz* eye. Ommatidia containing five outer photoreceptors (inset) are often seen. In ommatidia in which there are two small rhabdomeres, we infer the presence of supernumerary R7s. (**B**) A *sev^{0}; sev.lz* third instar larval disc stained for Elav, Runt and Cut. The cells in the R4 position most frequently express Cut. Occasionally they express Runt. (**C**) Schematic representation of the most common *sev^{0}; sev.lz* ommatidium. (**D**) Section through a *sev^{0}; sev.lz; sev.N** eye. Ommatidia containing only two large rhabdomere cells are frequently observed with an R8-like small rhabdomere cell. (**E**,**E′**) In *sev^{0}; sev.lz; sev.N** third instar eye discs the R8 (Runt/Elav expression) and R2/5 (Elav expression) precursors differentiate normally, whereas the R3/4, R1/6 and R7 precursors differentiate as cone cells (Cut expression). (**F**) The fates of a *sev^{0}; sev.lz; sev.N** ommatidium. (**G**) Section through a *sev.phyl* eye showing the multiple R7 phenotype. (**H**) An apical image at the cone cell level of a *sev.phyl* third instar eye disc showing cells in the anterior and posterior cone cell positions differentiating as R7s along with the native R7. (**I**) Schematic representation of a typical *sev.phyl* ommatidium. (**J**) Section through a *sev^{0}; sev-lz; sev.N*; sev.phyl* eye, showing four R7-like cells (arrowheads) and two large rhabdomere cells. (**K**) A *sev^{0}; sev.lz; sev.N*; sev.phyl* third instar eye disc, showing R3/4 and R1/6 cells frequently specified as R7s. (**L**) A *sev^{0}; sev.lz; sev.N*; sev.Rap** third instar eye disc showing the same phenotype as *sev^{0}; sev.lz; sev.N*; sev.phyl*. (**M**) Schematic representation of a *sev^{0}; sev.lz; sev.N*; sev.phyl* or *sev^{0}; sev.lz; sev.N*; sev.Rap** ommatidium.

we stained *sev^{0}; sev.N*; sev.lz* third instar discs for various markers, and observed the complete absence of photoreceptor markers from the R1/6/7 and R3/4 precursors. Instead, these cells stained positively for the cone cell marker Cut (Fig. 4E,E′) and for Ttk88 (not shown). Therefore, in the presence of Lz and high N levels, the R3/4 precursor cells critically require Sev; its presence specifies them as R7s and its absence directs them to the non-photoreceptor cone cell fate. This is the exact behavior of the native R7 precursor cell (Tomlinson and Ready, 1986) and suggests that the entire R7 specification mechanism can be recapitulated in the R3/4 cells.

High-level RTK activation can substitute for Sev in R3/4-to-R7 specification

Above we ascertained that when R3/4 precursors express Lz and have high N activity they critically require Sev for their specification as R7s; in its absence the cells fail to degrade Ttk and instead differentiate as cone cells. We inferred that Sev was providing the high RTK activity required to degrade Ttk and specify the photoreceptor fate. If this were correct, then supplying an independent high-level activator of the RTK pathway should rescue the absence of Sev. We used *sev.rap^{V12}*, which we previously showed to be a potent activator of the RTK pathway (Mavromatakis and Tomlinson, 2012), and examined *sev^{0}; sev.N*; sev.lz; sev.rap^{V12}* animals. The adult eyes of this genotype were too disorganized for any meaningful evaluation, but in third instar discs the R3/4 precursors now reverted back to the R7 fate (Fig. 4L). Thus, high-level RTK pathway activation is able to substitute for the absence of Sev. This suggests that the role of Sev in Lz-expressing R3/4 precursors counteracts the photoreceptor inhibiting activity of high level N activity by providing a potent activation of the RTK pathway.

Transcriptional activation of *phyl* can substitute for Sev in R3/4-to-R7 specification

In the native R7 precursor, the high-level RTK activity provided by Sev overcomes the N-imposed block on photoreceptor fate by activating the transcription of *phyl*. Phyl then acts to trigger Ttk degradation by linking it to the Sina E3 ubiquitin ligase (Li et al.,

1997; Tang et al., 1997). If the role of Sev activity in the R3/4 transformed cells was indeed to transcriptionally activate *phyl*, then supplying *phyl* transcription independently should replace the need for Sev. To this end we engineered and transformed a *sev.phyl* transgene and checked its efficacy by observing the multiple R7 phenotype in adult ommatidia (Fig. 4G) caused by the transformation of cone cell precursors into R7 types (Fig. 4H). When we crossed this into the experimental background (*sev^{0}; sev.N*; sev.lz; sev.phyl*), adult sections showed the restitution of R7-like cells (Fig. 4J, red arrowheads). In the eye discs, the cells in the R3/4 positions no longer expressed the cone cell marker Cut, but showed the characteristic R7 molecular features. Thus, expression of *phyl* compensates for the absence of Sev, and suggests that Sev functions to degrade Ttk by activating *phyl* transcription.

Specification of the R1/6 type in the Lz-expressing R3/4 cells

By manipulation of the RTK and N pathways in Lz-expressing R3/4 precursors we had generated the R7 and cone cell fates; the R1/6 fate, however, proved more elusive. Consider *sev.lz* eyes in which the R4s are transformed to R7s; we naively expected the R3 precursors to be specified as R1/6 types. This was not the case, as they expressed the R3/4/1/6 marker Svp but did not express the R1/6-specific marker Bar. However, R3/4 precursors have a much higher baseline level of N activity compared with their R1/6 counterparts, as evidenced, for example, by their Sev expression: high in R3/4, low in R1/6 (Fig. 1D). Furthermore, since *sev.lz* R3 precursors become R7s, albeit at low frequency, we infer that they normally experience high N activity, close to that required for R7 specification. We reasoned, therefore, that we should reduce N activity to allow the emergence of the R1/6 fate.

To reduce N activity we routinely use a *sev.Su(H)EnR* transgene that encodes a downregulator of the N pathway expressed under *sev* transcriptional control (Tomlinson and Struhl, 2001). The problem with this transgene is that it downregulates all *sev*-dependent transgenes (including itself – see the Discussion for an exposition of these effects) including *sev.lz*, the key construct in our experiments. We therefore defined a fragment of the *rough* (*ro*) gene that would drive expression in R2,3,4,5 precursors (Fig. 5A), and generated a *ro.Gal4* line. When Lz was expressed in this manner (*ro.Gal4; UAS.lz*) no adult animals emerged, preventing an analysis of the mature retina, but in third instar discs many R3/4 precursors showed the R7 transformation phenotype (Fig. 5C). Interestingly, the R2/5 cells appeared unaffected by their expression of Lz (as evidenced by normal Ro protein expression; data not shown). When N signaling was downregulated in the R3/4 cells [*ro.Gal4; UAS.lz; sev.Su(H)EnR*], many R3/4 precursors no longer appeared as R7s, but rather expressed both Svp and Bar – a molecular signature of the R1/6 types (Fig. 5E). Although in wild type both R3/4 and R1/6 precursors express Svp, they differ in that R3/4 persistently express it at high levels whereas in R1/6 its expression decays rapidly and frequently

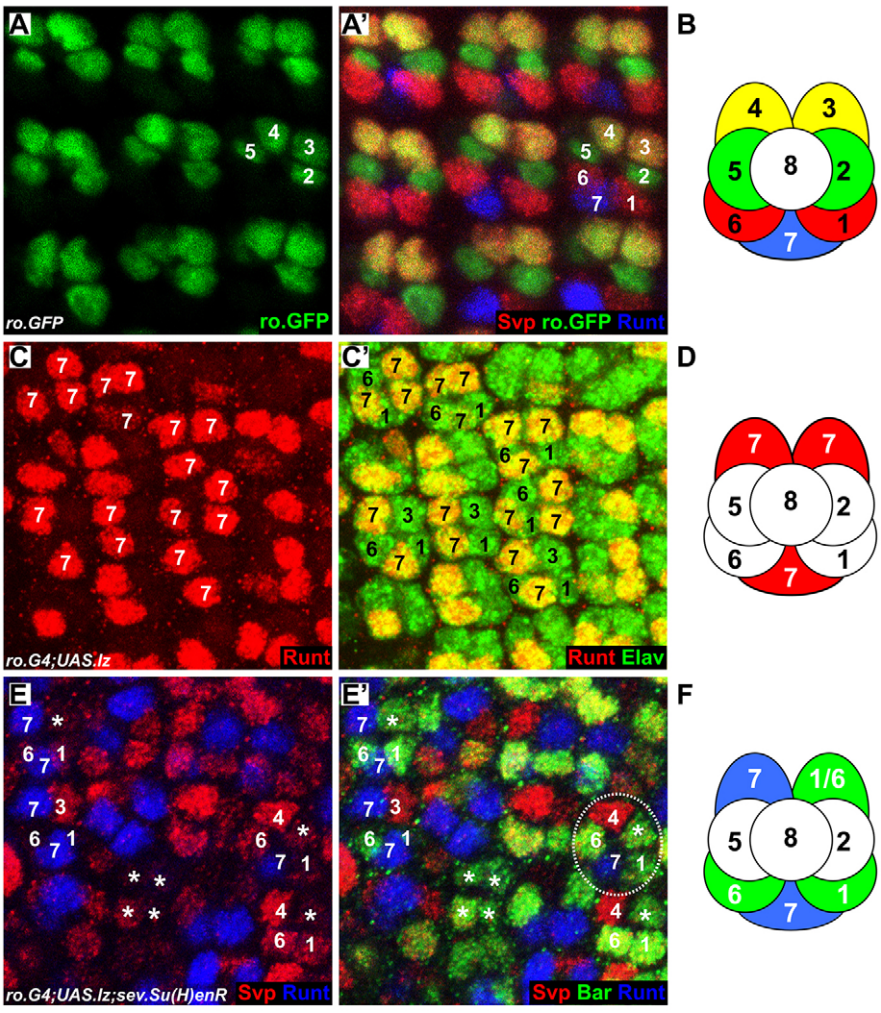

Fig. 5. The effects of lowering N activity in *sev.lz* R3/4 precursors. (**A**) The GFP staining of a *ro.GFP* third instar eye disc is restricted to the R2/3/4/5 precursors. (**A′**) Additional staining of Svp and Runt highlights the single R7 and paired R3/4, R2/5 and R1/6 precursors. (**B**) Schematic description of the staining shown in A′. (**C**,**C′**) A *ro.G4; UAS.lz* third instar eye disc showing many cells in the R3/4 positions expressing Runt and Elav, indicating their differentiation as R7s. (**D**) The frequently observed cell fates in *ro.G4; UAS.lz* ommatidia. (**E**,**E′**) When N activity is downregulated in R3/4 cells in *ro.G4; UAS.lz; sev.Su(H)enR* eye discs there is a frequent transformation of R3 precursors into Bar-expressing cells. The circle highlights an ommatidium in which the cell in the R3 position expresses Bar and reduced levels of Svp, with its neighboring R4 differentiating normally and expressing high levels of Svp (and no Bar). (**F**) The cell fates most frequently observed in *ro.G4; UAS.lz; sev.Su(H)enR* ommatidia.

appears at much lower levels than in R3/4. Consistent with these characteristics, in this experimental background we observed Bar-expressing cells in the R3/4 positions that showed the lower ephemeral Svp level (compare the Svp expression levels in the Bar-expressing R3/4 cell with the non-Bar-expressing R3/4 cell in the circled ommatidium in Fig. 5E′).

For technical reasons we were unable to evaluate Ro or Spalt expression to determine whether these cells concomitantly downregulated R3/4 markers as they expressed the R1/6 signatures. Accordingly, we focus on the emergence of Bar expression in these cells as the evidence that they transform into R1/6 types, and infer that, by the appropriate modulation of the RTK and N signaling pathways, the R7, R1/6 and cone cell fates could each be reproduced in Lz-expressing R3/4 cells.

Rescue of *lz* mutant second wave cells by *sev.lz*

The *sev* enhancer element drives expression in a number of cells of the developing ommatidia, including R3/4, R7 and the cone cells (Fig. 1E). In each of these cells the expression is transitory, occurring for only a few hours. When the R3/4 precursors are transformed into the various second wave fates by *sev.lz*, these transformations are thus induced by the transient expression of Lz. This suggests that Lz is only required at the time of cell fate specification and not for the maintenance of cell fate thereafter. This is surprising because Lz shows persistent expression in second wave cells, suggesting a prolonged requirement. To investigate this further we rescued *lz* gene function in second wave cells only at the time of their fate specification. This was achieved by examining the rescuing effects of *sev.lz* on lz^{77a7} second wave cells.

lz^{77a7} is a mutation caused by the deletion of an eye-specific enhancer element (Flores et al., 1998; Siddall et al., 2003) that selectively removes Lz expression from the developing eye, but leaves it in the antennal disc and throughout the remainder of the animal, resulting in healthy and fertile animals distinguished only by severely disrupted eyes (Flores et al., 1998; Siddall et al., 2003). Although no Lz protein can be detected in lz^{77a7} eye discs (Flores et al., 1998) we cannot assume that there is none, and provisionally regarded it as a strong hypomorph.

We engineered and transformed a *sev.DsRed* construct to monitor *sev* transcription, and confirmed that it showed the correct expression pattern in a wild-type background: high levels in R3/4/R7/cone cells and low levels in R1/6 (Fig. 6A). In lz^{77a7} eye discs, *sev.DsRed* expression in the cells in the R1/6/7 positions appeared normal: high in the R7 precursors and low in the R1/6 cells (Fig. 5B). Thus, the R1/6/7 precursor cells appear to be recruited to the precluster in a normal manner even in the absence of Lz. However, even though the cone cell precursors appear to express high levels of *sev*, as normal, they join the cluster in an aberrant manner and from this stage onward patterning defects were evident.

lz^{77a7}*; sev.lz* animals showed complete rescue of the second wave cells, as observed in sectioned adult eyes (Fig. 6C) and in third instar disc preparations (Fig. 6D). In fact, these eyes appeared completely wild type, with the exception of the effects engendered by the ectopic expression of Lz in the R3/4 precursors. Thus, by supplying *lz* gene function only at the time when their cell fate decisions are made, the *lz* mutant is fully rescued. This provides compelling evidence that Lz is not required for the later maintenance of the specified fates.

DISCUSSION

In this paper we have explored three inter-related themes bearing on the nature of the signals that specify the cell types in the *Drosophila* ommatidium. We examine the ability of a transcription factor to predispose the cellular responses to developmental signals, we validate the accuracy of the signaling code that represents these developmental signals, and we infer that both the intrinsic and extrinsic aspects are only required for a brief period of time. Below, we address each of these themes in sequence.

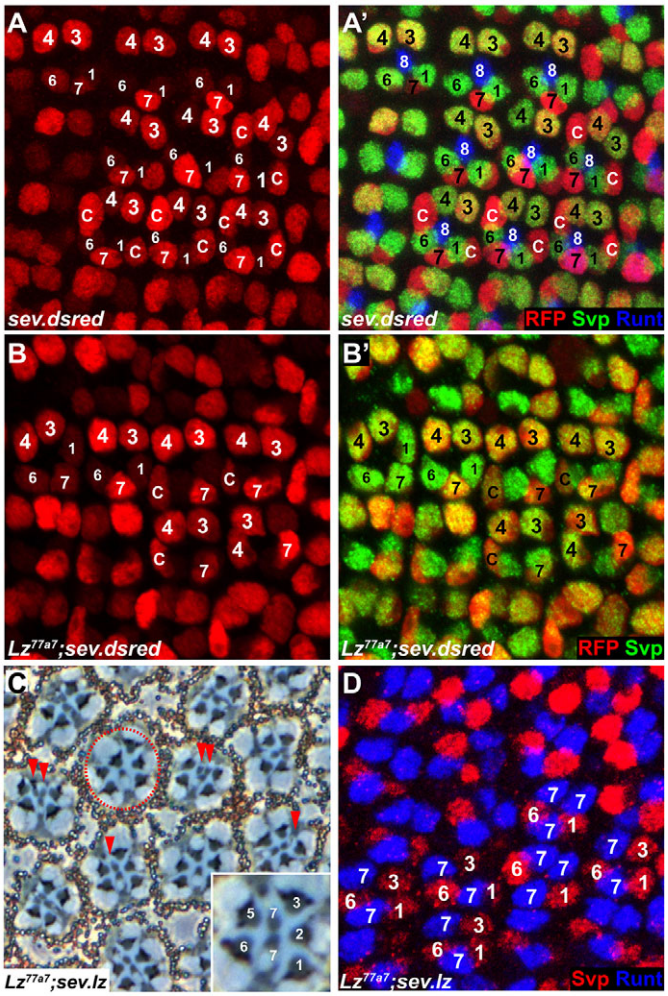

Fig. 6. *sev.lz* rescue of the lz^{77a7} mutant. (**A**) *sev.DsRed* eye discs showing the correct *sev* expression pattern: high in R3/4, R7 and cone cells but low in R1/6. (**A′**) The same image as A counterstained for Svp and Runt. (**B**) In lz^{77a7}eye discs *sev.DsRed* is initially expressed normally (high in R3/4 and R7, low in R1/6), but defects become evident as the strongly expressing cone cells join the cluster. (**B′**) The same image as B counterstained for Svp. (**C**) Tangential section through a lz^{77a7}*; sev.lz* eye, which is wild type except for the transformation of R3/4 precursors to R7 types (arrowheads). (**D**) A lz^{77a7}*; sev.lz* eye disc stained for Svp and Runt appears normal, except for the expression of Runt in R3/4 cells.

Lz and the predisposition of second wave cells

Lz had long been assumed to be a key factor that distinguishes how second wave cells differ from the precluster cells in their response to developmental signals. In this paper we rigorously tested this concept and reproduced features typical of the three second wave cell types in the R3/4 precluster cells by supplying ectopic Lz along with the appropriate RTK/N cell fate code. We thus infer that the presence of Lz in R3/4 precluster cells is sufficient to endow them with the second wave cell fate response repertoire. Below, we

discuss a number of issues related to these observations and their interpretations.

The N-Dl interaction in *sev.lz* R3/4 precursors

Normal R3/4 precursors undergo an N-Dl interaction that results in the R4 precursor experiencing much higher levels of N activity than the R3 precursor (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). When Lz was supplied to R3/4 precursors (*sev.lz*), the cell in the R4 position frequently transformed into an R7, consistent with the requirement of high N for R7 specification. Less frequently, both R3/4 precursors adopted the R7 fate, and sometimes it was the cell in the R3 position alone that generated an ectopic R7. These results suggest that in the *sev.lz* flies the R3/R4 N-Dl interaction does not occur correctly. When we used the *mδ0.5.lacZ* reporter line (Cooper and Bray, 2000) as a reporter of N activity (which in wild-type larvae is robustly upregulated in R4 precursors) an erratic pattern was observed, sometimes showing the wild-type pattern, sometimes showing both R3/4 cells with high levels of *lacZ* expression, and sometimes showing R3 alone with high levels (not shown). Hence, by expressing Lz in the R3/4 precursors we not only endowed them with second wave response abilities but also prevented them from executing their N-Dl interactions properly. Indeed, it was only when we artificially activated N to a high level (*sev.lz; sev.N**) that we potently induced the R7 fate in both R3/4 precursors.

R7 specification in the absence of *sev*

Native R7s critically require *sev* gene function; in its absence, they differentiate as cone cells. However, some ectopic R7s were able to differentiate when Lz was provided to the R3/4 precursors, even in the absence of *sev* (*sev^{0}; sev.lz*), suggesting that normal R7 specification was not fully reiterated here. Examination of these eye discs suggested that some R3/4 precursors differentiated as R7s whereas others became cone cells. Thus, these cells appear to be on the cusp of the R7/cone cell fate choice, and we even observed some cells expressing markers for both cell types. In the cells that became R7s, we infer the presence of sufficient RTK activity, which was likely to have been supplied by endogenous DER signaling active in the precluster cells. Only when N activity was raised in these cells (*sev^{0}; sev.lz; sev.N**) did their full *sev* dependence for the R7 fate emerge, when all R3/4 precursors differentiated as cone cells.

Specification of R1/6 types in R3/4 precursors

The *sev.N** construct is a very useful activator of the N pathway in developing eye cells. Since N activity drives *sev* expression, the *sev.N** transgene feeds back on itself and promotes its own expression, and by subsequent iterations of this effect the cells are left with potent N activity. This level is still within the physiological range, unlike that produced using Gal4/UAS techniques, and is therefore our choice method for activating the N pathway. The transgene that we routinely use to knock down N activity [*sev.Su(H)EnR*] has the opposite effect; it reduces its own expression, and mildly compromises N activity. This level of reduction in N activity is usually sufficient to trigger major effects without the disadvantage of the severe downregulation that can accompany the use of Gal4/UAS technology. Since the *sev.lz* construct would also be downregulated by *sev.Su(H)EnR*, we needed to ectopically express Lz in the precluster using another enhancer element, and to this end we generated the *ro.Gal4* line. When *UAS.lz* was expressed under *ro* control, the R3/4 precursors frequently differentiated as R7s, and crucially, when N activity was concomitantly reduced [*ro.Gal4; UAS.lz; sev.Su(H)EnR*] cells displaying R1/6 molecular features were now detected in the R3/4 precursors.

The insensitivity of R2/5 to ectopic Lz

The cells in the R2/5 positions in *ro.G4; UAS.lz* developing ommatidia appear to develop normally; they express Elav and Ro, but none of the other fate markers. This suggests that R2/5 cells are insensitive to the presence of Lz, and argues that there is a major molecular difference between these cells and the R3/4 precursors. Also noteworthy is the transformation of all *lz* mutant second wave cells into R3/4 types characterized by the expression of Svp (a marker that is not expressed in R2/5 precursors) and Elav. Thus, it appears that ectopic Lz selectively transforms R3/4 precursors of the precluster to the second wave fate, and second wave cells lacking Lz adopt the R3/4 fate. Hence, we suspect that Lz might not provide the intrinsic information that distinguishes the second wave cells from precluster cells per se, but rather distinguishes second wave cells from R3/4 types. We are currently undertaking experiments to evaluate this view.

A counter-argument emerges from the fate of the majority of cells in the R3 positions in *sev.lz* eyes, which do not switch their fate. Only when we activate or reduce N activity in these cells do we change their fates, and to be sure that the R2/5 cells are insensitive to Lz expression we would also need to correspondingly vary N activity in them. Our experiments to do this using *ro.Gal4* produced severely disrupted preclusters, presumably as a result of interference with N function at earlier stages of precluster formation. Since these clusters were largely uninterpretable, the issue of whether R2/5 cells are insensitive to Lz expression remains unresolved.

Validation of the cell specification code of second wave cells

For many years, the role of N in photoreceptor specification was confusing. In some contexts N appeared to oppose photoreceptor specification and in others N seemed to promote it, and this confusion prevented substantial progress in defining the fate codes that specified the different cell types. In recent work we identified three distinct roles for N in this process, and with that information inferred the cell fate codes for R1/6, R7 and the cone cells (Fig. 1C). A major goal of this current work has to been to test this code by its reiteration in the R3/4 cells of the precluster using Lz expression to endow them with second wave cell qualities. In these experiments, each of the cell codes induced the expected cell fates, providing cogent support for the validity of the code.

A short time window in which Lz and the developmental signals operate

DER is assumed to be ubiquitously expressed in the eye disc tissue, and its ligand, Spitz, diffusing from precluster cells, is thought to reach more distant cells with time. But the N and Sev signals are regulated in a different manner. Both their ligands are membrane bound, and their receptor activations only occur in immediate neighbors. Dl, the ligand for N, is expressed transiently by differentiating cells, and, accordingly, activates N in neighboring cells for only short periods of time (a few hours). *sev* is an N response gene and, in consequence, Sev is only expressed in cells for a short period of time. By contrast, Boss, the Sev ligand, is expressed for a prolonged period by the R8 precursor. Thus, both the N and Sev signaling systems are only available to the cells for restricted periods, with this restriction controlled by ligand expression in the N system and by receptor expression in the Sev system.

Although Lz is expressed in the second wave cells in a persistent manner in the eye disc, our experiments suggest that it, like the extrinsic signals, is required only for a brief developmental window. Consider the transformation of *sev.lz* R3/4 cells to the R7 fate. The *sev* enhancer is only active in these cells for a few hours and yet a complete transformation of the cells is achieved. The expression of specific cell type markers in the eye disc might erroneously indicate the transformation of a cell when only a transient effect occurs, but the presence of ectopic R7s in the adult retina argues otherwise and suggests that the transformations are potent and permanent. This view is further validated by the rescue of *lz* mutant second wave cells by the *sev.lz* transgene. This rescue is complete and is evident by the molecular markers expressed in the disc and by the morphology of the adult cells. Thus, we infer that Lz is only required during the same time window when the RTK/N signals are transduced, and we further infer that the combined activities of the RTK and N pathways, in concert with Lz, function in a short-lived manner to lock in the fate of the cells. How the presence of ephemeral extrinsic and intrinsic information is molecularly 'remembered' by the cells to allow their appropriate differentiation over a prolonged developmental period remains an intriguing question.

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The authors declare no competing financial interests.

Author contributions
Y.E.M. and A.T. designed the research, performed experiments, analyzed data and wrote the paper.

References

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.
Basler, K., Christen, B. and Hafen, E. (1991). Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing Drosophila eye. *Cell* **64**, 1069-1081.
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.
Bischof, J., Maeda, R. K., Hediger, M., Karch, F. and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. USA* **104**, 3312-3317.
Cooper, M. T. and Bray, S. J. (1999). Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. *Nature* **397**, 526-530.
Cooper, M. T. and Bray, S. J. (2000). R7 photoreceptor specification requires Notch activity. *Curr. Biol.* **10**, 1507-1510.
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.
Fanto, M. and Mlodzik, M. (1999). Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. *Nature* **397**, 523-526.
Flores, G. V., Daga, A., Kalhor, H. R. and Banerjee, U. (1998). Lozenge is expressed in pluripotent precursor cells and patterns multiple cell types in the Drosophila eye through the control of cell-specific transcription factors. *Development* **125**, 3681-3687.
Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. *Nature* **365**, 555-557.
Freeman, M. (1994). The spitz gene is required for photoreceptor determination in the Drosophila eye where it interacts with the EGF receptor. *Mech. Dev.* **48**, 25-33.
Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651-660.
Hafen, E., Basler, K., Edstroem, J. E. and Rubin, G. M. (1987). Sevenless, a cell-specific homeotic gene of Drosophila, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236**, 55-63.
Hofmeyer, K., Maurel-Zaffran, C., Sink, H. and Treisman, J. E. (2006). Liprin-alpha has LAR-independent functions in R7 photoreceptor axon targeting. *Proc. Natl. Acad. Sci. USA* **103**, 11595-11600.
Krämer, H., Cagan, R. L. and Zipursky, S. L. (1991). Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. *Nature* **352**, 207-212.
Kumar, J. P. (2010). Retinal determination the beginning of eye development. *Curr. Top. Dev. Biol.* **93**, 1-28.
Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B. Z. and Moses, K. (1998). Dissecting the roles of the Drosophila EGF receptor in eye development and MAP kinase activation. *Development* **125**, 3875-3885.
Li, S., Li, Y., Carthew, R. W. and Lai, Z. C. (1997). Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. *Cell* **90**, 469-478.
Li, S., Xu, C. and Carthew, R. W. (2002). Phyllopod acts as an adaptor protein to link the sina ubiquitin ligase to the substrate protein tramtrack. *Mol. Cell. Biol.* **22**, 6854-6865.
Mavromatakis, Y. E. and Tomlinson, A. (2012). The role of the small GTPase Rap in Drosophila R7 photoreceptor specification. *Proc. Natl. Acad. Sci. USA* **109**, 3844-3849.
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.
Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the Drosophila retina, a neurocrystalline lattice. *Dev. Biol.* **53**, 217-240.
Reinke, R. and Zipursky, S. L. (1988). Cell-cell interaction in the Drosophila retina: the bride of sevenless gene is required in photoreceptor cell R8 for R7 cell development. *Cell* **55**, 321-330.
Siddall, N. A., Behan, K. J., Crew, J. R., Cheung, T. L., Fair, J. A., Batterham, P. and Pollock, J. A. (2003). Mutations in lozenge and D-Pax2 invoke ectopic patterned cell death in the developing Drosophila eye using distinct mechanisms. *Dev. Genes Evol.* **213**, 107-119.
Tang, A. H., Neufeld, T. P., Kwan, E. and Rubin, G. M. (1997). PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism. *Cell* **90**, 459-467.
Tomlinson, A. and Ready, D. F. (1986). Sevenless: a cell-specific homeotic mutation of the Drosophila eye. *Science* **231**, 400-402.
Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in Drosophila ommatidium. *Dev. Biol.* **120**, 366-376.
Tomlinson, A. and Struhl, G. (1999). Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in establishing planar polarity in the Drosophila eye. *Development* **126**, 5725-5738.
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.
Tomlinson, A., Mavromatakis, Y. E. and Struhl, G. (2011). Three distinct roles for notch in Drosophila R7 photoreceptor specification. *PLoS Biol.* **9**, e1001132.