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Interdependent regulation of stereotyped and stochastic photoreceptor fates in the fly eye

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Abstract

Diversification of neuronal subtypes often requires stochastic gene regulatory mechanisms. How stochastically expressed transcription factors interact with other regulators in gene networks to specify cell fates is poorly understood. The random mosaic of color-detecting R7 photoreceptor subtypes in *Drosophila* is controlled by the stochastic on/off expression of the transcription factor Spineless (Ss). In Ss^{ON} R7s, Ss induces expression of Rhodopsin 4 (Rh4), whereas in Ss^{OFF} R7s, the absence of Ss allows expression of Rhodopsin 3 (Rh3). Here, we find that the transcription factor Runt, which is initially expressed in all R7s, is sufficient to promote stochastic Ss expression. Later, as R7s develop, Ss negatively feeds back onto Runt to prevent repression of Rh4 and ensure proper fate specification. Together, stereotyped and stochastic regulatory inputs are integrated into feedforward and feedback mechanisms to control cell fate.

Keywords

Spineless; Runt; Feedforward loop; Feedback; Photoreceptor; Stochastic

1. Introduction

Nervous systems are extremely complex, with some organisms having thousands of different neuronal subtypes. Sensory systems often diversify cell fates through stochastic cell fate decisions (Johnston and Desplan, 2010; Urban and Johnston, 2018). In the human retina, for example, the final choice between the red and green cone fates appears to occur by chance: a noncoding regulatory DNA element called a Locus Control Region (LCR) is hypothesized to randomly loop to the promoter of either the red or green opsin gene, activating its expression and preventing expression of the other opsin (Smallwood et al., 2002). Similar LCR elements are thought to control the stochastic selection of one of 1300 odorant receptors for

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Appendix A. Supplementary data

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expression in olfactory neurons in mice (Marken-scoff-Papadimitriou et al., 2014; Monahan and Lomvardas, 2015; Serizawa et al., 2003). Stochastic cell fate specification is even observed in the olfactory system of the nematode *C. elegans,* whose development is highly stereotyped: Ca^{2+}-mediated lateral inhibition randomly specifies fates of the two AWC olfactory neurons (Alqadah et al., 2016; Chuang et al., 2007; Troemel et al., 1999). Thus, stochastic mechanisms are widely utilized to diversify neuronal subtypes. We are interested in understanding how stochastic mechanisms are incorporated into gene regulatory networks to control cell fate.

The R7 photoreceptor (PR) subtypes of the fly eye comprise a random mosaic (Fig. 1A)(Bell et al., 2007). This random distribution is controlled by the stochastic expression of the bHLH transcription factor Spineless (Ss). Ss is expressed in ~65% of R7s and induces yellow R7 (yR7) fate, including activation of Rhodopsin 4 (Rh4) and repression of Rhodopsin 3 (Rh3)(Fig. 1B). In the complementary ~35% of R7s where Ss is not expressed, R7s take on pale R7 (pR7) fate, marked by expression of Rh3 and absence of Rh4 (Fig. 1B) (Anderson et al., 2017; Johnston and Desplan, 2014; Wernet et al., 2006). In yR7s, Ss activates Rh4 directly and represses Rh3 by activating the transcriptional repressor Defective Proventriculus (Dve) (Fig. 1B). In pR7s, the absence of Ss and Dve allows expression of Rh3 and prevents expression of Rh4 (Fig. 1B). The Spalt transcription factors (Sal) are expressed in all R7s and activate stereotyped expression of the general R7 fate gene Prospero (Pros) in all R7s and stochastic expression of Ss (Fig. 1B). Sal also feeds forward to repress Dve and activate Rh3 (Fig. 1B) (Johnston, 2013; Johnston et al., 2011; Thanawala et al., 2013; Yan et al., 2017). Here, we investigate how this stochastic regulatory mechanism is integrated into the gene regulatory network that specifies R7 fate.

The Runt (Run) transcription factor is expressed in R7 and R8 PRs and induces aspects of their fates, including the generation of small rhabdomeres and axonal targeting to the medulla (Kaminker et al., 2002). Ectopic expression of Run in the outer PRs (i.e. R1–R6) induces Rh3 reporter expression, but not Rh4 reporter expression (Edwards and Meinertzhagen, 2009), leading us to speculate that Run might participate with Ss in specifying R7 subtype fate.

Here, we describe the regulatory relationship between Ss and Run that specifies PR subtypes in the fly eye and identify a complex gene regulatory logic that controls stochastic cell fate specification. We find that Run is expressed early in all R7s, preceding stochastic Ss. We show that ectopic expression of Run endows additional PRs with the ability to express Ss independent of general R7 fate. Negative feedback from Ss is necessary and sufficient to restrict Run expression to pR7s. Perturbing this feedback by extending Run expression leads to misregulation of Rhodopsin expression, specifically the loss of Rh4 in yR7s. Our studies reveal that PR specification in the fly eye is achieved via a complex interplay between stereotyped and stochastic regulators.

2. Results

2.1. Ss and Run expression dynamics suggest regulatory interactions

We first examined the temporal dynamics of Ss and Run expression in 3rd instar larvae. The developing fly eye is comprised of dividing un-differentiated cells. A morphogenetic furrow moves from posterior to anterior, followed by specification of PRs. As a consequence, within a single larval eye, each row of developing PRs is ~1.5 h older than the row immediately anterior to it. We examined Run and Ss expression in larval eyes using immunohistochemistry and quantified on/off expression in individual R7s. Immediately following R7 recruitment, Run is expressed in all R7s, but Ss expression has not yet begun (Fig. 2A). Eleven rows (~16 h) later, Ss expression becomes detectable in ~65% of R7s, in addition to Run (Fig. 2A, C)(Johnston and Desplan, 2014). For more than 12 h, yR7s continue to co-express Ss and Run, while pR7s express Run only (Fig. 2C). At 12 h after puparium formation (APF), Run expression begins to turn off in Ss-expressing yR7s (Fig. 2C). By 48 h APF, Ss and Run expression are mutually exclusive, leaving two classes of R7 cells within the main part of the retina: yR7s that express Ss only and pR7s that express Run only (Fig. 2B–C). This successive pattern of Run and Ss expression in R7s (summarized in Fig. 2D) motivated us to test whether Run and Ss regulate one another during R7 subtype specification.

2.2. Run is sufficient to activate stochastic expression of Ss in outer PRs

We first tested whether *run* regulates stochastic Ss expression. To do so, we disrupted Run expression by creating homozygous mutant *run* clones. We found that *run* mutant clones had a normal proportion of R7s expressing Rh3 and Rh4 (Fig. 3A and B), suggesting that *run* is not required for Ss expression or the subsequent regulation of Rh3 and Rh4. However, as run^2 is a strong hypomorphic allele (Torres and Sanchez, 1992), it remains possible that completely removing *run* causes an effect. As an alternative approach, we created whole eye clones that were homozygous for a mutation in *Brother (Bro)*, which encodes a Run co-factor (Kaminker et al., 2001; Li and Gergen, 1999). *Bro* mutant R7s also displayed wild-type Rh3 and Rh4 expression (Fig. S1A–B). We conclude Run is likely not required to regulate stochastic Ss expression in R7s. However, these results do not rule out a role for Run in this process: *Bro* has been shown to be redundant with the related *Big brother* gene (Kaminker et al., 2001), and *run* itself is adjacent to two *run*-related genes, *RunxA* and *RunxB*, with which it may be redundant.

To further test whether *run* regulates Ss expression, we next turned to tests of sufficiency. We first wanted to determine whether variations in Run expression levels in developing R7s might bias their stochastic Ss^{ON}/Ss^{OFF} choice. To test this hypothesis, we overexpressed Run early and specifically in all R7s using the PM181-Gal4 driver (Maurel-Zaffran et al., 2001). We observed no effect on the ratio of Ss^{ON} to Ss^{OFF} R7s (Fig. 3C), indicating that Run is not sufficient to bias the outcome of the stochastic Ss^{ON}/Ss^{OFF} choice in R7s.

Finally, we tested whether Run is capable of endowing outer PRs with the ability to express Ss. We ectopically expressed Run early in all PRs and analyzed Ss expression. As a comparison, we ectopically expressed Sal, which activates both Ss and the R7 marker Pros

(Johnston et al., 2011), and we removed *seven-up* (*svp*), which normally prevents the stochastic specification of R7 and R8 fates in subsets of outer PRs (Miller et al., 2008; Mlodzik et al., 1990). We found that each of the three manipulations increased the number of Ss^{ON} cells per ommatidium, without increasing the total number of PRs (Fig. 3D–H, J). We conclude that, like ectopic Sal and loss of *svp*, ectopic Run is sufficient to induce expression of Ss. Consistent with the previously reported conversion of outer PRs to an R7-like fate, the number of $Pros^{ON}$ cells per ommatidium increased in all three conditions (Fig. 3D–G, I), and the number of Ss^{OFF} $Pros^{OFF}$ cells was reduced (Fig. S1C). We note that all three manipulations resulted in Ss^{ON} $Pros^{OFF}$ cells, indicating that Ss and Pros expression can be induced independently (Fig. 3D–G, K, S1D–F). This result is consistent with our previous findings that Ss expression is independent of Pros expression (Johnston et al., 2011) and that an enhancer in the *ss* gene locus drives expression independent of R7 fate (Johnston and Desplan, 2014).

We conclude that Run is neither required nor sufficient to modulate the frequency of stochastic Ss expression in R7s but is sufficient to promote general R7 fate and stochastic Ss expression independently in outer PRs.

2.3. Ss restricts Run expression to Ss^{OFF} cells later in development

We next turned to the question of whether Ss later regulates Run. Given the mutually exclusive expression of Ss and Run at 48 h APF, we speculated that Ss feeds back to repress Run expression. If so, Run should be derepressed in *ss* mutants. Indeed, in whole eye null mutant clones of *ss,* Run was expressed in all R7s (Fig. 4A and B). To test the cell autonomy of this effect, we examined *ss* null single-cell clones and observed nearly complete derepression of Run in *ss* mutant R7s (Fig. 4C, E). We saw similar effects on Rhodopsin expression in adult R7 photoceptors: individual *ss* null mutant R7s displayed loss of Rh4 and gain of Rh3 expression (Fig. 4F, H). Thus, Ss is required in yR7s to repress Run.

As Ss is necessary and sufficient for yR7 fate (Johnston et al., 2011; Wernet et al., 2006), we predicted that Ss would be sufficient to repress Run in R7s. We found that ectopic expression of Ss in individual pR7s caused near-complete repression of Run (Fig. 4D and E), induced Rh4 expression, and repressed Rh3 expression (Fig. 4G and H). Together, these data show that Ss is necessary and sufficient to repress Run in yR7s later in development.

2.4. Run is repressed in Ss^{ON} R7s to allow Rh4 expression

We next tested the relationship between Ss, Run, and Rh expression. Ectopic early expression of Run in all PRs was initially reported to have no effect on rhodopsin expression (Kaminker et al., 2002). Subsequent work showed that Run is sufficient to induce Rh3 expression (Edwards and Meinertzhagen, 2009), indicating that Run regulates R7 subtype fate. To confirm this result, we used a PR-specific version of the *GMR-Gal4* driver, *lGMR-Gal4* (Wernet et al., 2003), to express Run early in all PRs. We found that ectopic Run not only induces ectopic expression of the pR7 marker Rh3 but also completely eliminates Rh4 expression (Fig. 5A and B).

As early ectopic Run in all PRs induced Pros and Ss (Fig. 3E, H–I, K) yet produced retinas with only Rh3 (Fig. 5A and B), we hypothesized that Run is sufficient to perform two

parallel functions: (1) induce stochastic Ss expression and R7 fate and (2) act downstream of Ss to repress Rh4. This hypothesis would explain why Ss must repress Run in Ss^{ON} R7s: to allow Rh4 expression.

To test the second part of this hypothesis and assess whether repression of Run by Ss in yR7s is required for proper Rh3 and Rh4 expression, we used the *panR7-Gal4* driver (Chou et al., 1999) to ectopically express Run late in all R7s, at the time when they initiate rhodopsin expression. Forcing yR7s to express Run in this way caused significant repression of Rh4, leading to "empty" R7s that expressed neither Rh3 nor Rh4 in the main part of the retina, where Rh3 and Rh4 expression is normally mutually exclusive (Fig. 5C–E). In the specialized dorsal third region, where Rh3 is normally co-expressed with Rh4 in yR7s (Fig. 5F, H)(Mazzoni et al., 2008), ectopic Run expression also led to a loss of Rh4, resulting in "pseudo pR7s" that expressed Rh3 (Fig. 5G and H). We conclude that repression of Run by Ss is necessary to prevent repression of Rh4 in yR7s (Fig. 5I and J).

3. Discussion

Our characterization of the regulatory relationship between the transcription factors Ss and Run reveals a surprising complexity in the logic controlling stochastic PR specification. Run is sufficient to endow cells with the ability to express Ss. Ss, in turn, feeds back to repress Run and prevent Rh4 repression.

Our data support a temporally dynamic model of stochastic R7 subtype specification. Initially, Sal and Run are expressed in all R7s. They activate the expression of Ss in a random subset of R7s. In yR7s, Ss induces Dve, a transcriptional repressor that turns off Rh3 expression. Ss directly induces Rh4 and feeds back to repress Run, allowing derepression of Rh4. In pR7s lacking Ss, Sal represses Dve and feeds forward to activate Rh3 expression. In the absence of Ss, Run remains expressed (Johnston et al., 2011; Thanawala et al., 2013). This model supports a complex interplay between stereotyped factors like Run and stochastic factors like Ss (Fig. 6).

Both Run and Sal are sufficient to activate Ss expression in other cell types, yet neither appears to regulate the frequency of Ss expression. The only known regulator of Ss expression frequency is Klumpfuss (Klu), a transcriptional repressor (Anderson et al., 2017). It appears that activating inputs promote Ss in R7s and R8s, supported by the roles of Run and Sal and the presence of an enhancer within *ss* that drives expression in all R7s and R8s (Johnston and Desplan, 2014; Johnston et al., 2011). Activation is countered by parallel repressive pathways, including regulation by Klu, to limit Ss to a subset of R7s.

Though both Run and Sal are sufficient to enable cells to express Ss, they differ in their response to Ss itself. Sal is expressed in all R7s throughout development, is indifferent to Ss expression, and is critical for Rh3 expression in pR7s (Johnston et al., 2011) and the generation of small rhabdomeres in all R7s (Mollereau et al., 2001). Run, on the other hand, is repressed by Ss in yR7s to allow Rh4 expression. The network is further complicated since Sal activates expression of Run (Domingos et al., 2004).

How Sal and Run genetically interact with Ss to regulate Rh3 and Rh4 differs in complex ways. Sal activates Ss to repress Rh3 (via Dve) and Sal also feeds forward to activate Rh3. Run is sufficient to induce stochastic Ss expression to induce Rh4 and also feeds forward to repress Rh4. Both Run and Sal interact with Ss in incoherent feedforward loops, suggesting that stereotyped and stochastic mechanisms are in direct competition for PR fate (Fig. 6).

4. Materials and methods

4.1. Drosophila genotypes and stocks

Flies were raised on standard cornmeal-molasses-agar medium and grown at 25°C.

Short Genotype	Complete Genotype	Figures	Source
wild type	*yw; +; +* or *+; +; +*	1B, 2A–C, 3D, 3H–K, 5A, 5C, 5E–F, 5H, S1B–F	
all PRs > run	*yw; lGMR-Gal4/+; UAS-run/+*	3E, 3H–K, 5B, S1C–F	
all PRs > sal	*yw, UAS-sal; lGMR-Gal4/+; +*	3F, 3H–K, S1C–F	Kuhnlein and Schuh (1996)
svp mutant		3G–K, S1C–F	
	ey-Flp; +; FRT82b svp^{2}/FRT82b GMR-hid, cL		(Stowers and Schwarz, 1999) BL5253
all R7s > control	*yw; PM181-Gal4/+; +/+*	3C	Maurel-Zaffran et al. (2001)
all R7s > run	*yw; PM181-Gal4/+; UAS-run/+*	3C	
run mutant clones	*run^{2}/(Mi ET1)MB01026*	3A–B	
ss mutant eye	*ey-Flp; +; FRT82b ss$^{d115.7}$/FRT82b* GMR-*hid, cL*	4A–B	(Duncan et al., 1998; Stowers and Schwarz, 1999) BL5253
ss mutant R7s	*GMR-Flp/w; +/Act-Gal4, UAS-mCD8-GFP; FRT82b ss$^{d115.7}$/FRT82b, tub-Gal80*	4C, 4E–F, 4H	
ectopic *ss* in R7s	*GMR-Flp/w; UAS-ss/Act-Gal4, UAS-mCD8-GFP;FRT82b/FRT82b, tub-Gal80*	4D–E, 4G–H	
panR7>runt	*yw; panR7-Gal4/+; UAS-run/+* or *yw; panR7-Gal4/+; UAS-run/IroC-LacZ* Note: two different drivers and two different *UAS-run* transgenes were used for these experiments (total 4 combinations)	5D–E, 5G–H	Mazzoni et al., 2008) (Chou et al., 1999)
Bro mutants	*ey-Flp; +; FRT2A Bro^{w4}/FRT2A GMR > hid, cL*	S1A–B	(Stowers and Schwarz, 1999) BL6343

4.2. Generation of run^{2} clones

Because the *run* locus is more proximal to the centromere than *FRT19*, we created *run^{2}* mutant clones by radiation-induced mitotic recombination. To do so, we collected 0–24 h old embryos that were heterozygous for *run^{2}* and a closely linked Minos-GFP enhancer trap ((*Mi ET1) MB01026* - which is expressed in all R7s), waited 24 h, and then exposed them to 2 kRs of gamma-radiation. *run^{2}* mutant clones were indicated by the absence of GFP.

5. Antibodies

Antibodies were used at the following dilutions: mouse anti-Rh3 (1:100) (gift from S. Britt, University of Colorado), rabbit anti-Rh4 (1:100) (gift from C. Zuker, Columbia University), guinea pig anti-Ss (1:500) (gift from Y.N. Jan, University of California, San Francisco), anti-Run (1:250) (gift from E. Wieschaus, Princeton University), guinea pig anti-Run (1:800) (gift from P. Gergen, Stony Brook University), sheep anti-GFP (1:500) (BioRad), chicken anti-β-gal (1:800) from Abcam (Cambridge, MA), rat anti-ELAV (1:50) (DSHB), mouse anti-Pros (1:10) (DSHB), and Alexa 488 Phalloidin (1:80) (Invitrogen). All secondary antibodies were Alexa Fluor-conjugated (1:400) and made in donkey or goat (Molecular Probes, Eugene, OR).

6. Antibody staining

Adult, mid-pupal, and larval retinas were dissected as described (Hsiao et al., 2012) and fixed for 15 min with 4% formaldehyde at room temperature. Retinas were rinsed three times in PBS plus 0.3% Triton X-100 (PBX) and washed in PBX for >2 h. Retinas were incubated with primary antibodies diluted in PBX overnight at room temperature and then rinsed three times in PBX and washed in PBX for >4 h. Retinas were incubated with secondary antibodies diluted in PBX overnight at room temperature and then rinsed three times in PBX and washed in PBX for >2 h. Retinas were mounted in SlowFade Gold Antifade Reagent (Invitrogen). Images were acquired using a Zeiss LSM 700 confocal microscope or a Leica SP2 microscope.

6.1. Quantification of expression

Number of N/retinas and n/ommatidia or R7s counted are provided within the figure legends. Expression was scored as on or off based on the presence or absence of detectable signal.

Ss and Run expression in larval and pupal R7s: Expression of Ss, Run, or both in R7s were manually scored in larval (3rd instar larval) or pupal stages (6–48 h APF). Rows were numbered based on the first anterior row of Pros-expressing R7s in 3rd instar larval retinal discs. Pupal stages were determined based on time after pupation (i.e. formation of white pupae).

6.2. Ss and Pros expression in mid-pupal photoreceptors

Expression of Ss and Pros in PRs was assessed in mid-pupal retinas. Ommatidia were counted using Elav staining marking all PRs. The number of Ss^{OFF} $Pros^{OFF}$, Ss^{ON} $Pros^{ON}$, Ss^{OFF} $Pros^{ON}$, and Ss^{ON} $Pros^{OFF}$ cells within each ommatidia were counted manually. Graphs represent average counts of each combo + - SD. Genotypes were compared to wildtype with a one-way ANOVA using a Bonferroni multiple comparison test.

6.3. Rh3 and Rh4 expression in adult R7s

Frequency of Rh3 (Ss^{OFF}) and Rh4 (Ss^{ON}) expression in R7s was scored in adults manually. Dorsal third R7s were identified by the expression of *IroC-LacZ*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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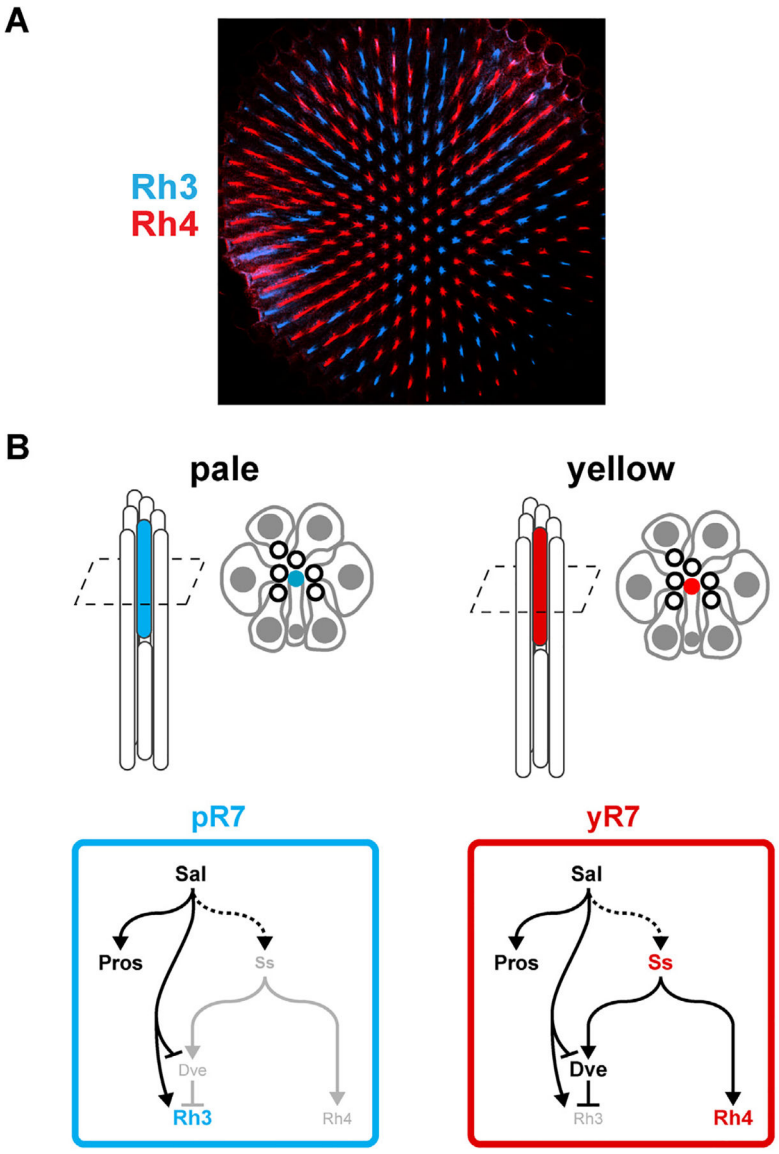

Fig. 1.

Photoreceptor subtype specification in *Drosophila melanogaster*. A. Stochastic pattern of Rh4-expressing yR7s and Rh3-expressing pR7s in a wild type adult fly retina. Rh4 (red), Rh3 (blue).

B. Schematic and cross section of a single ommatidia comprising eight PRs, R1–R8 cells. R7 cells denoted with Rh4 (red) or Rh3 (blue). Gene regulatory pathways responsible for pale (p) vs. yellow (y) R7 fate.

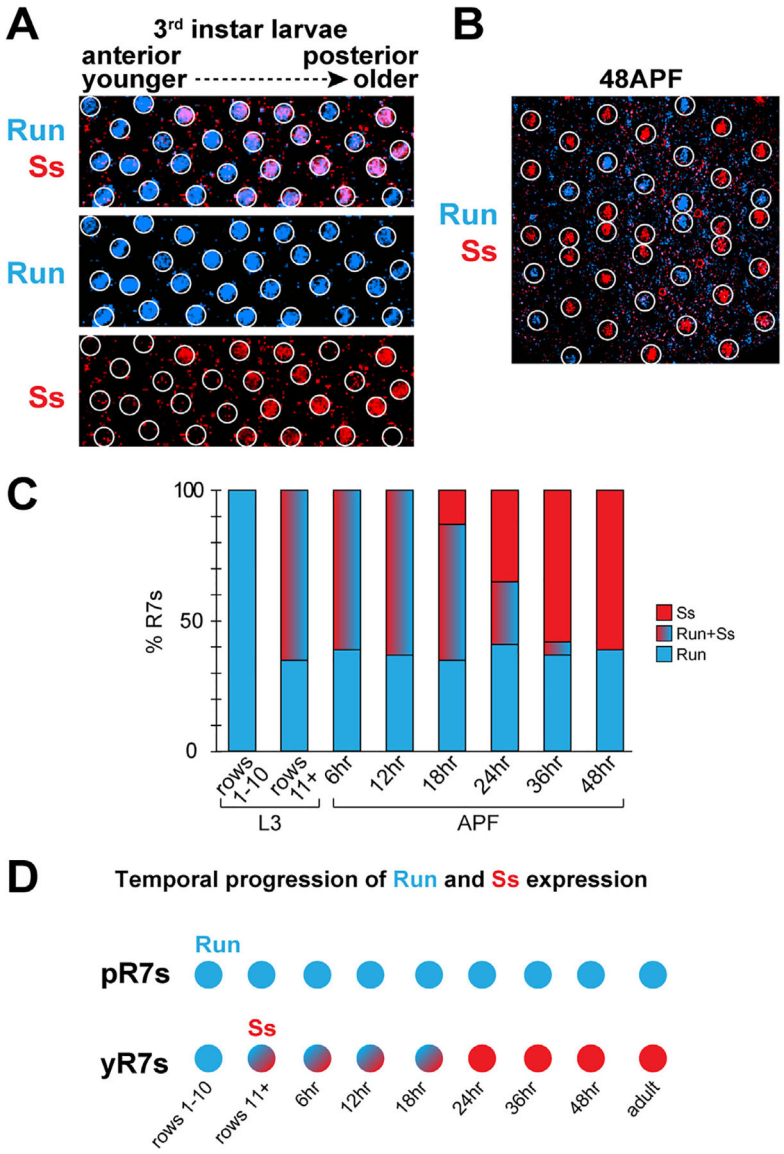

Fig. 2. Run expression in R7s precedes Ss but becomes restricted to Ss^{OFF} R7s.

For A-B, white circles indicate individual R7s expressing Run and/or Ss. A. Run (blue) is expressed in several rows of R7s before Ss (red) is expressed in a subset of R7s in the 3rd instar larval retina. Cell differentiate from the posterior to anterior direction. Cells on the right are the oldest, while cells on the left are the youngest. Each row of developing PRs is ~1.5 h older than the row immediately anterior to it. Rows 7–20 of a late 3rd instar larval retina are shown.

B. By 48 h APF, Run and Ss are expressed in mutually exclusive subsets of R7s. C. Quantification of the temporal dynamics of Ss and Run expression. Run^{ON} Ss^{OFF}, Run^{ON} Ss^{ON}, and Run^{OFF} Ss^{ON} R7s were scored for each retina.

For 3rd instar larval rows, 1–10, N = 3 retinas, n = 147 R7s.

For 3rd instar larval rows >10, N = 3 retinas, n = 172 R7s.

For 6 h APF, N = 4 retinas, n = 956 R7s.

For 12 h APF, N = 5 retinas, n = 959 R7s.
For 18 h APF, N = 5 retinas, n = 578 R7s.
For 24 h APF, N = 6 retinas, n = 390 R7s.
For 36 h APF, N = 3 retinas, n = 416 R7s.
For 48 h APF, N = 4 retinas, n = 408 R7s.

D. Temporal dynamics of Ss and Run expression in pR7s and yR7s during development.

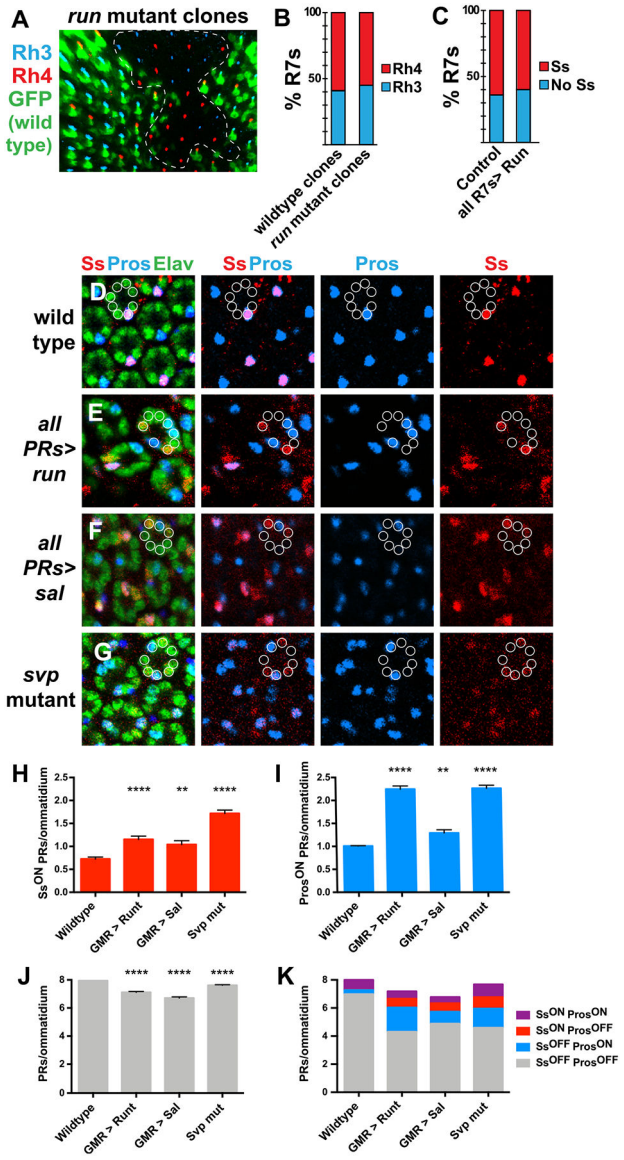

Fig. 3.

Run is sufficient to induce Pros and stochastic Ss expression independently early in development.

A. Rh3 and Rh4 expression is unaffected in homozygous *run* loss-of-function clones. GFP + marks non-mutant clone; GFP- marks a *run* homozgyous mutant clone. Dotted line indicates clone boundary.

B. Quantification of L. N = 5 retinas, n = 654 *wild type* (*run*$^{+/+}$ or *run*$^{+/-}$) R7s, n = 183 *run* (*run*$^{-/-}$) mutant R7s.

C. Ectopic expression of Run in R7s does not affect the on/off ratio of stochastic Ss expression. For *Control,* N = 3 retinas, n = 346 R7s. For *all R7s > Run*, N = 3 retinas, n = 270 R7s.

D-G. Ss (red); Elav (green) indicates PRs; Pros (blue) indicates general R7 fate; white circles denote PRs in one representative ommatidium per genotype.

E-G. Ectopic expression of Run, ectopic expression of Sal, or loss of *svp* induce Ss and Pros, resulting in a decrease in Ss^{OFF} $Pros^{OFF}$ cells.

E. Ectopic expression of Run.

F. Ectopic expression of Sal.

G. *svp* mutants.

For H–K, quantification of D-G. For *wild type*, N = 8 retinas, n = 120 ommatidia. For *all PRs* > *Run*, N = 7 retinas, n = 105 ommatidia. For *all PRs* > *Sal*, N = 5 retinas, n = 75 ommatidia. For *svp* mutant, N = 8 retinas, n = 120 ommatidia.

H. Ss^{ON} PRs per ommatidium.

I. $Pros^{ON}$ PRs per ommatidium.

J. Total PRs per ommatidium.

K. Combinations of Ss and Pros expression in PRs per ommatidium. Purple indicates Ss^{ON} $Pros^{ON}$. Red indicates Ss^{ON} $Pros^{OFF}$. Blue indicates Ss^{OFF} $Pros^{ON}$. Gray indicates Ss^{OFF} $Pros^{OFF}$.

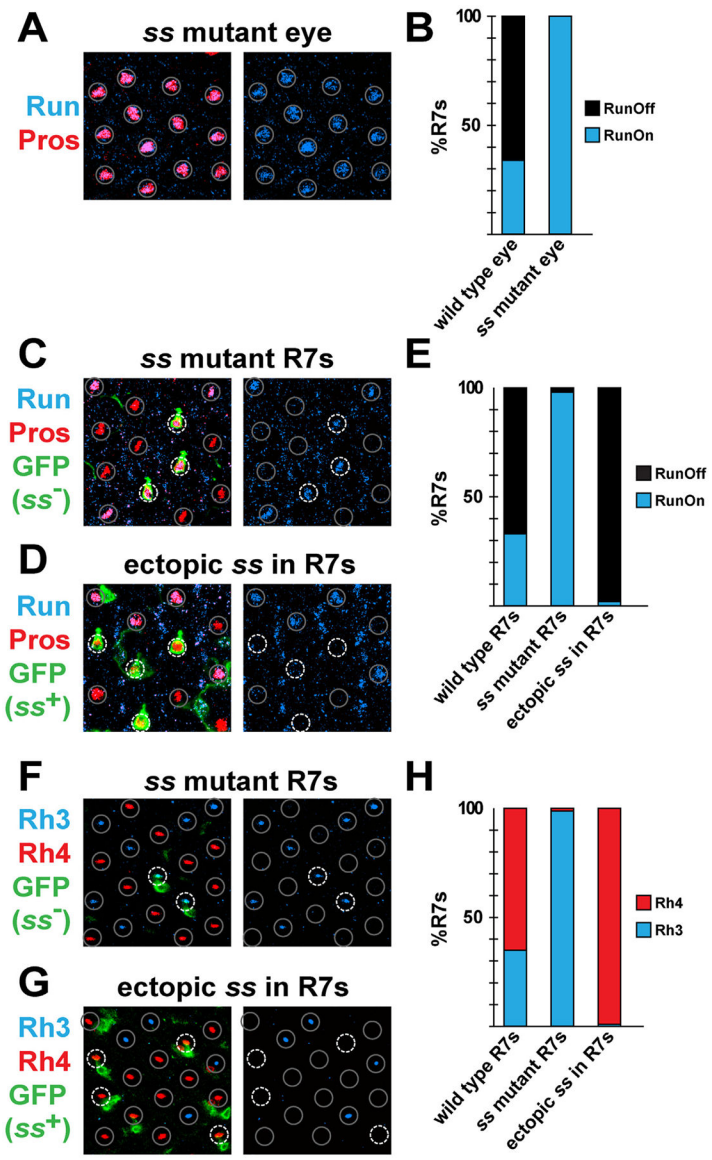

Fig. 4.

Ss restricts Runt to Ss^{OFF} cells.

For A-E, Anti-Run antibody staining (blue) was assessed at 48 h APF and scored as on or off. R7s were labeled with anti-Pros (red).

A. Run was expressed in all R7s in *ss* null mutant eyes. R7 cells marked by gray circles. Gray circles indicate R7 cells, which are labeled with anti-Pros antibodies (red). Anti-Run staining is in blue.

B. Quantification of R7 cells in A. For wild type eye, N = 2 retinas, n = 142 R7s. For *ss* mutant eye, N = 2 retinas, n = 103 R7s.

For C-G, GFP marks single *ss* loss-of-function or gain-of-expression single cell clones.

C. Run was derepressed in *ss* null mutant R7s. Wild type R7 cells are indicated with gray circles. GFP (dashed white circles) marks single cell *ss* mutant R7s. Single cell clones also occur in non-R7 cells (GFP, no circle).

D. Run was repressed in R7s with ectopic Ss expression. Wild type R7 cells are indicated with gray circles. GFP (dashed white circles) marks single R7s ectopically expressing Ss. Single cell clones also occur in non-R7 cells (GFP, no circle).

E. Quantification of C and D. For wild type R7s, N = 4 retinas, n = 79 wild type R7s. For *ss* mutant R7s, N = 4 retinas, n = 59 ss mutant R7s. For ectopic *ss* in R7s, N = 7 retinas, n = 209 R7s with ectopic *ss* expression. For F–H, Rh3 (blue) and Rh4 (red) expression was assessed in adults. GFP marked *ss* loss-of-function or gain-of-expression single cell clones.

F. Rh3 was derepressed in *ss* null mutant R7s. Wild type R7 cells are indicated with gray circles. GFP (dashed white circles) marks single cell R7 *ss* mutant clones. Single cell clones also occur in non-R7 cells (GFP, no circle).

G. Rh3 was repressed in R7s with ectopic Ss expression. Wild type R7 cells are indicated with gray circles. GFP (dashed white circles) marks single cell ectopic expression of Ss in R7s. Single cell clones also occur in non-R7 cells (GFP, no circle).

H. Quantification of F and G. For wild type R7s, N = 3 retinas, n = 85 wild type R7s. For *ss* mutant R7s, N = 5 retinas, n = 193 ss mutant R7s. For ectoptic *ss* in R7s, N = 5 retinas, n = 160 R7s with ectopic *ss* expression.

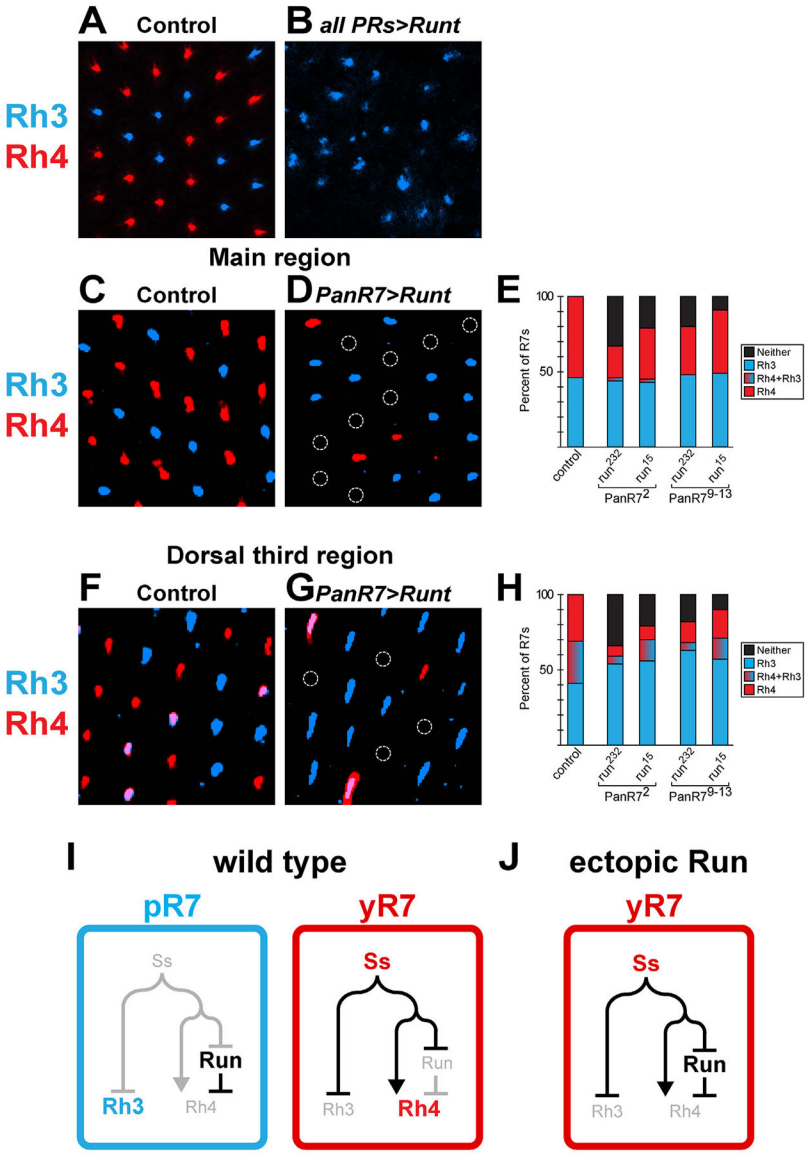

Fig. 5.

Runt is repressed in Ss^{ON} cells to allow Rh4 expression.

A. Rh3 and Rh4-expressing R7s are distributed randomly in the wild type adult retina.

B. Expression of Run in all PRs induces Rh3 and represses Rh4.

For C–H, Rh3 (blue) and Rh4 (red) expression was assessed in adult retinas and scored as on or off. The *panR7-Gal4* driver induces ectopic expression at the time of Rhodopsin expression at ~72 h APF.

C. In the main region of the adult retina, Rh3 and Rh4 expression is mutually exclusive.

D. *panR7-Run* represses Rh4, leaving "empty" R7s in the main region of the retina (dashed white circles). For E and H, two different drivers (*panR7*^{2}*-Gal4* and *panR7*$^{9–13}$*-Gal4*) and two different *UAS-run* transgenes (*UAS-Run*^{232} and *UAS-Run*^{15}) were used for these experiments for a total of four combinations.

E. Quantification of C-D.

For *control*, N = 18 retinas, n = 3741 R7s.
For *panR7^{2}>run^{232}*, N = 21 retinas, n = 3587 R7s.
For *panR7^{2}>run^{15}*, N = 19 retinas, n = 3435 R7s.
For *panR7$^{9-13}$>run^{232}*, N = 20 retinas, n = 4028 R7s.
For *panR7$^{9-13}$>run^{15}*, N = 20 retinas, n = 3760 R7s.
F. In the dorsal third region of the retina, Rh3 is normally co-expressed with Rh4 in yR7s.
G. *panR7-Run* represses Rh4 expression in the dorsal third region, generating Rh3-expressing “pseudo pR7s”.
H. Quantification of F-G.
For *control*, N = *17* retinas, n = 1575 R7s.
For *panR7^{2}>run^{232}*, N = 21 retinas, n = 1585 R7s.
For *panR7^{2}>run^{15}*, N = 19 retinas, n = 2078 R7s.
For *panR7$^{9-13}$>run^{232}*, N = 18 retinas, n = 1576 R7s.
For *panR7$^{9-13}$>run^{15}*, N = 20 retinas, n = 1836 R7s.
I. Model for the relationship between Ss, Run, Rh3, and Rh4 expression late in R7 subtype specification.
J. Model for how extending Run expression in yR7s leads to Rh4 repression. Run normally turns off in yR7s by 48 h APF. Extension of expression results in an increase in the number of empty R7s by repressing Rh4.

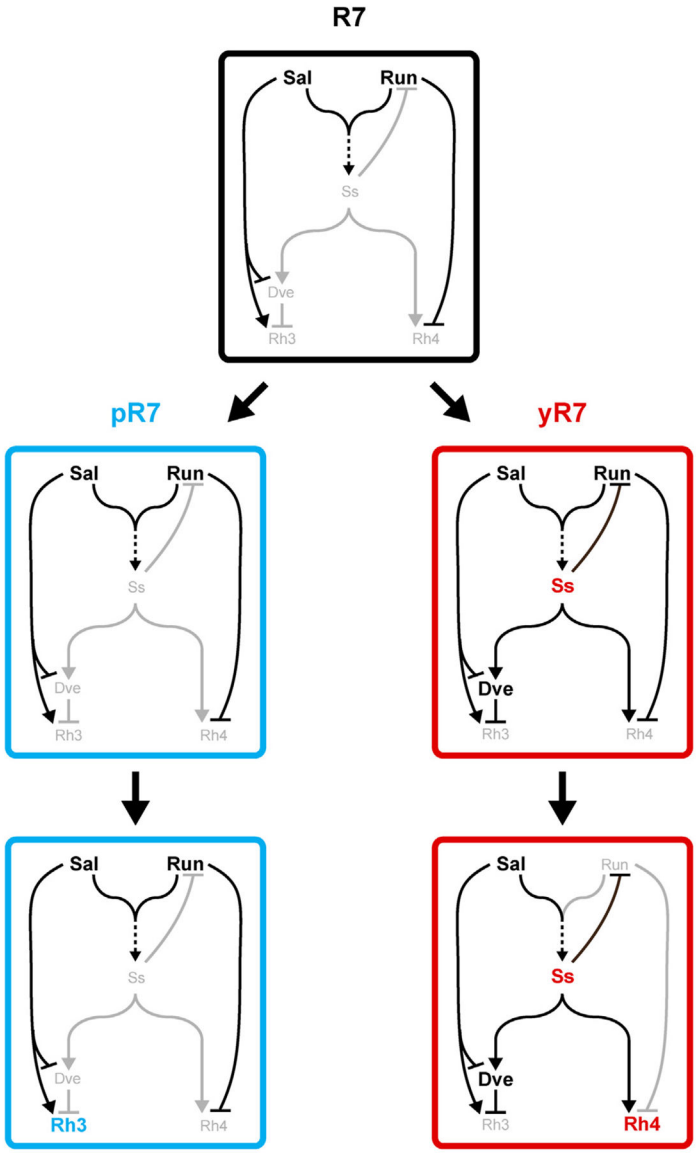

Fig. 6.

Model for temporal specification of R7 subtypes.

Sal and Run are expressed in larval R7s preceding stochastic Ss expression. Sal and Run then activate stochastic Ss expression in larval R7s. During pupal development, Ss interacts with Sal and Run in incoherent feed forward loops to specify pR7 vs. yR7 fate. Ss feeds back to repress Run in yR7s to allow Rh4 expression.