Development 135, 707-715 (2008) doi:10.1242/dev.016386

Loss of *seven-up* from *Drosophila* R1/R6 photoreceptors reveals a stochastic fate choice that is normally biased by Notch

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Recent evidence suggests that stochasticism is important for generating cell type diversity. We have identified a novel stochastic fate choice as part of the mechanism by which Delta/Notch (Dl/N) signaling specifies R7 fate in the *Drosophila* eye. The equivalence of R1/R6/R7 precursors is normally broken by the activation of N, which specifies the R7 fate. The orphan nuclear hormone receptor Seven-up (Svp) is necessary and sufficient to direct R1/R6/R7 precursors to adopt the R1/R6 fate. A simple model, therefore, is that N represses Svp, which otherwise prevents adoption of the R7 fate. However, we have found that R1/R6s lacking *svp* stochastically adopt either the R7 or the R8 fate with equal likelihood. We show that N specifies the R7 fate by a novel branched pathway: N represses Svp expression, thereby exposing an underlying stochastic choice between the R7 and R8 fates, and then tips this choice towards the R7 fate.

KEY WORDS: Stochastic, Seven-up, Notch, Photoreceptor, *Drosophila*

INTRODUCTION

Although many cell fate choices are biased by extracellular signals or inherited determinants, there is increasing evidence that some occur stochastically. For example, only a subset of cells within an *E. coli* culture infected with lambda phage are lysed, and only a subset of cells within a *B. subtilis* culture become competent to accept DNA; this variability is derived from the noise inherent in cellular processes such as transcription and translation, causing genetically identical cells to express different levels of the same gene product (Arkin et al., 1998; Ozbudak et al., 2002; Elowitz et al., 2002; Suel et al., 2006; Maamar et al., 2007). It is thought that the diversity generated by these random binary choices might enhance the survival of unicellular organisms in unpredictable environments. Stochasticism is also important for cell fate specification during the development of multicellular organisms. During N-mediated lateral inhibition, for example, stochastic differences in the ability of two initially equivalent cells to provide and receive N signals are amplified by feedback loops, ultimately causing the cells to adopt different fates (reviewed by Simpson, 1997). Stochasticism also generates the scattered distribution of sensory neuron types within receptive fields (Wernet et al., 2006; Bell et al., 2007; Serizawa et al., 2003; Shykind, 2005; Lomvardas et al., 2006). We have identified a normally hidden stochastic fate choice that occurs during the development of the *Drosophila* eye; our work suggests that stochastic binary choices may underlie even fate choices that are induced by signals or lineage.

The fly eye is an important paradigm of fate specification (Voas and Rebay, 2004). Each of its ~750 ommatidia contains eight photoreceptor neurons (R1-R8) that are specified by reiterative cell-cell signaling. First, proneural gene expression is restricted to regularly spaced cells by N-mediated lateral inhibition, generating the founder R8 neurons. Next, each R8 recruits neighboring cells as the R2/R5 and R3/R4 neuron pairs. The surrounding undifferentiated cells then undergo a final round of mitosis [the 'second mitotic wave' (SMW)], generating the pool of equipotent cells from which the R1/R6 neuron pair is then recruited, followed by R7 and the non-neuronal cone and pigment cells.

In the adult, R1-R6 are thought to function as motion-detectors: they have large light-sensing rhabdomeres, express the broadly tuned Rh1 rhodopsin, and send their axons to the first optic ganglion, the lamina (reviewed by Cook and Desplan, 2001). R7 and R8 probably sense color: they have small rhabdomeres, express more narrowly tuned rhodopsins and send their axons to two different layers within the second optic ganglion, the medulla (Cook and Desplan, 2001). Despite the divergent mechanisms by which R7 and R8 are initially specified, recent work has shown that these fates are closely related: both require the redundant zinc-finger transcription factors Spalt major (Salm) and Spalt related (Salr) (together referred to as Sal) (Domingos et al., 2004a; Mollereau et al., 2001), and R7s lacking the homeodomain transcription factor Prospero (Pros) adopt a mixture of both R7 and R8 fates (Kauffmann et al., 1996; Cook et al., 2003).

In this paper, we focus on the binary fate choice faced by members of the R1/R6/R7 equivalence group: R1/R6/R7 precursors in which N is activated adopt the R7 fate but otherwise become R1/R6s (Tomlinson and Struhl, 2001; Cooper and Bray, 2000). Previous work has suggested that N might promote the R7 fate by repressing expression of the Svp orphan nuclear hormone receptor (Mlodzik et al., 1990; Hiromi et al., 1993; Begemann et al., 1995; Kauffmann et al., 1996; Cooper and Bray, 2000; Tomlinson and Struhl, 2001). In this paper, however, we demonstrate that *svp* mutant R1/R6 precursors stochastically and with approximately equal likelihood adopt either the R7 fate or the R8 fate. This result has two broad implications. First, it reveals an unexpected stochastic binary choice between the R7 and R8 fates. We show that *svp* mutant R1/R6s transition from an initially mixed R7/R8 fate to discrete R7 or R8 fates, suggesting that the stochasticism and bistability of this choice is derived from mutual negative feedback between the two programs. Second, our results suggest that if N normally represses

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Accepted 28 November 2007

Svp in R7 precursors, a second pathway must then prevent them from stochastically adopting the R8 fate. We provide evidence that N itself both represses Svp, exposing the stochastic R7 versus R8 choice, and tips this otherwise stochastic choice towards the R7 fate. This type of gene regulatory strategy has not previously been described.

MATERIALS AND METHODS

Genetics

z4 and *z5* were identified in a screen of mosaic animals in which ~15% of retinal cells derived from the SMW were homozygous for randomly mutagenized right arms of chromosome 3; mosaic animals were generated using *GMR-FLP* and MARCM (Lee et al., 2001). Mutations were induced with ethylmethane sulfonate by standard methods (Ashburner, 1989). *z4* and *z5* failed to complement the lethality of svp^{e22} (Mlodzik et al., 1990; Begemann et al., 1995). The svp^{e22} null allele was used for all data presented.

Homozygous wild-type, svp^{e22}, or $pros^{17}$ R1s, R6s and R7s were created by *GMR-FLP*-induced mitotic recombination between *FRT82* chromosomes (Lee et al., 2001). Homozygous cells were labeled by the MARCM technique (Lee and Luo, 1999) with either *act-Gal4 UAS-Synaptotagmin (Syt)-GFP* (axon terminals) or *act-Gal4 UAS-mCD8-GFP* (cell bodies). The $pros^{17}$ chromosome was obtained from T. Cook (Cincinnati Children's Hospital). *Rh3-lacZ*, *Rh4-lacZ*, *Rh5-lacZ* and *Rh6-lacZ* stocks were obtained from C. Desplan (New York University). Mosaic animals lacking R7s were males hemizygous for a *sev, GMR-FLP* chromosome; as previously hypothesized (Mlodzik et al., 1990), *svp* mutant R1/R6s were unaffected by loss of *sev*. Individual homozygous wild-type or svp^{e22} R3s, R4s and MCs were created by $ey\text{-}FLP^{3.5}$ (from Iris Salecker, NIMR, London) and labeled by MARCM with *act-Gal4 UAS-mCD8-GFP*. Wholly wild-type or svp^{e22} mutant eyes were created by $ey\text{-}FLP^{3.5}$ and the EGUF/hid method (Stowers and Schwarz, 1999). $sev\text{-}N^{act}$ was obtained from G. Doughts (MGH, Boston).

Histology

Fixation was in 4% PLP at room temperature for 25 minutes (adult retinas) or 20 minutes (all other tissue), and antibody staining was as described by Lee et al. (Lee et al., 2001). Confocal images were collected on a Leica SP2 microscope and analyzed with Leica or ImageJ software.

We obtained mouse anti-Chaoptin (24B10; 1:200), mouse anti-Elav (9F8A9; 1:10) and rat anti-Elav (7E8A10; 1:5) from the Developmental Studies Hybridoma Bank; rabbit anti-Rh1 (1:1000) from D. Ready (Purdue University); mouse anti-Rh3 (1:10), anti-Rh4 (1:10), anti-Rh5 (1:10) and anti-Rh6 (1:50) from S. Britt (UCHSC, Denver); rabbit anti-Rh4 (1:300) and anti-Rh6 (1:1000) from C. Desplan (New York University); rabbit anti-Salm (1:500) from R. Barrio (Universidad Autónoma de Madrid); guinea pig anti-Sens (1:1000) from H. Bellen (Baylor College of Medicine); mouse anti-Pros (mR1A, 1:1000) from C. Doe (University of Oregon); and mouse anti-Svp (1:500) from Y. Hiromi via C. Doe; chicken anti-GFP (1:500) and anti-β-gal (1:800) from Abcam (Cambridge, MA); and rabbit anti-GFP (1:1000), phalloidin conjugated to Alexa Fluor 555 (1:10) and all secondary antibodies [goat IgG coupled to Alexa Fluor 488, 555 or 633 (1:250)] from Molecular Probes (Eugene, OR).

RESULTS

svp mutant R1 and R6 axons terminate in either the R8 or the R7 target layer

To identify genes required for layer-specific targeting of R axons, we performed a genetic screen using mosaic animals in which ~15% of the cells derived from the SMW, including R1s, R6s and R7s, were homozygous for a randomly mutagenized chromosome (Lee et al., 2001). We isolated two lethal and non-complementing mutations, *z4* and *z5*, that caused mutant R1 or R6 axons to terminate in the R8 target layer (Fig. 1B, arrowhead). When we used a *sevenless* (*sev*) mutation to remove all R7s (Tomlinson and Ready, 1986; Mlodzik et al., 1990), we found that a similar number of mutant R1 or R6 axons terminated in the R7 target layer (Fig. 1D).

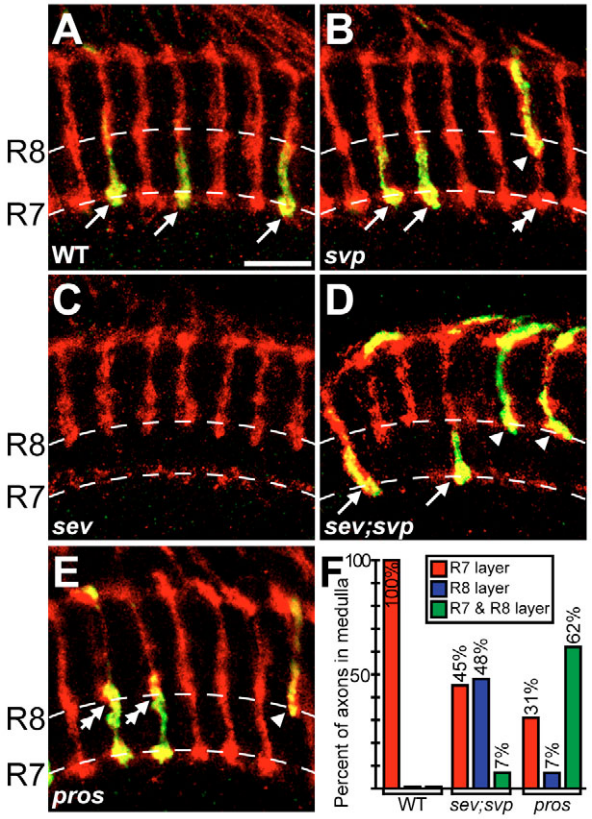

Fig. 1. *svp* mutant R1 and R6 axons terminate in either the R8 or R7 target layer. Adult medullas in which the axon terminals of individual homozygous R1s, R6s and R7s created by *GMR-FLP*-mediated mitotic recombination were labeled with the synaptic vesicle marker synaptotagmin-GFP (green) using MARCM. All R axons were labeled with mAb24B10 (red). The approximate positions of the R8 recipient layer, M3, and the R7 recipient layer, M6, are indicated by broken lines. (**A**) Wild-type (*FRT82*) R7s terminate in the R7 recipient layer (arrows). (**B**) Some *svp* mutant R1/R6s terminate in the R8 recipient layer (arrowhead); a wild-type R7 is present in the same column (double arrow). Labeled axons in the R7 recipient layer (arrows) originate from R7s or from R1/R6s. (**C**,**D**) We used the *sev* mutation to remove R7s (residual mAb24B10 staining in the R7 recipient layer is derived from medulla neurons). (C) No wild-type R1/R6s terminate in the medulla. (D) *svp* mutant R1/R6s terminate in either the R8 (arrowheads) or the R7 recipient layer (arrows) with approximately equal frequency. (**E**) *pros* mutant R7s form synaptic boutons at both the R8 and R7 recipient layers (double arrows), but sometimes appear wild-type (not shown) or terminate in the R8 recipient layer (arrowhead). (**F**) Quantification of A,D,E. Red bars represent homozygous axons that form synaptic boutons in the R7 recipient layer only, blue bars represent those that terminate in the R8 recipient layer, and green bars represent those that form boutons in both the R8 and R7 recipient layers. Scale bar: 10 μm.

z4 and *z5* are alleles of *svp* (see Materials and methods); we therefore performed all analyses (including Fig. 1B,D) using the *svp* null allele *e22*.

svp encodes several isoforms of an orphan nuclear hormone receptor and controls fate in a variety of cell types, including photoreceptors, neuroblasts, cardioblasts and kidney cells (Mlodzik et al., 1990; Kanai et al., 2005; Lo and Frasch, 2001; Kerber et al., 1998). Within the fly eye, *svp* is expressed specifically in the R3/R4 and R1/R6 neuron pairs, where it was previously reported to repress the R7 cell fate (Mlodzik et al.,

1990). We found that about half (48%; 72/151) of *svp* mutant R1/R6 axons that reached the medulla terminated precisely in the R8 target layer and half (45%; 68/151) in the R7 target layer (Fig. 1F), suggesting either that *svp* mutant R1s or R6s are transformed into R7s whose axons fail to target correctly or that some *svp* mutant R1s or R6s adopt R8 fates.

svp mutant R1s and R6s stochastically express R8 or R7 rhodopsins with approximately equal likelihood

To test whether *svp* mutant R1s or R6s can indeed adopt R8 fates, we examined their phenotype in adult retinas. As previously reported, most *svp* mutant R1s and R6s (~95%) lost expression of the R1-R6-specific rhodopsin Rh1 (Zuker et al., 1985) (data not shown) and, like R7 and R8, elaborated small rhabdomeres in the center of the ommatidium (Fig. 2) (Mlodzik et al., 1990). Previous work demonstrated that *svp* mutant eyes contained an increased number of cells expressing the R7-specific rhodopsin Rh4, but the R8 rhodopsins had not yet been identified (Mlodzik et al., 1990). Because available antibodies did not allow us to distinguish all four R7 and R8 rhodopsins simultaneously, we examined R8 rhodopsin expression (Rh5 and Rh6) and R7 rhodopsin expression (Rh3 and Rh4) in separate experiments. We found that 52% (71/136) of *svp* mutant R1s and 53% (73/138) of *svp* mutant R6s had small rhabdomeres that contained R8 rhodopsins (Fig. 2A-E); the nuclei of *svp* mutant R1s and R6s expressing R8 rhodopsins were, like those of R8s, located proximal to wild-type R1, R6 and R7 nuclei (data not shown). These results suggest that both *svp* mutant R1s and R6s can adopt R8 fates, including expression of R8 rhodopsins, proximal positioning of the nucleus and axon termination in the R8 target layer. In the same experiment, 44% (60/136) of *svp* mutant R1s and 44% (60/138) of *svp* mutant R6s had small rhabdomeres that did not contain R8 rhodopsins (Fig. 2E). We hypothesized that these may instead express R7 rhodopsins. Indeed, in a separate experiment we found that 42% (28/67) of *svp* mutant R1s and 41% (26/63) of *svp* mutant R6s had small rhabdomeres that contained R7 rhodopsins (Fig. 2F-J) and nuclei that were not displaced proximally (data not shown). Together, our results support a model in which approximately half of *svp* mutant R1s and R6s express R8 fates and half express R7 fates.

Neither the *svp* mutant R1s and R6s that expressed R8 rhodopsins nor those that expressed R7 rhodopsins formed a regular spatial pattern (data not shown), suggesting that the decision between the two fates was stochastic. To test whether R1 and R6 might somehow influence one another's decision, we examined ommatidia in which both the R1 and R6 neuron lacked *svp*. When staining for R8 rhodopsins, we observed approximate 1:2:1 ratios of ommatidia in which both *svp* mutant R1 and R6 expressed R8 rhodopsin, ommatidia in which only one expressed R8 rhodopsin, and ommatidia in which neither expressed R8 rhodopsin (data not shown); we observed the same ratios when staining for R7 rhodopsins (data not shown). We therefore conclude that *svp* mutant R1s and R6s make their decisions to express R7 or R8 rhodopsins independently of one another. The lack of correlation between fate transformations within an ommatidium provides additional evidence that this fate decision is not influenced by the ommatidium's position in the retina. Because the effects on R1 and R6 were indistinguishable, we henceforth refer to them as R1/R6s.

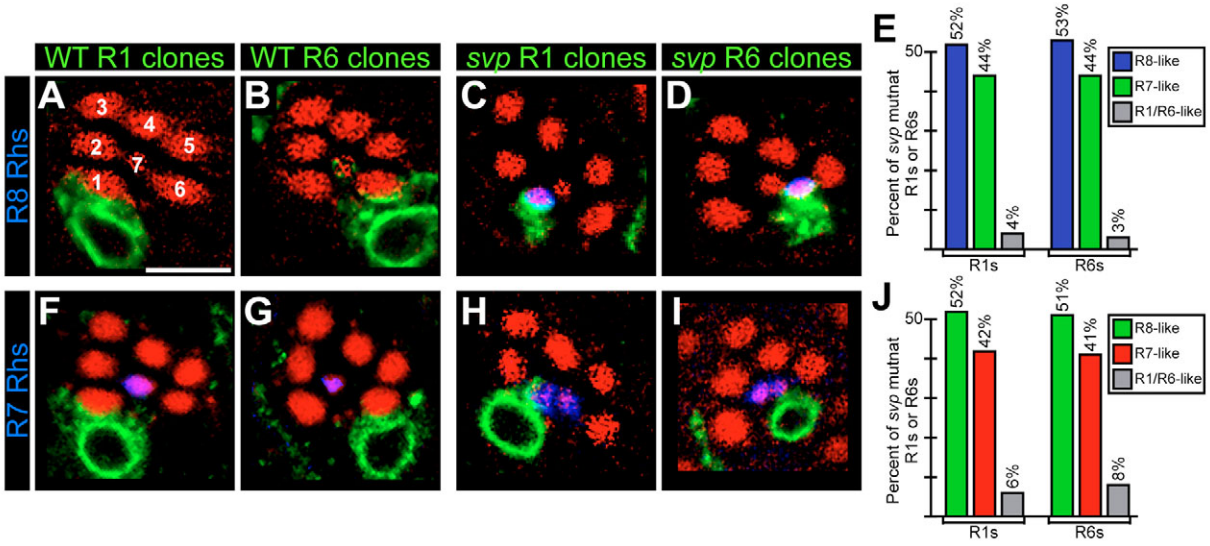

Fig. 2. *svp* mutant R1s and R6s express R8 or R7 rhodopsins with approximately equal likelihood. Adult retinas in which individual homozygous R1s and R6s created with *GMR-FLP* were labeled with mCD8-GFP (green) using MARCM. Rhabdomeres are visualized with phalloidin (red). Images are from the distal region of the retina, which contains the R1-R7 (identified in A) but not R8 rhabdomeres. (A-D) Retinas were labeled with antibodies against the two R8-specific rhodopsins Rh5 and Rh6 (both in blue). (**A**) Wild-type (*FRT82*) R1s and (**B**) R6s do not express R8 rhodopsins. Some *svp* mutant R1s (**C**) and R6s (**D**) express R8 rhodopsins. (**E**) Quantification of the experiment sampled in C and D. Blue bars represent mutant cells that adopted three R8 characteristics: a small, central rhabdomere, expression of Rh5 or Rh6 and a proximal nucleus. Green bars represent mutant cells that adopted three R7 characteristics: a small, central rhabdomere, a failure to express Rh5 or Rh6, and a distal nucleus. Gray bars represent mutant cells that retained R1/R6 characteristics. (**F-I**) Retinas were labeled with antibodies against the two R7-specific rhodopsins, Rh3 and Rh4 (both in blue). (F) Wild-type (*FRT82*) R1s and (G) R6s do not express R7 rhodopsins. Some *svp* mutant R1s (H) and R6s (I) express R7 rhodopsins. (**J**) Quantification of the experiment sample in H and I. Green bars represent mutant cells that adopted three R8 characteristics: a small central rhabdomere, a failure to express Rh3 or Rh4 and a proximal nucleus. Red bars represent mutant cells that adopted three R7 characteristics: a small central rhabdomere, expression of Rh3 or Rh4 and a distal nucleus. Gray bars represent mutant cells that retained R1/R6 characteristics. Scale bar: 5 μm.

svp mutant R1/R6s adopt either R8 or R7 fates but not both

Although these experiments were consistent with a model in which R1/R6s adopt R8 or R7 fates stochastically and with approximately equal likelihood, they did not directly address whether *svp* mutant R1/R6s cleanly adopt either R7 or R8 fates, or might instead adopt partial or mixed R7/R8 fates as *pros* mutant R7s do (Cook et al., 2003). To test this, we first determined whether *svp* mutant R1/R6s co-express R7 and R8 rhodopsins. We examined expression of pairwise combinations of one R7 rhodopsin and one R8 rhodopsin (Rh3 and Rh6; Rh4 and Rh5; and Rh4 and Rh6; we did not examine Rh3 with Rh5 because working antibodies made in different organisms were not available). We found that only 7% (12/172) of *svp* mutant R1/R6s that expressed Rh4 or Rh6 expressed both; 0% (0/136) of *svp* mutant R1/R6s that expressed Rh4 or Rh5 expressed both, and 6% (7/109) that expressed Rh3 or Rh6 expressed both. By contrast, 25% (32/129) of *pros* mutant R7s that expressed Rh4 or Rh6 expressed both. These results suggest that most *svp* mutant R1/R6s do not adopt mixed R7/R8 fates. We note that the decision by *svp* mutant R1/R6s (unlike that made by wild-type R8s) to express Rh5 or Rh6 does not strictly correlate with the presence or absence, respectively, of Rh3-expressing R7s within the same ommatidium (data not shown) (Chou et al., 1996; Papatsenko et al., 1997).

We next assessed *svp* mutant R1/R6 axon targeting. As described above, we observed that >90% of *svp* mutant R1/R6 axons cleanly target either the R7 or R8 target layer (Fig. 1D,F), consistent with a discrete transformation into either an R7 or an R8. By contrast, we have found that most *pros* mutant R7 axons (62%, 72/118) have synaptic bouton-like thickenings at both R8 and R7 target layers (Fig. 1E,F). To determine whether the choice of axon target of a *svp* mutant R1/R6 is appropriately correlated with the rhodopsin it expresses, we used *Rh-lacZ* transgene reporter constructs that reflect normal rhodopsin expression patterns (Papatsenko et al., 2001); whereas rhodopsin proteins are restricted to the retina, β-galactosidase expression is detectable in the axon, allowing us to score target selection and *Rh-lacZ* expression simultaneously. In wild type, 30% of R7s express Rh3 and 70% express Rh4, while 30% of R8s express Rh5 and 70% express Rh6 (Zuker et al., 1987; Montell et al., 1987; Fryxell and Meyerowitz, 1987; Fortini and Rubin, 1990; Chou et al., 1996; Papatsenko et al., 1997; Huber et al., 1997; Chou et al., 1999). We found that 67% (94/141) of *svp* mutant R1/R6s that target the R8 layer expressed *Rh6-lacZ* and 47% (30/64) expressed *Rh5-lacZ* (Fig. 3A-D,I); only 12% (10/83) expressed *Rh3-lacZ* and 3% (2/62) expressed *Rh4-lacZ* (Fig. 3I), indicating a tight correlation between selection of the R8 target layer and R8 rhodopsin expression (Fig. 3I). Similarly, we found that 64% (106/166) of *svp* mutant R1/R6s that target the R7 layer expressed *Rh4-lacZ* and 39% (99/255) expressed *Rh3-lacZ* (Fig. 3E-H,I); only 2% (4/172) expressed *Rh5-lacZ* and 11% (47/447) expressed *Rh6-lacZ*, indicating a tight correlation between selection of the R7 target layer and R7 rhodopsin expression (Fig. 3I). Together, our results suggest that most *svp* mutant R1/R6s either express R7 rhodopsins and select the R7 target layer or express R8

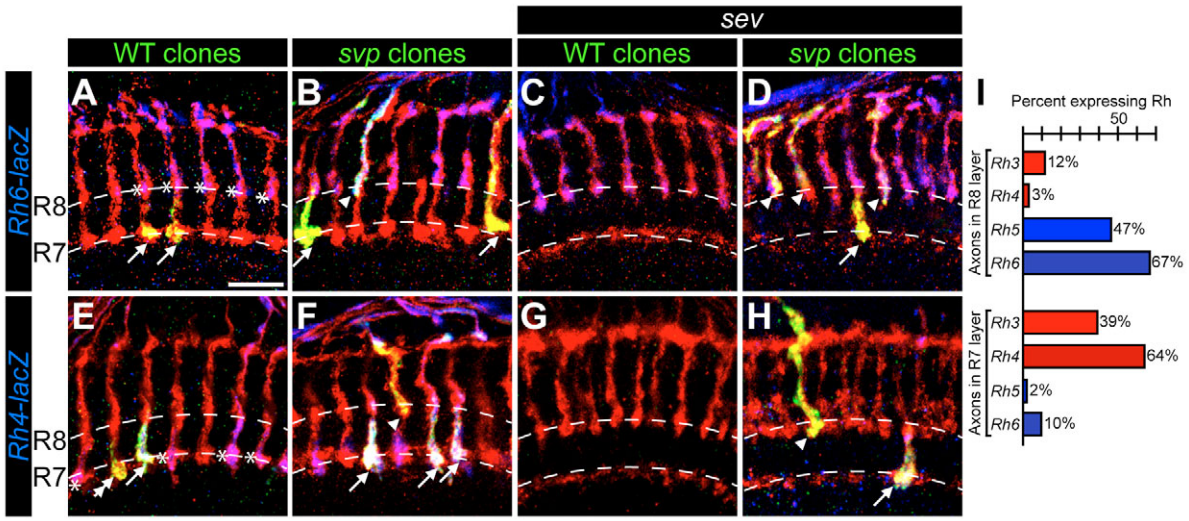

Fig. 3. Rhodopsin expression and target selection are correlated in *svp* mutant R1/R6s. Adult medullas in which the axon terminals of individual homozygous R1s, R6s and R7s created with *GMR-FLP* were labeled with synaptotagmin-GFP (green) using MARCM. All R axons were labeled with mAb24B10 (red). The approximate positions of the R8 recipient layer, M3, and the R7 recipient layer, M6, are indicated by broken lines. (A-D) Animals contained a transgene expressing *lacZ* under the control of the *Rh6* promoter (*Rh6-lacZ*; blue). (**A**) Wild-type (*FRT82*) R7s never express *Rh6-lacZ* (arrows), whereas ~70% of wild-type R8s do (asterisks). (**B**) Approximately 70% of *svp* mutant R1/R6 axons that terminate in the R8 recipient layer express *Rh6-lacZ* (arrowhead), whereas most *svp* mutant R1/R6 and R7 axons that terminate in the R7 recipient layer do not (arrows). (C,D) We used the *sev* mutation to remove R7s. (**C**) Wild-type R8s all express *Rh6-lacZ* when R7s are absent. (**D**) *svp* mutant R1/R6 axons that terminate in the R7 recipient layer do not express *Rh6-lacZ* (arrow), while those that terminate in the R8 recipient layer do (arrowheads). (E-H) Animals contained a transgene expressing *lacZ* under the control of the *Rh4* promoter (*Rh4-lacZ*; blue). (**E**) Approximately 70% of wild-type (*FRT82*) R7s express *Rh4-lacZ* (arrow; asterisks indicate heterozygous R7s expressing *Rh4-lacZ*); those that do not (double arrow) presumably express Rh3. (**F**) Approximately 70% of *svp* mutant R1/R6 and R7 axons that terminate in the R7 recipient layer express *Rh4-lacZ* (arrows), whereas most *svp* mutant R1/R6 axons that terminate in the R8 recipient layer do not (arrowhead). (**G**,**H**) We used the *sev* mutation to remove R7s. (G) When R7s are removed in wild type, there is no *Rh4-lacZ* expression. (H) Approximately 70% of *svp* mutant R1/R6s that terminate in the R7 recipient layer express *Rh4-lacZ* (arrow), while those that terminate in the R8 recipient layer do not (arrowheads). (**I**) Quantification of the experiments sampled in B and F, as well as of analogous experiments using *Rh5-lacZ* and *Rh3-lacZ*. *Rh-lacZ* expression was quantified in axons terminating in the R7 or R8 target layers of the medulla; each *Rh-lacZ* was examined in a separate experiment. Scale bar: 10 μm.

rhodopsins and select the R8 target layer; we therefore conclude that they adopt either the R7 or the R8 fate and not a mixed R7/R8 fate.

svp mutant R3s and R4s can also become R8s

svp is also expressed in the R3/R4 neuron pair. We therefore examined whether removal of *svp* from these cells might similarly cause a stochastic transformation into R7 or R8. Because the genotypes of R3 and R4 are unaffected by *GMR-FLP*, we used *ey$^{3.5}$-FLP* to induce mutant clones (Nern et al., 2005; Newsome et al., 2000). We found that 76% (96/126) of *svp* mutant R4s in otherwise wild-type ommatidia had small rhabdomeres that lacked Rh1; approximately half expressed R8 rhodopsins and half R7 rhodopsins (Fig. 4B,D,F,H), suggesting that *svp* mutant R4s adopt the R7 or R8 fate with equal likelihood. Although 77% (54/70) of *svp* mutant R3s in otherwise wild-type ommatidia retained a large outer rhabdomere that expressed Rh1, the remainder adopted either R7 or R8 fates with equal likelihood (Fig. 4D,H), as did half of the *svp* mutant mystery cells (MCs) that adopted R neuron fates (data not shown). These results suggest that *svp* mutant R3s, R4s and MCs can also stochastically adopt either the R7 or the R8 fate. We noticed that most *svp* mutant R3s with large outer rhabdomeres were in ommatidia with reversed chirality, indicating that they had adopted the R4 fate (Fig. 4C,G); this reversal in chirality has been previously observed, although not explicitly attributed to a transformation of *svp* mutant R3s into R4s (Fanto et al., 1998; Domingos et al., 2004b).

svp mutant R neurons are transformed shortly after recruitment and transition from a mixed R7/R8 fate to a discrete R7 or R8 fate

svp mutant R1/R6 and R3/R4 neurons were previously reported not to adopt R8 fates because they fail to express the larval R8 marker Bride of Sevenless (Hiromi et al., 1993). This suggested to us that *svp* mutant R neurons might not adopt R8 fates upon initial recruitment into the ommatidium but instead undergo a later transformation. We therefore sought to define the developmental timecourse of *svp* mutant R neuron transformation. Because of Gal80 protein perdurance, the GMR-FLP/MARCM technique does not result in GFP-labeling of mutant cells until ~15 hours after R neuron recruitment. We therefore used *ey$^{3.5}$-FLP* and the EGUF/hid method (Stowers and Schwarz, 1999) to create entirely *svp* mutant eyes and first confirmed that they contained the transformations predicted from our analysis of single clones; indeed, we observed an average of 2.2 R7s (n=112) and 2.5 R8s (n=232) per ommatidium in adult *svp* mutant retinas. We therefore analyzed wholly *svp* mutant larval eye discs for the expression of three early R7- and R8-specific markers: Salm, Pros and Senseless (Sens). The redundant Sal transcription factors are expressed in R7 and R8, where they prevent adoption of R1-R6-like fates (Domingos et al., 2004a; Mollereau et al., 2001); Salm is also briefly expressed in R3 and R4 (Domingos et al., 2004b). The Pros transcription factor is expressed in R7, where it prevents co-expression of R8 fates (Kauffmann et al., 1996; Cook et al., 2003); and Sens is a zinc-finger transcription factor expressed in R8s, where it prevents adoption of R2/R5-like fates (Frankfort et al., 2001). We co-stained with antibodies against the neuron-specific

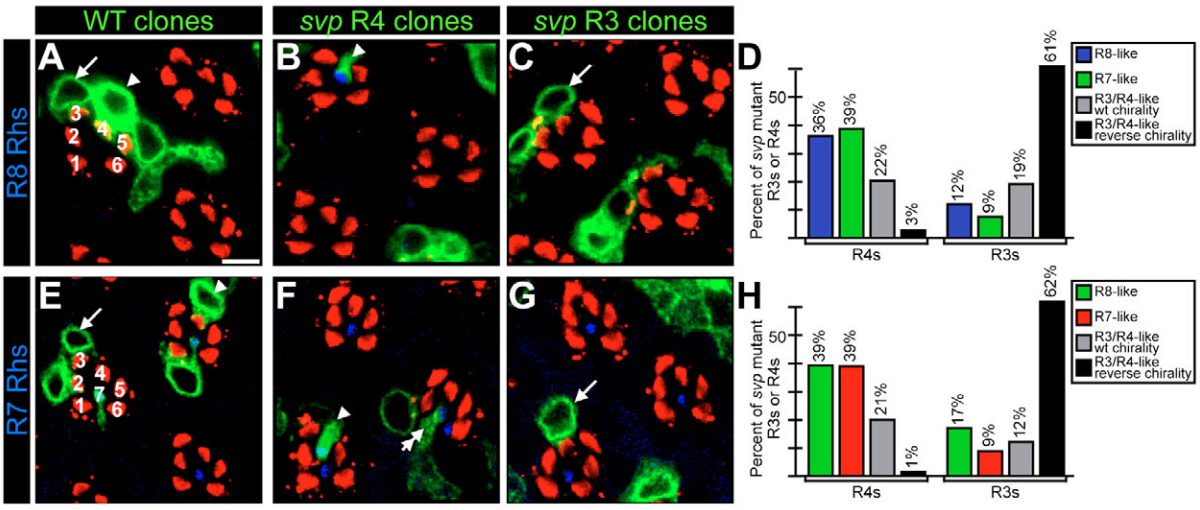

Fig. 4. *svp* mutant R3s and R4s can become either R7s or R8s. Adult retinas in which individual homozygous cells created with *ey$^{3.5}$-FLP* were labeled with mCD8-GFP (green) using MARCM. The R1-R6-specific rhodopsin Rh1 was visualized with antibodies (red). Images are all from the distal region of the retina, which contains the R1-R7 (labeled in A and E) but not R8 rhabdomeres. (**A-C**) Retinas were labeled with antibodies against the two R8-specific rhodopsins, Rh5 and Rh6 (both in blue). (A) Wild-type (*FRT82*) R3s (arrow) and R4s (arrowhead) have large outer rhabdomeres that express Rh1 but not R8 Rhs. (B) Most *svp* mutant R4s (arrowhead) have small central rhabdomeres that no longer express Rh1, of which approximately half gain expression of R8 rhodopsins. (C) Most *svp* mutant R3s (arrow) retain large outer rhabdomeres that express Rh1 but occupy R4-like positions in ommatidia with reversed chirality. (**D**) Quantification of the experiment sampled in B and C. Blue and green bars represent mutant cells that adopted R8 or R7 characteristics, respectively (see Fig. 2E; in addition these lost Rh1 expression). Gray and black bars represent mutant cells that retained large outer rhabdomeres expressing Rh1 in ommatidia with normal or reversed chirality, respectively. (**E-G**) Retinas were labeled with antibodies against the two R7-specific rhodopsins, Rh3 and Rh4 (both in blue). (E) R7s but not wild-type R3s (arrow) or R4s (arrowhead) express R7 rhodopsins. (F) Approximately half of *svp* mutant R4s with small central rhabdomeres (arrowhead) express R7 rhodopsins (quantified in H). *svp* mutant R1/R6s generated by *ey$^{3.5}$-FLP* resemble those generated by *GMR-FLP* (double arrow indicates an R1). (G) As in C, most *svp* mutant R3s (arrow) retain large, outer rhabdomeres that express Rh1 but occupy R4-like positions in ommatidia with reversed chirality (quantified in H). (**H**) Quantification of the experiment sampled in F and G. Green and red bars represent mutant cells that adopted R8 or R7 characteristics, respectively (see Fig. 2J; in addition these lost Rh1 expression). Gray and black bars are as in D. Scale bar: 5 μm.

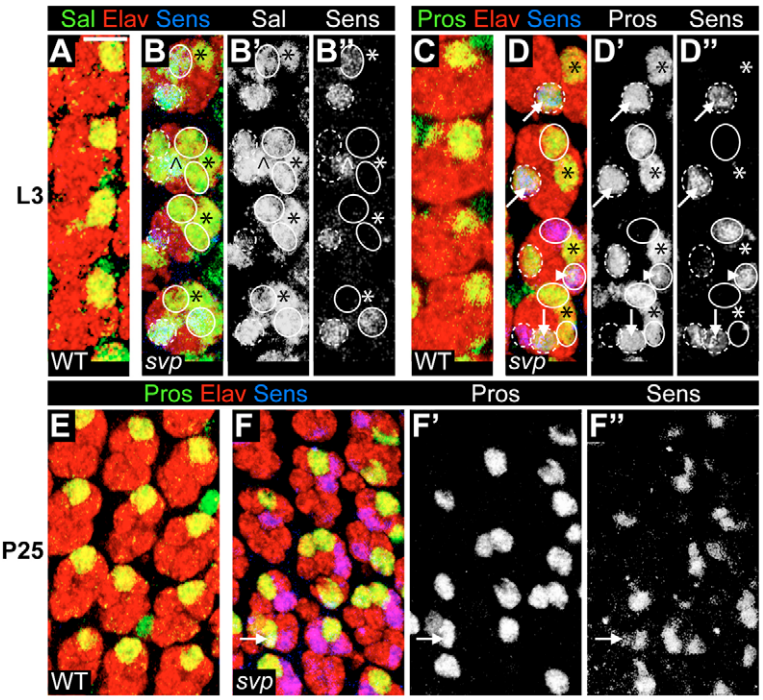

Fig. 5. *svp* mutant R neurons co-express R7 and R8 markers during larval but not pupal development. Eye discs from wandering third instar larvae (L3; **A-D″**) or pupae 25 hours after puparium formation (P25; **E-F″**). *ey-FLP*$^{3.5}$ and the EGUF/hid method (Stowers and Schwarz, 1999) were used to create eyes homozygous for a wild-type (*FRT82*; A,C,E) or *svp* mutant (B-B″,D-D″,F-F″) chromosome. R cell nuclei were visualized with anti-Elav antibodies (red). Images are all from the apical region of the disc, which contains the R7 nuclei; R8 nuclei are not visible, except as noted in B. A proportion of R1/R6 and R3/R4 nuclei are visible. (A-B″) L3 eye discs were labeled with antibodies against the R7 and R8 marker Sal (green) and the R8 marker Sens (blue). Images are from row 20. (A) In wild-type L3 eye discs, R7s express Sal but not Sens, and R1/R6s and R3/R4s express neither. R8s (not visible) express both. (B-B″) In *svp* mutant L3 eye discs, most R1/R6s (solid circles) express Sal; some co-express Sens. R7s (asterisks) express Sal but not Sens, and R8s (the single visible R8 is marked with a carat) express Sal and Sens, as in the wild type. R3/R4/MCs (dashed circles) can also express either both Sal and Sens or Sal alone. (C-D″) L3 eye discs were labeled with antibodies against the R7 marker Pros (green) and the R8 marker Sens (blue). Images are from row 20. (C) In wild-type L3 eye discs, R7s express Pros but not Sens, R1/R6s and R3/R4s express neither, and R8s (not visible) express Sens but not Pros. (D-D″) In *svp* mutant L3 eye discs, most R1/R6s (solid circles) express Pros or Sens, and some express both (arrowhead). R7s (asterisks) express Pros only, and R8s express Sens only (not shown). R3/R4/MCs (dashed circles) can also co-express Pros and Sens (arrows). (E-F″) P25 eye discs were labeled with antibodies against Pros (green) and Sens (blue). (E) In wild-type P25 eye discs, R7s express Pros, and R8s (not shown) express Sens. (F-F″) In *svp* mutant P25 eye discs, *svp* mutant R neurons express either Pros or Sens but only rarely both (arrow). The disorganization of the *svp* disc at this stage prevents unambiguous identification of R1/R6 versus R3/R4 versus R7 neurons, but each ommatidium contains extra Pros or Sens-expressing R cells (see text). Scale bar: 5 μm.

marker Elav to distinguish R cells from non-neuronal cone cells, which also express Pros and Salm. We found that *svp* mutant R1/R6s expressed Salm by row 14-15 posterior to the morphogenetic furrow (Fig. 5B-B″, solid circles), and that approximately half expressed Pros by row 15 (Fig. 5D-D″, solid circles), and approximately half expressed Sens by row 15-16 (Fig. 5B-B″,D-D″, solid circles). We also found that, as previously observed, *svp* mutant R3/R4s failed to turn off Salm expression (Fig. 5B-B″, dashed circles) (Domingos et al., 2004b), but that while some expressed Pros (Fig. 5D-D″, dashed circles), a similar number instead expressed Sens (Fig. 5B-B″,D-D″, dashed circles). These results indicate that *svp* mutant R1/R6s and R3/R4s are directed towards R7 or R8 fates shortly after their recruitment during larval development.

We were surprised to find that many *svp* mutant R1/R6s and R3/R4s expressed both Pros and Sens (Fig. 5D-D″, arrowhead and arrows). We have never detected co-expression of Pros and Sens in wild-type R neurons at any stage (Fig. 5C,E). That most adult *svp* mutant R neurons do not adopt mixed R7/R8 fates suggested either that this initially mixed Pros/Sens expression is later resolved into mutually exclusive Pros or Sens expression, or that R neurons expressing both Pros and Sens can nonetheless adopt discrete R7 or R8 fates. To distinguish between these possibilities, we examined Sens, Pros and Elav expression 25 hours after puparium formation and found that <5% of Sens- or Pros-expressing R neurons expressed both (six out of the 157 Sens-positive and 105 Pros-positive R neurons found in 54 ommatidia); an example is shown in Fig. 5F-F″ (arrow). These results indicate that most Pros/Sens co-expression is ultimately resolved, presumably allowing those R neurons that express Pros to become R7s and those that express Sens to become R8s.

N both represses Svp and prevents adoption of R8 fates in the R1/R6/R7 equivalence group

Our discovery that *svp* mutant R1/R6s stochastically become either R7s or R8s has important implications for the normal specification of wild-type R7s. The R1/R6/R7 precursor cells have previously been shown to form an equivalence group (Tomlinson and Struhl, 2001; Cooper and Bray, 2000); however, R7s fail to express Svp and yet never adopt R8 fates. Why?

The equivalence of the R1/R6/R7 precursors is broken by Dl/N signaling: R1 and R6 redundantly use Dl to activate N in the next recruited precursor, causing it to become an R7 (Tomlinson and Struhl, 2001; Cooper and Bray, 2000). Loss of N from R7 precursors causes them to adopt the R1/R6 fate, whereas ectopic N activation in R1/R6 precursors causes at least some to adopt the R7 fate (Tomlinson and Struhl, 2001; Cooper and Bray, 2000) and to lose expression of a *svp* enhancer trap (Kauffmann et al., 1996). To confirm that N is sufficient to repress Svp in R1/R6/R7 precursors, we directly examined Svp protein expression in larval eye discs of *sev-N*^{act} animals, in which the constitutively active N intracellular domain is expressed in R1/R6, R3/R4, R7 and cone cells (Basler et al., 1989; Bowtell et al., 1989; Fortini et al., 1993; Tomlinson and Struhl, 1999; Tomlinson and Struhl, 2001); indeed, *sev-N*^{act} caused a loss of Svp from R1 and R6 (Svp expression in R3/R4s was unaffected) (Fig. 6B), suggesting that the activation of N in wild-type R7s is sufficient to explain their lack of Svp.

How do wild-type R7s avoid stochastically adopting the R8 fate despite their lack of Svp? One possibility is that activated N might itself repress both *svp* expression and R8 fates by parallel mechanisms. However, it is also possible that some N-independent property of the R7 precursor, not possessed by the R1/R6 precursors,

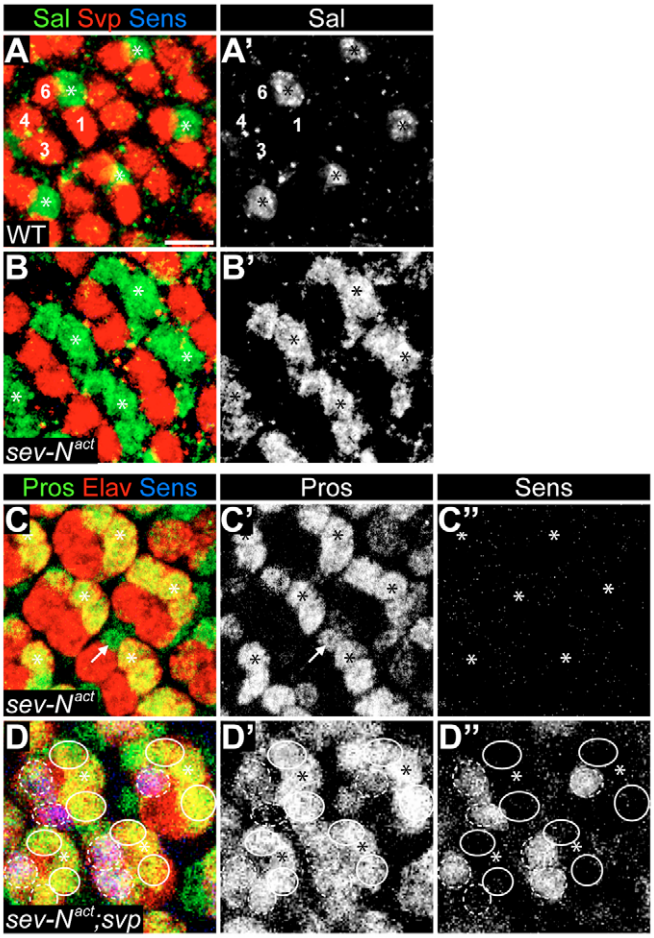

Fig. 6. Notch activation in R1/R6s represses both Svp and Sens expression. L3 eye discs. Images are all from the apical region of the disc, containing the R3/R4 and R1/R6 nuclei (labeled in A), and the R7 nuclei (asterisks) but not the R8 nuclei; all panels are presented in the same orientation. Images are from rows 15-17. (**A-B'**) Wild-type or *sev-N^{act}* L3 eye discs were stained with antibodies against Sal (green), Svp (red) and Sens (blue). (A,A′) In wild-type L3 eye discs, R7s express Sal, R1/R6s and R3/R4s express Svp, and R8s (not shown) express Sens. (B,B′) The *sev-N^{act}* transgene expresses the intracellular domain of N in R1/R6s, R3/R4s and R7s. R1/R6s expressing activated N lose Svp expression and, like *svp* mutant R1/R6s, gain Sal expression. Unlike *svp* mutant R1/R6s, they never express Sens. (Activated N does not affect Svp expression in R3/R4s.) (**C-D″**) *sev-N^{act}* or *sev-N^{act}*; *svp* mutant L3 eye discs were stained with antibodies against Pros (green), Elav (red) and Sens (blue). (C-C″) All R1/R6s expressing activated N gain Pros expression but never Sens, consistent with adoption of the R7 fate (compare with wild-type R1/R6s in Fig. 5C). A small number of R1/R6s gain Pros but also lose the neuronal marker Elav, consistent with adoption instead of the cone cell fate (arrow). R3/R4s gain neither Pros nor Sens expression, consistent with their continued expression of Svp and failure to express Sal (see B,B′). (D-D″) In *svp* mutant L3 eye discs, R1/R6s (solid circles) expressing activated N always express Pros and not Sens. Activated N does not affect the ability of *svp* mutant R3/R4s (dashed circles) to express Sens. Scale bar: 5 μm.

might block R8 fates. To determine whether ectopic N activation in R1/R6 is sufficient to prevent adoption of R8 fates despite causing loss of Svp, we examined Sens expression in *sev-N^{act}* larval discs. *sev-N^{act}* was previously shown to transform at least some R1/R6s into R7s or cone cells, but R8 markers were not examined

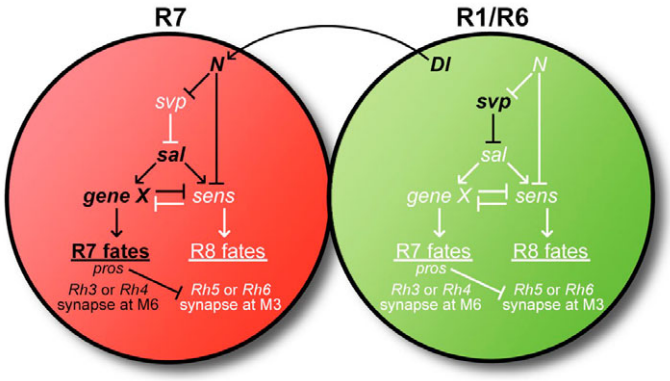

Fig. 7. Proposed model for the specification of fates in the R1/R6/R7 equivalence group. Because N is not activated in the R1 and R6 precursors, they express Svp, which prevents expression of Sal (and likely other determinants of R7 and R8 fate), resulting in adoption of the default R1/R6 fate. By contrast, Dl in R1 and R6 activates N in the R7 precursor. N represses *svp*, allowing expression of Sal (and other determinants of R7 and R8 fate), which promotes both R7 and R8 fates; mutual negative feedback between the two programs would result in stochastic adoption of either the R7 or the R8 fate, but, in parallel, N represses *sens*, resulting in exclusive adoption of the R7 fate. See text for details.

(Tomlinson and Struhl, 2001; Cooper and Bray, 2000). We found no examples of ectopic Sens expression by *sev-N^{act}* R1/R6s (or by any cell other than R8) (0/161 ommatidia) (Fig. 6B,C,C″). Instead, nearly all *sev-N^{act}* R1/R6s expressed Salm and Pros, and most co-expressed Elav, indicating a transformation into R7s (Fig. 6B-C″). A small number expressed Salm and Pros but not Elav, consistent with adoption of the cone cell fate, as has been previously observed (Cooper and Bray, 2000); this is probably caused by higher than normal levels of N, which antagonizes Ras (Rohrbaugh et al., 2002) (reviewed by Doroquez and Rebay, 2006), transforming cells that would otherwise adopt the R7 fate into cone cells (Fortini et al., 1992; Tomlinson and Struhl, 2001) (Fig. 6C,C′, arrow). To confirm that N activation is sufficient to repress Sens in R1/R6s lacking Svp, we examined the effect of *sev-N^{act}* on *svp* mutant L3 eye discs created by *ey$^{3.5}$-FLP* and the EGUF/hid method (Stowers and Schwarz, 1999). As expected, all *sev-N^{act}*; *svp* mutant R1/R6s expressed Salm and Pros but not Sens, indicating a transformation into R7s (Sens expression in *sev-N^{act}; svp* mutant R3/R4s was unaffected) (Fig. 6D-D″). We conclude that N activation is sufficient to repress both Svp and Sens in R1/R6 precursors, allowing them to adopt the R7 fate. We therefore suggest that N normally directs R7 precursors to adopt the R7 fate by repressing Svp expression, thereby exposing the stochastic choice between the R7 and R8 fates, and in parallel preventing adoption of the R8 fate (Fig. 7).

DISCUSSION

We have found that loss of *svp* causes R1/R6 precursors stochastically to adopt either the R7 or the R8 fate with approximately equal likelihood. *svp* mutant R1/R6s are transformed shortly after their recruitment as photoreceptors but transition from a mixed R7/R8 fate to discrete R7 or R8 fates. Precursors in which N is activated fail to express *svp* and yet exclusively adopt the R7 fate. Our work therefore reveals a novel branched pathway in which N both represses *svp*, thereby exposing an underlying stochastic choice between the R7 and R8 fates, and also tips this stochastic choice towards the R7 fate (Fig. 7).

How does loss of *svp* cause a stochastic fate choice?

We have found that *svp* mutant R1/R6s (and R3/R4/MCs) misexpress Salm, which is sufficient to downregulate the R1/R6-specific transcription factor BarHI and to induce expression of Pros in larval, Sens in pupal, and R7 and R8 rhodopsins in adult R neurons (Domingos et al., 2004a). Retinas lacking both *svp* and *sal* resemble those lacking *sal* only: all R rhabdomeres have an R1-R6-like morphology and express Rh1 (Domingos et al., 2004b), indicating that the transformation of *svp* mutant R neurons into R7s and R8s requires misexpression of Sal. These results suggest that Svp normally represses expression of Sal, which otherwise turns on R7 and R8 determinants, including Pros and Sens (Fig. 7), although Sal is likely not the only regulator of R7 and R8 fate that is repressed by Svp, as Sal has only a limited effect on axon connectivity (Domingos et al., 2004a; Mollereau et al., 2001).

How might misexpression of Sal (and other determinants) cause discrete yet stochastic adoption of either the R7 or R8 fate? Theoretical models suggest that stochasticism can arise from intrinsic noise within the underlying gene regulatory network (reviewed by Kaerns et al., 2005; Raser and O'Shea, 2005); this noise is particularly influential in networks whose gene products exist at very low levels. Bistability (i.e. 'switch-like' behavior between two discrete fates) can arise from feedback within the network (reviewed by Ferrell, 2002). We have found that some *svp* mutant R1/R6s initially express both the R8 marker Sens and the R7 marker Pros but later express only one or the other. This result supports a model in which their switch-like ability to adopt exclusively R8 or exclusively R7 fates is derived from mutual negative feedback between the two programs; the winning fate can emerge more quickly (for example, in those cells that never appear to co-express Pros and Sens) or more slowly. We hypothesize that expression of R7 determinants and R8 determinants in *svp* mutant R1/R6s initiates approximately simultaneously, such that negative feedback between the fate pathways begins when the relevant molecules are at low levels. The intrinsic variability caused by low molecule numbers might therefore cause the observed stochasticism of the final fate choice, as well as the variability in the length of time required for a final choice to emerge.

Which genes mediate mutual negative feedback between the R7 and R8 fates?

One obvious candidate is *pros*, which prevents R7s from also adopting R8 fates. However, we have found that Pros misexpression is not sufficient to prevent *svp* mutant R1/R6s from adopting the R8 fate (data not shown), and others have found that Pros cannot repress Sens in wild-type R8s (Cook et al., 2003). These results suggest that as yet unidentified genes ('gene X' in Fig. 7) mediate the negative feedback that turns off Sens, thereby reinforcing R7 fate in *svp* mutant R1/R6s. Pros instead directly represses Rh5 and Rh6 expression (Cook et al., 2003) and, we have shown here, prevents axon targeting to the R8 recipient layer.

By contrast, we have found that forced Sens expression is sufficient to cause *svp* mutant R1/R6s, as well as wild-type R7s, to express R8 rhodopsins and project axons that terminate in the R8 target layer (data not shown). This result suggests that Sens can mediate the negative feedback that reinforces the R8 fate in *svp* mutant R1/R6s (Fig. 7) and that wild-type R8s never adopt R7 fates because they already express a substantial amount of Sens by the time they turn on Sal.

How does N prevent R7 precursors from adopting R8 fates?

Theoretically, N might either promote the R7 fate or repress the R8 fate, but because N has a well-known earlier role in repressing R8 fates (reviewed by Frankfort and Mardon, 2002; Hsiung and Moses, 2002), we favor the latter model. In particular, during the initial specification of R8 neurons by lateral inhibition, N activation prevents cells from becoming R8s by turning on expression of the *Enhancer of Split* complex [*E(Spl)-C*] (Ligoxygakis et al., 1998); during N-mediated lateral inhibition in sensory organ development *E(Spl)-C* genes have been shown to repress Sens expression directly (Jafar-Nejad et al., 2003). A parsimonious model of R7 specification is therefore that activated N in the R7 precursor represses both Svp and Sens in parallel to induce adoption of the R7 fate (Fig. 7).

We thank R. Barrio, H. Bellen, S. Britt, C. Desplan, C. Doe, G. Doughts, Y. Hiromi, D. Ready, L. Zipursky, T. Cook, I. Salecker, S. Stowers, the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank for flies and antibodies. We also thank C. Doe, K. Guillemin, R. Herman and T. Stevens for discussion and comments on the manuscript. This work was funded by a Burroughs-Wellcome Career Development Award to T.G.H. and by an institutional NIH NRSA Genetics Training Grant (5-T32-GM007413) to A.C.M.

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