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Regulation of R7 and R8 differentiation by the *spalt* genes ☆

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Abstract

Photoreceptor development begins in the larval eye imaginal disc, where eight distinct photoreceptor cells (R1–R8) are sequentially recruited into each of the developing ommatidial clusters. Final photoreceptor differentiation, including rhabdomere formation and rhodopsin expression, is completed during pupal life. During pupation, *spalt* was previously proposed to promote R7 and R8 terminal differentiation. Here we show that *spalt* is required for proper R7 differentiation during the third instar larval stage since the expression of several R7 larval markers (*prospero*, *enhancer of split mδ0.5*, and *runt*) is lost in *spalt* mutant clones. In R8, *spalt* is not required for cell specification or differentiation in the larval disc but promotes terminal differentiation during pupation. We show that *spalt* is necessary for *senseless* expression in R8 and sufficient to induce ectopic *senseless* in R1–R6 during pupation. Moreover, misexpression of *spalt* or *senseless* is sufficient to induce ectopic *rhodopsin 6* expression and partial suppression of *rhodopsin 1*. We demonstrate that *spalt* and *senseless* are part of a genetic network, which regulates *rhodopsin 6* and *rhodopsin 1*. Taken together, our results suggest that while *spalt* is required for R7 differentiation during larval stages, *spalt* and *senseless* promote terminal R8 differentiation during pupal stages, including the regulation of *rhodopsin* expression.

Keywords: *spalt*; *senseless*; Rhodopsin; *Drosophila*; Photoreceptor; Eye development

Introduction

The *Drosophila* adult eye is composed of approximately 800 ommatidia. Each ommatidial unit contains eight photoreceptor cells (PRCs) and 12 accessory cells, including cone, pigment, and bristle cells. PRCs can be divided into two classes distinguishable by functional, morphological, and molecular criteria: (1) the six outer PRCs (R1–R6) allow image formation (for a review, see Hardie, 1985) and project to the lamina layer of the optic lobe. They have large rhabdomeres, the microvillar structures that capture light, which span the whole depth of the retina. R1–R6 cells express Rhodopsin 1 (Rh1), a light sensing protein, which accumulates in the rhabdomeres. (2) The two inner PRCs (R7 and R8) are sensitive to UV and green light, respectively, and project to the medulla layer of the optic lobe (for a review, see Cook and Desplan, 2001). The R7 rhabdomere spans only the apical half and the R8 rhabdomere the basal half of the retina. R7 expresses Rh3 or Rh4 while R8 expresses Rh5 or Rh6 (Chou et al., 1996; Chou et al., 1999; Fortini and Rubin, 1990; Huber et al., 1997; Montell et al., 1987; Papatsenko et al., 1997).

Two classes of ommatidia can be distinguished based on rhodopsin expression in R7 and R8: the "yellow" (y) class in which the R7 cell expresses Rh4 and the underlying R8 expresses Rh6, and the "pale" (p) class in which the R7 cell expresses Rh3 and the underlying R8 expresses Rh5. These two classes are randomly distributed throughout the retina and are represented in the proportion of 70%/30% (y/p) (Chou et al., 1996; Chou et al., 1999; Huber et al., 1997; Papatsenko et al., 1997).

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PRC development begins in the third larval instar with the progression of the morphogenetic furrow, which sweeps anteriorly across the eye imaginal disc (Ready et al., 1976; Tomlinson and Ready, 1987). Posterior to the furrow, PRCs are sequentially recruited into the developing ommatidia. Each PRC is distinguishable by the order in which it is recruited and by its position in the cluster. The R8 cell is specified first and then R2/R5, R3/R4, and R1/R6 are successively recruited into the cluster by reiterative stimulation of the epidermal growth factor receptor (EGFR) signaling pathway (Dominguez et al., 1998; Freeman, 1996). R7 is the last PRC to be recruited in a process which, in addition to the activation of the EGFR, also requires signaling through the Sevenless (Sev) (Hafen et al., 1987; Tomlinson and Ready, 1986) and the Notch receptors (Cooper and Bray, 2000; Tomlinson and Struhl, 2001).

The differentiation of inner PRCs requires *senseless* (*sens*) and the *spalt* (*sal*) genes. *sens* encodes a zinc finger transcription factor that is necessary and sufficient for peripheral nervous system development in *Drosophila* (Nolo et al., 2000). In *sens* mutant clones in the larval eye disc, *rough* (*ro*) is induced in R8 causing the transformation of R8 into the R2/R5 subtype. The other PRCs are recruited normally with the exception of R7, which does not differentiate presumably due to the absence of *bride of sevenless* (*boss*) expression in R8 (Frankfort et al., 2001). The *sal* genes encode related zinc finger transcription factors, which are involved in several developmental processes, as well as the human disease, Townes–Brocks syndrome (Cantera et al., 2002; de Celis et al., 1996; Dong et al., 2000; Elstob et al., 2001; Kohlhase et al., 1998; Kuhnlein et al., 1994; Nishinakamura et al., 2001; Rusten et al., 2001; Sweetman et al., 2003). In the *Drosophila* eye, deletion of the *sal* complex, which includes *sal major* (*salm*) and *sal related* (*salr*), causes the transformation of R7 and R8 into outer PRCs (Mollereau et al., 2001). In the absence of the *sal* complex, both the expression of rhodopsins and the rhabdomere morphology of R7 and R8 change to become identical to those of R1-6. These results, combined with the observation that *salm* is expressed in R7 and R8 from the late pupal stage through adulthood, led to the proposal that *sal* regulates R7 and R8 terminal differentiation during pupation (Mollereau et al., 2001). However, the precise molecular mechanisms by which *sal* regulates R7 and R8 differentiation are still poorly understood. Our present results show that *sal* has distinct roles during R7 and R8 differentiation. We found that *sal* is required for R7 differentiation during larval stages since the expression of R7 larval markers (*pros*, *E(spl)mδ0.5*, and *runt*) is lost in *sal* mutant clones. However, the majority of *sal* mutant presumptive R7 cells do not get transformed into the outer PRC subtype during larval stages since the expression of outer PRC markers (*seven-up*, *ro*, and *barH1*) is not induced (Fig. 2) and the R7 marker *H214-klg* is still present. In R8, we show that the expression of larval markers is not affected in *sal* mutant clones. However, by midpupation, *sal* is necessary for *sens* expression in R8, and misexpression of *sal* is sufficient to induce ectopic *sens* in R1–R6. These results indicate that, in R8, *sal* is not required for differentiation during larval stages but is essential for terminal differentiation during late pupal stages. Finally, we show that *sal* and *sens* are part of a genetic network that activates the expression of *rh6* and suppresses *rh1* in the terminal differentiating R8 cell.

Materials and methods

Transgenic construct, fly stocks, and generation of salm or salr mutant clones

The *Ola* region, a 5.76-kb *Eco*R1 fragment from digestion of the G5.2 phage (between the breakpoints FCK-20 and FCK-73) and located 18 kb upstream of the *salr* transcription start (Barrio et al., 1999), was cloned in the pCasper3 vector upstream of the *hsp70* minimal promoter and the *Gal4* sequence. Transgenic flies were generated using standard procedures. The following fly stocks were used: *w;Ola78-Gal4* (this work), *w; Df(2L)32FP5F40A/CyO* (deficiency spanning both *salm* and *salr*; Barrio et al., 1999), *w; sensE1 FRT80B/TM6B* (Frankfort et al., 2001), *eyflp; P{w+mC=ubi-GFP}61EF M(3)i(55) P{w+}70C FRT80B/TM6B* (Frankfort et al., 2001), *hs-Flp1.2; Ubi-GFP FRT40A* (a gift from J. Treisman), *ey-FLP; GMR-hidCLFRT40* (Stowers and Schwarz, 1999), *GMR-Gal4, Rh3(−247/+18)-lacZ* (Tahayato et al., 2003), *Rh1-Gal4* (gift from J. Treisman, Mollereau et al., 2000), *w; UAS-salm* (line RS90, Kuhnlein and Schuh, 1996), *w; UAS-sens* (line c5c, Nolo et al., 2000), *H214-lacZ* (Mlodzik et al., 1992), svp^{rA028}/TM3 (Hoshizaki et al., 1994), and *E(spl)mδ0.5* (Cooper and Bray, 1999).

Mosaic clones were generated by Flipase-mediated mitotic recombination (Golic, 1991). Flipase expression was induced under the control of the *ey* promoter or by heat-shocking larvae (37°C, 1 h) 48 h after egg laying. For pupal dissections, white prepupae (0 h) were collected and maintained at 25°C.

Immunohistochemistry

Larval, pupal, and adult eyes, as well as pupal optic lobes, were dissected in 1× PBS, fixed in a 1× PBS + 4% formaldehyde solution for 20 min at room temperature and washed three times with PBX (1× PBS + 0.3% Triton X-100). Primary antibodies were incubated in BNT (1× PBS, 1% BSA, 0.1% Tween 20, 250 mM NaCl) overnight at 4°C under gentle agitation. The antibodies and dilutions used were as follows: rabbit anti-Salm 1/100 (Salm serum, a gift from R. Schuh and R. Kühnlein, affinity purified in our lab), rat anti-Salm 1/100 (Barrio et al., 1999), guinea pig anti-Sens 1/500 (a gift from H. Bellen; Nolo et al., 2000), rabbit

anti-BarH1 (a gift from K. Saigo; Higashijima et al., 1992), guinea pig anti-Runt 1/300 (a gift from P. Gergen; Duffy et al., 1991), mouse anti-Boss 1/300 (a gift from H. Kramer), rabbit anti-β-galactosidase 1/500 (Cappel), mouse 22C10 1/10, mouse anti-Pros 1/10, mouse and rat anti-Elav 1/100 (Developmental Studies Hybridoma Bank), and rabbit anti-GFP 1/500 (Molecular Probes). Samples were then washed with PBX and incubated with appropriate secondary antibodies (Cy3, Cy5, and FITC from Jackson Immuno-Research Laboratories) for 2 h at room temperature. Samples were mounted in Vectashield (Vector Laboratories).

For whole-mount retina preparations, adult eyes were dissected in 1× PBS, fixed in a 1× PBS + 4% formaldehyde solution, and stained as described above for imaginal and pupal eye discs. Primary antibodies used were as follows: mouse anti-Rh1 1/20 (DSHB), mouse anti-Rh3 1/10, mouse anti-Rh4 1/10 and mouse anti-Rh5 1/20 (a gift from S. Britt; Chou et al., 1999), and rabbit anti-Rh6 1/2000 (a gift from C. Desplan; Mollereau et al., 2001). Secondary antibodies were as follows: conjugated goat anti-mouse and rabbit Cy5 (1/500; Jackson Immuno-Research Laboratories), Alexa 488 (1/200-Molecular Probes), and phalloidin-TRITC (1/1000; Sigma). Samples were mounted in 70% glycerol in a bridge formed by three cover slips to prevent the samples from being crushed while analyzed on a Zeiss LSM 510 confocal microscope.

Results

Salm expression in R7 and R8

Here we show that *salm* is expressed in R7 and R8 in the larval eye imaginal disc (Fig. 1), as was recently shown by Wernet et al. (2003). In a previous report, we claimed that the expression of *sal* in R7 and R8 starts only at 60 h pupation, remaining throughout adult stages (Mollereau et al., 2001). In the larval eye imaginal disc, *salm* expression was observed in R3, R4, and cone cells but not in R7 or R8. At the time, costainings with R7 and R8 markers were not done and it was not recognized that *salm* is expressed in these cells due to the high level of Salm staining in the cone cells, which are above R7 and R8. Here we determine the onset of *salm* expression in the developing imaginal disc by performing costainings with antibodies against Salm, various PRCs markers, and the neural antigen 22C10 (Tomlinson and Ready, 1987). Salm expression in R3 and R4 starts at row 3 and progressively fades away between rows 7 and 11 (Fig. 1A). The onset of Salm expression in R7 is detected by row 9 (Fig. 1A), although some weaker expression is visible in the basal part of the eye disc at row 7 (data not shown). We can identify the presumptive R7 cell, stained with Prospero (Pros; Kauffmann et al., 1996) and Salm, between the R1/R6 Seven-up (Svp; Mlodzik et al., 1990) positive cells (Fig. 1B). The onset of Salm and Pros expression in R7 is concomitant. Salm is expressed in R8 as visualized by costaining with Sens (Fig. 1C). Salm expression in R8 starts one to two rows after its onset in R7. Salm is also expressed in the anterior and posterior cone cells by row 11 and in the four cone cells by rows 14–15 (Fig. 1; and data not shown). At 24 h pupation, Salm is still expressed in R7 and R8 (Figs. 1D and E) and continues to be expressed in both cells during pupation and adulthood (Mollereau et al., 2000, 2001; Wernet et al., 2003).

sal is required for R7 but not for R8 differentiation during larval stages

In *salm/salr* mutant ommatidia, inner PRCs are transformed into outer PRCs, which was proposed to occur during late pupal stages (Mollereau et al., 2001). However, the larval imaginal disc expression of Salm in R7 and R8 (Fig. 1) raised the possibility that the phenotype observed in *salm/salr* mutant ommatidia is due to defects in R7 and R8 differentiation earlier in development. To address this possibility, we analyzed the expression of R7 and R8 markers in larval imaginal discs where *salm/salr* mutant clones were induced (Fig. 2).

The expression in R7 of the markers Pros and Runt (Run) is lost in *salm/salr* mutant clones (Figs. 2A and B). Pros is normally expressed in R7 (from rows 7 to 8) and in the four cone cells (from rows 11 to 12; Kauffmann et al., 1996). In *salm/salr* mutant clones in the larva, the presumptive R7 does not express Pros, while the Pros expression in cone cells is reduced but not lost (Fig. 2A). Run is expressed in R8 immediately posterior to the furrow and starts in R7, six to seven rows posterior to the furrow (Kaminker et al., 2002). In *salm/salr* mutant clones, Run expression is maintained in R8, but its expression in R7 is lost in the majority of ommatidia (87.6%, $n = 194$; Fig. 2B).

The level of *H214-klg* expression is reduced in R7 cells mutant for *salm/salr* (Fig. 2C). *H214-klg* is a *lacZ* enhancer trap insertion of the *klg* gene, which is normally expressed in R7 beginning in rows 12–13 and progressively increasing in more posterior rows (Mlodzik et al., 1992). In *salm/salr* mutant clones, the initiation of *H214-klg* expression is not affected but we observe a reduction in more posterior rows (arrowheads in Fig. 2C′). This result suggests that some commitment to R7 differentiation is independent of *sal* genes. In addition, the expression of the neuronal marker Elav is maintained in *salm/salr* mutant R7 cells (Fig. 2C), indicating that these cells are not transformed into non-neuronal cone cells.

Since several R7 larval markers are lost in *sal* mutant clones, we next asked whether R7 is already transformed into an outer PRC fate during larval stages in *sal* mutants. We analyzed the expression of BarH1 (R1 and R6; Higashijima et al., 1992) and Svp (R1, R3, R4, and R6). In the majority of *salm/salr* mutant ommatidia, the presumptive R7 does not express BarH1 (Fig. 2D) or Svp (data not shown). However, a small number of presumptive R7 cells (4.8% of mutant ommatidia, $n = 187$) express BarH1 (Fig.

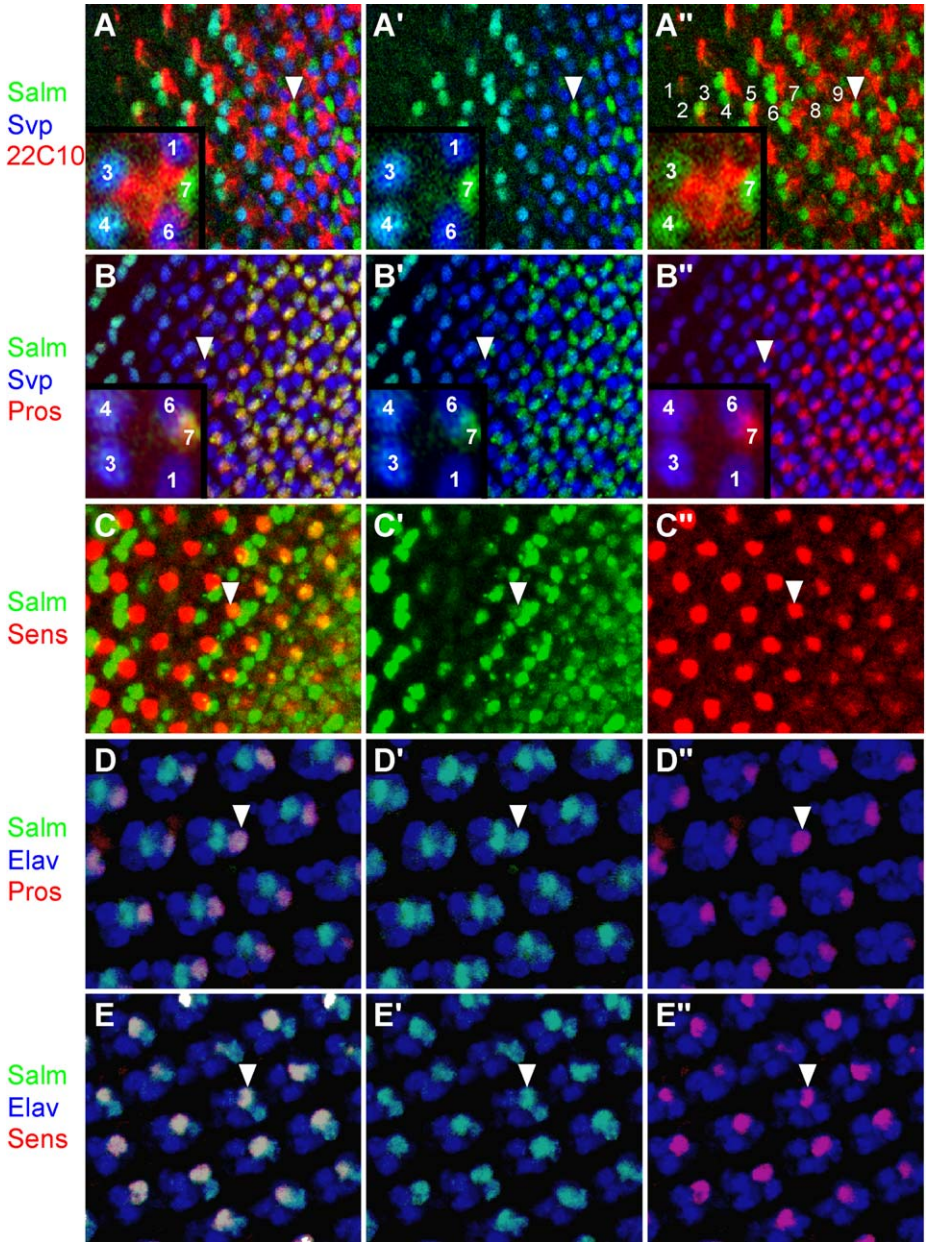

Fig. 1. Salm is expressed in R7 and R8 in the larval eye imaginal disc and in the 24-h pupal eye. Panels (A–C) represent third larval instar eye imaginal discs where posterior is to the right. The insets in A and B are magnifications of the ommatidia indicated by the white arrowheads. Numbers in the insets indicate the identity of each PRC. (A) Salm (green) is expressed in R7 by row 9. R7 localizes between the Svp (blue) positive R1 and R6. R3 and R4 are also Svp positives. The number of rows is determined by staining with 22C10 (red) and is indicated in (A″) above or below the respective ommatidium. (B) Salm colocalizes with the R7 marker Pros (red). (C) Salm colocalizes with the R8 marker Sens (red). The white arrowhead indicates the onset of Salm expression in R8. In 24-h pupal eyes (D and E), Salm is still expressed in R7 and R8. (D) The white arrowhead indicates an R7 cell, which is Salm and Pros positive. (E) The white arrowhead indicates an R8 cell, which is Salm and Sens positive.

2D) and Svp (data not shown). We did not observe ectopic expression of Rough (R2, R3, R4, and R5; Kimmel et al., 1990) in *salm/salr* mutant R7 or R8 cells (data not shown). These results demonstrate that the great majority of the *sal* mutant R7 cells are not transformed into an outer PRC subtype during larval stages.

The transformation of R7 into outer PRCs, observed in adult *salm/salr* mutant retina, also occurs when activation of the Notch signaling pathway is impaired (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). This led us to investigate if *sal* is necessary for activation of the Notch signaling pathway in R7. Activation of Notch signaling promotes the transcription of the *enhancer of split* (*E(spl)*) genes (Artavanis Tsakonas et al., 1999) and can be visualized in R4 and R7 by the *E(spl)mδ0.5* transgenic line (Cooper and Bray, 1999, 2000). In *salm/salr* mutant clones, *E(spl)mδ0.5* staining is lost in all R7 and most R4 cells (Fig. 2E). This result shows that *sal* is required for the transcriptional activation of

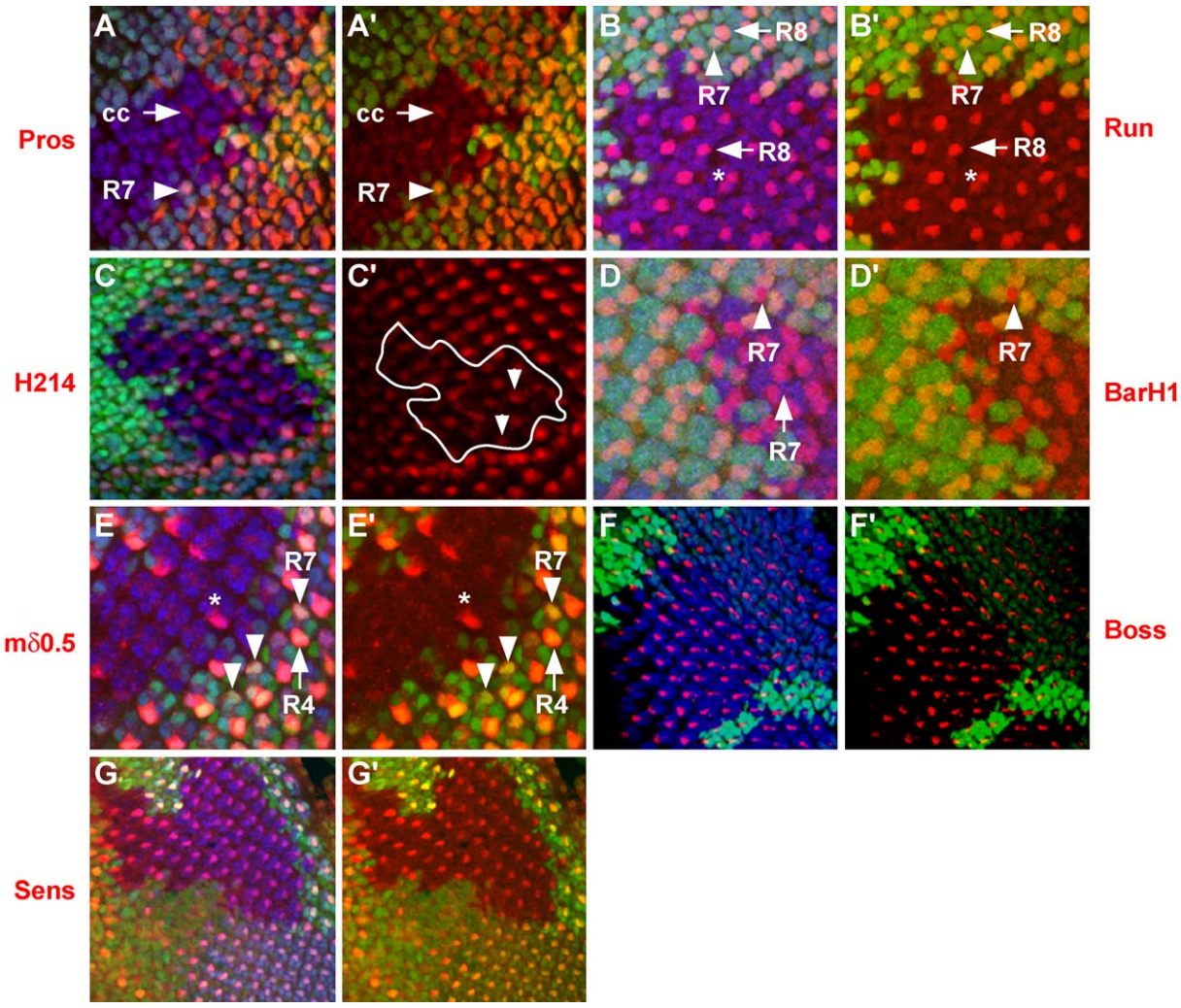

Fig. 2. *sal* is required for the expression of R7 but not R8 larval imaginal disc markers. All panels represent larval eye imaginal discs in which *salm/salr* mutant clones (*hs-flp*; *Df(2L)32FP5 FRT40A/Ubi-GFP FRT40*A) were induced and are labeled by the absence of ubi-GFP (green). Posterior is to the right. Elav (blue) was used to label photoreceptor cells. (A) Pros expression (red) in R7 is lost in *salm/salr* mutant clones. In nonmutant tissue, R7 cells express Pros and Elav and stain pink (white arrowhead). In *salm/salr* mutant tissue, no cells coexpressing Pros and Elav are observed. Expression of Pros in cone cells (cc-Elav negative) is reduced but not completely lost (white arrow). (B) Expression of Run (red) is lost in R7 but not in R8 (white arrow) in *salm/salr* mutant clones. The indicated ommatidium in the nonmutant area shows Run expression in both R7 (white arrowhead) and R8 (white arrow). A minority of ommatidia in the mutant area (13.4%, $n = 194$) still express some level of Run in R7 (white star). (C) *H214* expression (red) in R7 is reduced but not lost (white arrowheads). The white line in (C′) marks the *salm/salr* mutant clone. Elav expression in R7 is not affected in *salm/salr* mutant clones. (D) In wild-type tissue, BarH1 (red) labels R1 and R6. In the majority of *salm/salr* mutant ommatidia, the presumptive R7 is BarH1 negative and Elav positive (white arrow). BarH1 is expressed in the presumptive R7 (white arrowhead) in a minority of *salm/salr* mutant ommatidia (4.8%, $n = 187$). (E) *sal* is required for the expression of *mδ0.5* (red), a read out of Notch signaling activation. In nonmutant tissue, *mδ0.5* expression is observed in R4 (white arrow) and R7 (white arrowheads). In *salm/salr* mutant clones, *mδ0.5* expression in R4 and R7 is lost. Some ommatidia show expression in R4 (white star) but not in R7. (F) Boss expression (red) in R8 is identical in mutant and nonmutant tissue. (G) Sens expression (red) in R8 is identical in mutant and nonmutant tissue.

E(spl)mδ0.5 in R4 and R7 and activation of the Notch signaling pathway in these cells. The requirement of *salm/salr* for Notch signaling in R7 may explain the loss of R7 markers and the occasional ectopic expression of R1/R6 markers. These results further demonstrate that *salm/salr* are necessary for R7 differentiation during larval stages, although the loss of *E(spl)mδ0.5* observed in *sal* mutants is not sufficient to respecify the presumptive R7 into an outer PRC during larval development. In contrast to R7, expression of the R8 markers Run (Fig. 2B), Boss (Fig. 2F), and Sens (Fig. 2G) is maintained in *salm/salr* mutant larvae. These results indicate that *sal* is not required for early R8 differentiation in the larval eye imaginal disc.

sal is required for expression of Sens at mid pupation

Expression of the larval R8 markers Run, Boss, and Sens is not affected in *salm/salr* mutant clones (Fig. 2). However, both R7 and R8 are transformed into the outer PRC subtype in the adult retina mutant for *salm/salr* (Mollereau et al., 2001). To determine the point during eye development when *sal* is required for R8 differentiation, we analyzed the

expression of Sens in *salm/salr* mutant clones during pupal stages. In wild-type animals, Sens expression is maintained in R8 throughout pupal and adult stages (data not shown). In *salm/salr* mutants, Sens is still present in R8 at 24 h pupation (Fig. 3A). However, by 48 h, Sens is no longer observed in R8 (Fig. 3B) and it is still absent in the adult retina (data not shown). These results show that although *sal* is dispensable for Sens expression during larval stages (Fig. 2G), *sal* is required for Sens expression and R8 differentiation during pupal stages.

sal is sufficient to induce R7 larval markers and R8 pupal markers

Our analysis of *salm/salr* loss of function mutants (Figs. 2 and 3) suggests that *sal* is required for R7 differentiation during larval stages and for R8 differentiation during pupal stages. To further characterize the role of *sal* in R7 and R8 differentiation, we undertook a gain of function approach. We ectopically expressed the *sal* genes under the control of *GMR-Gal4* or *rh1-Gal4* and stained these eyes with various PRC markers (Figs. 4 and 5).

The *GMR* enhancer drives expression in all differentiating retinal cells posterior to the morphogenetic furrow, from larval stages until adulthood. When *salm* is expressed under the control of *GMR-Gal4*, BarH1 expression is repressed in R1/R6 (Fig. 4B), Pros is ectopically expressed in several additional PRCs (Fig. 4D), but the expression of Sens remains unaffected (Fig. 4F) in the larval eye disc. These results further demonstrate a role for *sal* in R7 but not R8 differentiation during larval stages. Moreover, the misexpression of *salm* under GMR induces ectopic *Rh3-lacZ* (R7 rhodopsin) expression in outer PRCs, which keep their axonal projections into the lamina (Fig. 4H). Since *sal* misexpression does not cause outer PRCs to project their axons to the medulla, *sal* is sufficient to determine the R7 subtype identity after the cell has been instructed to become an outer photoreceptor neuron.

Expression of *rh1* in outer PRCs starts around 70% pupation (approximately 67 h at 25°C) and stays on throughout adulthood. (Kumar and Ready, 1995; Sheng et al., 1997). We used the *rhodopsin 1-Gal4* (*rh1-Gal4*) driver to misexpress *salm* or *salr* in outer PRCs during late pupal stages of development. Misexpression of *salm* or *salr* under the control of *rh1-Gal4* induces ectopic Sens expression in outer PRCs (Figs. 5C and D; and data not shown). In contrast, misexpression of *sens* with *rh1-Gal4* does not induce ectopic expression of Salm (data not shown). These results indicate that both *salm* and *salr* are sufficient to induce ectopic *sens* expression during the late pupal stages of PRC differentiation.

Misexpression of sal or sens induces ectopic expression of Rh6 and partial suppression of Rh1

The initiation of rhodopsin expression occurs during late pupal stages and is a key event in terminal PRC differentiation. In both *salm/salr* and *sens* mutants, the

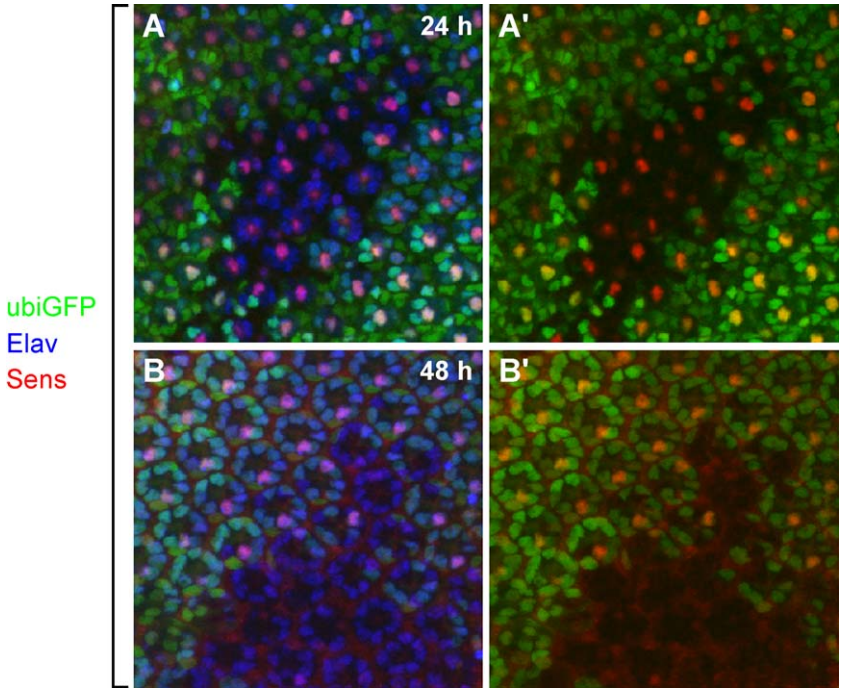

Fig. 3. Sens expression requires *salm/salr* at midpupation. Panels represent 24 h (A) or 48 h (B) pupal eye discs where *salm/salr* mutant clones (*hs-flp*; *Df(2L)32FP5 FRT40A/Ubi-GFP FRT40*A) were induced. The mutant area is labeled by the absence of ubi-GFP (green) and Elav (blue) was used to label photoreceptor cells. (A) At 24 h pupation, Sens expression (red) in R8 is similar in *salm/salr* mutant and nonmutant areas. (B) At 48 h pupation, Sens expression in R8 is lost in *salm/salr* mutant area.

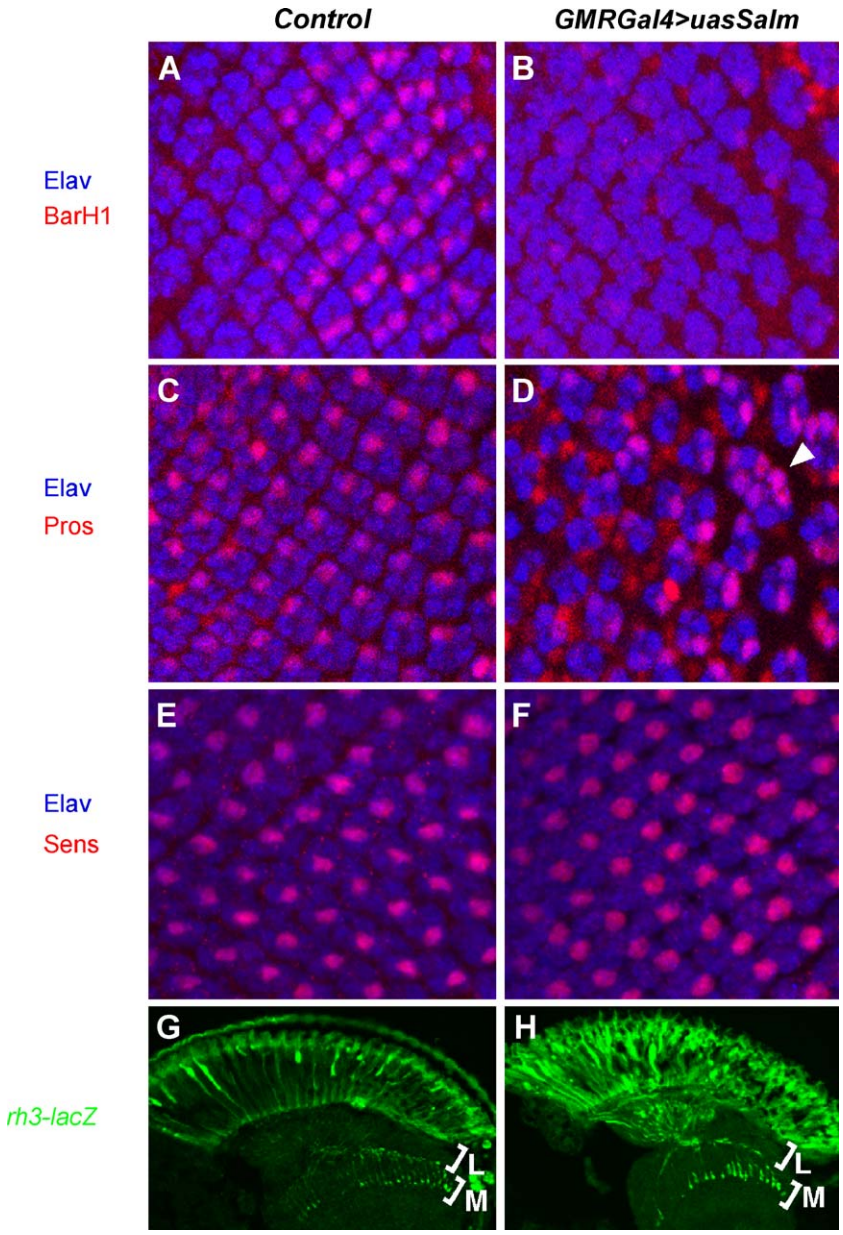

Fig. 4. Misexpression of *salm* in the eye imaginal disc is sufficient to induce ectopic R7 but not R8 markers. Salm was misexpressed under the control of *GMR-Gal4* in all differentiating cells in the larval eye disc (B, D, F, and H). Larval eye discs were stained for BarH1 (A and B), Pros (C and D), and Sens (E and F). Misexpression of *salm* represses BarH1 in R1/R6 (B), induces ectopic expression of Pros in additional PRCs (arrowhead in D), but does not affect expression of Sens (F). (G and H) Horizontal sections of adult wild-type (G) and *GMR-Gal4 > uas-salm* (H) flies carrying *Rh3-lacZ*. (G) In wild-type flies, *Rh3-lacZ* is expressed in a subset of R7 cells (pale) in the upper layer of the retina and it is possible to distinguish the R7 *pale* projections in the medulla (M). (H) In *GMR-Gal4 > uas-salm* eyes, *Rh3-lacZ* staining is expanded to outer PRCs as visualized by anti-*lacZ* staining in the entire retina and also in the lamina (L), where outer PRCs project.

expression of rhodopsins changes so that all PRCs express *rh1* but not *rh3*, *rh4*, *rh5*, and *rh6* (R7 or R8 rhodopsins; Mollereau et al., 2001; and data not shown). In *sens* mutants, this change in the expression of rhodopsins can be explained by the early transformation of R8 into a cell of the R2/R5 subtype and the failure of R7 to differentiate in the eye imaginal disc (Frankfort et al., 2001). Similarly, for *salm/salr* mutants, this change in the expression of rhodopsins in R7 can be explained by an early defect in R7 differentiation during larval stages (Fig. 2). However, since the expression of both *salm* and *sens* continues during pupal stages, it is possible that these genes also regulate R7 and R8 terminal differentiation, including rhodopsin expression.

To test this hypothesis, we misexpressed *salm*, *salr*, and *sens* in outer PRCs using the *rh1-Gal4* driver (Fig.

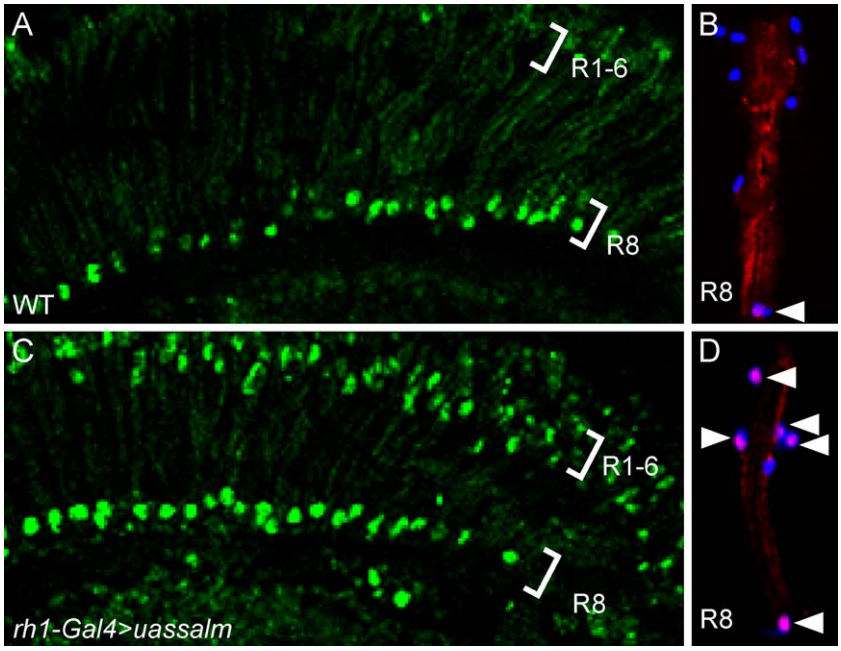

Fig. 5. Misexpression of *salm* during pupal stages is sufficient to induce ectopic *sens* expression. Horizontal sections of wild-type (A) and *rh1-Gal4* > *uas-salm* (C) eyes were stained for Sens (green). Dissociated ommatidia of wild-type (B) and *rh1-Gal4* > *uas-salm* (D) eyes were stained for Sens (red) and DAPI (blue) to reveal the position of nuclei. In all panels, the apical part of the retina is at the top. (A) In wild-type eyes, Sens staining is restricted to the R8 nuclear layer. (B) A wild-type ommatidium stains for Sens in R8 only (arrow). (C) In *rh1-Gal4* > *uas-salm* eyes, Sens staining is observed in the R8 and R1–R6 nuclear layer. (D) A *rh1-Gal4* > *uas-salm* ommatidium stains for Sens in R8 (arrow) and four additional nuclei that are in R1–R6 nuclear layer (arrowheads).

6). Misexpression of *salm* or *salr* (data not shown) induces ectopic expression of Rh6 (Figs. 6O and R) and a partial suppression of Rh1 (Figs. 6C and R). Many ommatidia contain three to five PRCs that express Rh6, and the coexpression of Rh1 and Rh6 is frequently observed (Fig. 6R). Misexpression of *sens* induces a more moderate expansion of Rh6 (Figs. 6N and Q) and reduction of Rh1 expression (Figs. 6B and Q). Many ommatidia show two to three PRCs that express Rh6 in photoreceptors in which Rh1 expression is lost (Fig. 6Q). Misexpression of *salm* or *sens* with *rh1-Gal4* does not induce the ectopic expression of Rh3, Rh4, or Rh5 (Figs. 6E, F, H, I, K, and L). Since ectopic expression of *salm* or *sens* represses *rh1* expression, we misexpressed *salm* or *sens* using a different driver than *rh1-Gal4* (*Ola-Gal4*; described in Material and Methods). We used this driver because it is expressed in all PRCs around 70% pupation where it is maintained throughout adulthood (Supplemental Fig. 1). We obtained similar results with both the *Ola-Gal4* and *rh1-Gal4* drivers (data not shown). In addition, the regulation of Rh1 and Rh6 by Sal and Sens occurs at the transcriptional level since misexpression of *sal* or *sens* suppressed *rh1-lacZ* and induced *rh6-lacZ* reporters (data not shown).

These results show that *sal* is not sufficient to induce ectopic R7 rhodopsins during pupation (*rh1-Gal4*), although the misexpression of *sal* during larval stages (*GMR-Gal4*) induced ectopic expression of Rh3 (Fig. 4H). The expansion of Rh3 expression to outer PRCs, obtained with the *GMR-Gal4* driver, is probably a secondary consequence of the earlier role of *sal* in R7 differentiation. However, *sal* and *sens* do regulate terminal differentiation of R8, particularly the induction of Rh6 and the suppression of Rh1.

sal and sens are part of a genetic network that regulates Rh1 and Rh6

We have demonstrated that *sal* is necessary and sufficient for *sens* expression during pupation (Figs. 3 and 5) and that *sal* and *sens* are sufficient to suppress Rh1 and induce Rh6 (Fig. 6). These results suggest that *sens* acts downstream of *sal* in the regulation of Rh1 and Rh6. To test this hypothesis, we asked whether the regulation of Rh1 or Rh6 by *sens* requires *sal*. We analyzed *salm/salr* mutant retina in which *sens* was misexpressed in all PRCs under the control of *rh1-Gal4*. In these eyes, Rh1 is partially suppressed and ectopic Rh6 is induced (Figs. 7B and F) to an extent similar to that observed by misexpression of *sens* in wild-type eyes (Figs. 6B, N, and Q). These results show that *sens* regulation of Rh1 and Rh6 does not require *sal*, and that *sens* acts downstream of *sal*.

Next we asked whether *sal* can regulate Rh1 and Rh6 expressions independently of *sens*. We analyzed *sens* mutant retina in which *salm* was misexpressed under the control of *rh1-Gal4*. We observed partial suppression of Rh1 and ectopic expression of Rh6 (Figs. 7D and H). However, the

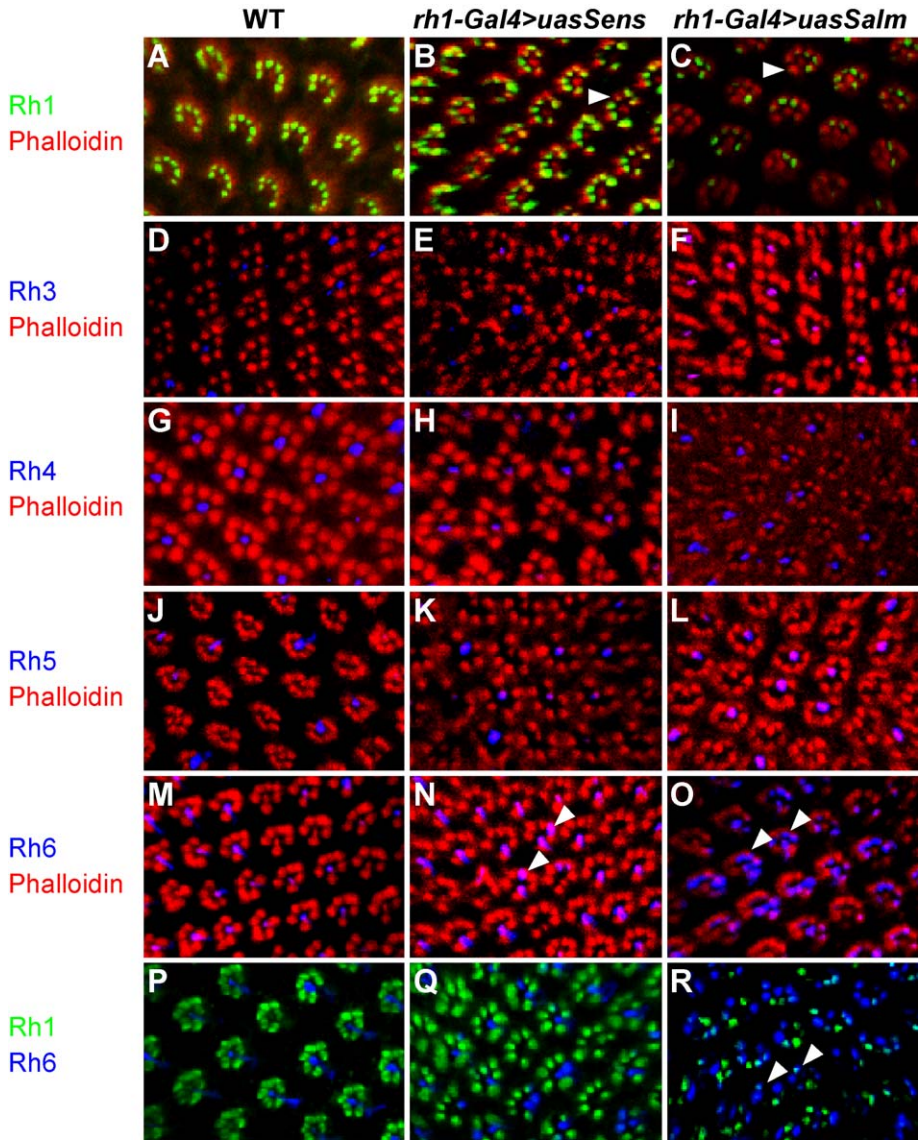

Fig. 6. Misexpression of *salm* or *sens* induces ectopic expression of Rh6 and partial suppression of Rh1. All panels represent whole-mount preparations of adult eyes. Phalloidin (red; A–O) was used for visualization of the rhabdomeres together with Rh1 (green; A–C), Rh3 (blue; D–F), Rh4 (blue; G–I), Rh5 (blue; J–L), and Rh6 (blue; M–O). Double staining with Rh1 (green) and Rh6 (blue) is presented in (P–R). Wild-type eyes (A, D, G, J, M, and P) show normal staining with Rh1 in the six outer PRCs, Rh3, or Rh4 in R7 and Rh5 or Rh6 in R8. In eyes where *sens* (B, E, H, K, N, and Q) or *salm* (C, F, I, L, O, and R) are misexpressed under the control of *Rh1–Gal4*, ectopic Rh6 expression is observed in outer PRCs (arrowheads in N and O) and Rh1 is partially suppressed (arrowheads in B and C). Arrowheads in R indicate rhabdomeres where coexpression of Rh1 and Rh6 is observed.

extent of these effects is reduced in comparison with the misexpression of *salm* in wild-type eyes (Figs. 6C, O, and R). These results suggest that there is a strong dependence on *sens* for *sal*-mediated induction of *rh6* expression and *rh1* suppression, although a less efficient *sens*-independent pathway also exists.

Discussion

PRC development in *Drosophila* has been used as a paradigm to understand neuronal specification and differentiation. Previously, we have shown that in the absence of the *sal* genes, R7 and R8 cells are transformed into the outer PRC subtype, and this phenotype was interpreted as a result of the role of *sal* in R7 and R8 terminal differentiation during pupal stages (Mollereau et al., 2001). As a consequence, a model was proposed in which PRC differentiation occurs as a two-step process. In the first step, during larval stages, the cells adopt their fate as neurons, become committed and send specific axonal projections. In the second step, during pupal stages, these neurons execute their terminal differentiation program and become mature photoreceptors. In this model, *sal* is required for the second step of differentiation in R7 and R8. Here, we show that *sal* has distinct roles during R7 and R8 differentiation. In R7, *sal* is

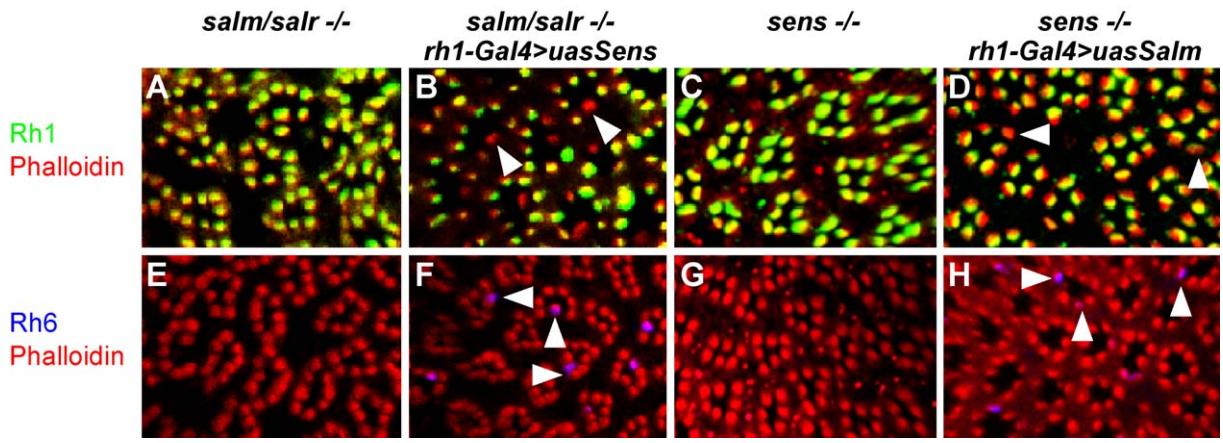

Fig. 7. *sal* acts upstream of *sens* in the regulation of *rh1* and *rh6*. All panels represent whole-mount preparations of adult eyes that were double stained with Phalloidin (red) and Rh1 (green; A, B, C, and D) or Phalloidin (red) and Rh6 (blue; E, F, G, and H). In whole *salm/salr* mutant eyes (*eyflp*; *Df(2L)32FP5 FRT40A/GMR-hid CL FRT40*A), all rhabdomeres express Rh1 (A) and Rh6 expression is not observed (E). In whole *salm/salr* mutant eyes where *sens* is misexpressed under the control of *Rh1-Gal4*, Rh1 is partially suppressed (arrowheads in B) and Rh6 expression is observed in some PRCs (arrowheads in F). In *sens* null mutant eyes (*eyflp*; $sens^{E1}$ *FRT80B/ubi-GFP M(3)i(55) FRT80B*—clones generated in this manner occupy almost the entire eye due the presence of the *minute* mutation), all rhabdomeres express Rh1 (C) and Rh6 expression is not observed (G). In *sens* null mutant eyes where *salm* is misexpressed under the control of *rh1-Gal4*, Rh1 is partially suppressed (arrowheads in D) and Rh6 expression is observed in some PRCs (arrowheads in H).

necessary for the expression of the larval markers *pros*, *E(spl)mδ0.5*, and *run* (Fig. 2). In addition, misexpression of *sal* during larval stages (Fig. 4) is sufficient to induce ectopic expression of Pros (R7 marker) and suppress BarH1 (R1/R6 marker). These results demonstrate that *sal* is required for R7 differentiation during larval stages. However, the majority of *sal* mutant presumptive R7 cells do not get transformed into the outer PRC subtype during larval stages since the expression of outer PRC markers (Svp, Ro, and BarH1) is not induced (Fig. 2). Moreover, R7 specification is not disrupted in *sal* mutants since R7 still acquires a neuronal fate (expresses Elav), expresses detectable levels of the R7 marker *H214-klg* (Fig. 2), and projects to the medulla (Mollereau et al., 2001). Therefore, we conclude that the requirement for *sal* during R7 differentiation occurs soon after R7 specification in a continuum rather than in two temporally distinct steps as previously suggested (Mollereau et al., 2001).

In R8, *sal* is not required for specification or early differentiation in the larval imaginal disc (Fig. 2) but is necessary for its terminal differentiation during pupation (Figs. 3, 5, and 6). During pupal stages, *sal* is necessary for *sens* expression in R8 (Fig. 3) and is sufficient to induce ectopic *sens* in R1–R6 (Fig. 5). Misexpression of *salm*, *salr*, or *sens* is sufficient to induce ectopic expression of Rh6 and partial suppression of Rh1 in the outer PRCs (Fig. 6). Furthermore, our results place *sens* genetically downstream of *sal* during R8 pupal development and show that the regulation of Rh1 and Rh6 by *sal* can occur both via *sens*-dependent and -independent mechanisms (Fig. 7). These findings raise a number of interesting issues with respect to the differentiation of R7 during larval stages, the terminal differentiation of R8 at pupation, and the role of *sal* and *sens* in these processes.

The role of sal in R7 differentiation

Current models account for three developmental stimuli in R7 specification and differentiation during larval stages: EGFR pathway activation, which is required for neuronal differentiation (Dominguez et al., 1998; Freeman, 1996); Sevenless (Sev) receptor signaling, which is required for R7 fate assumption since in Sev mutants the presumptive R7 is transformed into a nonneural cone cell (Hafen et al., 1987; Tomlinson and Ready, 1986); and Notch signaling, which is also required for R7 fate assumption since loss of Notch function causes the presumptive R7 to be transformed into the R1/R6 subtype (Cooper and Bray, 2000; Tomlinson and Struhl, 2001).

We have determined that in *salm/salr* mutant tissue, the presumptive R7 becomes a neuron since it expresses Elav (Fig. 2). This result implies that activation of EGFR and Sev signaling is not significantly affected by the loss of *salm/salr* function, placing *sal* downstream of EGFR and Sev activation during R7 differentiation. We have shown that *sal* is required for activation of the Notch signaling pathway in R7 since expression of *E(spl)mδ0.5* is lost in *salm/salr* mutants (Fig. 2E). However, since expression of *H214-klg* is only partially suppressed in *salm/salr* mutants and BarH1 is ectopically expressed in only 4.8% of the mutant ommatidia, it is possible that some residual Notch signaling is present in *salm/salr* mutant R7 cells. Following Notch loss of function, all presumptive R7 cells that are transformed into the R1/R6 subtype show ectopic BarH1 and complete loss of *H214-klg* expression in larvae (Tomlinson and Struhl, 2001). Thus, in *salm/salr* loss of function, the expression of *E(spl)mδ0.5* is lost in R7 but this is not sufficient to respecify the presumptive R7 into R1/R6 subtype as is observed in Notch loss of function mutants

(Cooper and Bray, 2000; Tomlinson and Struhl, 2001). Only later, during pupal development, does the presumptive R7 mutant for *salm/salr* acquire features of outer PRCs, including large rhabdomere size and expression of *rh1* (Mollereau et al., 2001).

The role of sal and sens in R8 terminal differentiation

Previous studies led to a model for R7 and R8 rhodopsin regulation in the "yellow" and "pale" ommatidial subtypes where the "yellow" subtype (Rh4 in R7 and Rh6 in R8) corresponds to the default state and the "pale" subtype (Rh3 in R7 and Rh5 in R8) corresponds to the acquired state (Chou et al., 1996, 1999; Papatsenko et al., 1997). This model was based on the observation that, in *sev* mutants where R7 is absent, all R8 cells express Rh6, suggesting that communication between an R7 expressing Rh3 and the underlying R8 is responsible for the repression of Rh6 and the induction of Rh5 in R8. Here, we show that although *salm* and *sens* are expressed in all R8 cells, misexpression of these genes in outer PRCs under the control of the *rh1* promoter induces ectopic expression of Rh6 but not Rh5 (Fig. 6). Based on the models described above, our results suggest that *sal* and *sens* regulate the default state of rhodopsin expression in R8 ("yellow" subtype) and that additional factors may be required to repress Rh6 and activate Rh5 expression in the R8 "pale" subtype. Our results allow us to present a model for the regulation of rhodopsin by *sal* and *sens* in R8 during pupal stages (Fig. 8). In this model, *sal* regulates *sens* expression, which in turn suppresses Rh1 and induces Rh6 expression. In addition, *sal* can also regulate Rh1 and Rh6 independently of *sens*, in a direct manner or in conjunction with other target genes.

sal is normally expressed in both R7 and R8, which raises the question as to why *sal* does not also induce Rh6 expression in R7. A possible explanation for the absence of Rh6 in R7 could be the presence of an Rh6 repressor in R7. In accordance with this hypothesis, it has recently been shown that in *pros* mutant adult retinae, Rh5 and Rh6 expression expands to R7 and that *pros* is a direct repressor of *rh5* and *rh6* (Cook et al., 2003). In *pros* mutants, *salm* but not *sens* is expressed in R7 (Cook et al., 2003). These results indicate that in the absence of *pros*, induction of Rh6 expression in R7 occurs independently of *sens*, and that *sal* may be involved in this process. Moreover, in R7 cells mutant for *pros*, since *sal* is not sufficient to induce *sens*, factors other than *pros* should repress *sens* expression in R7.

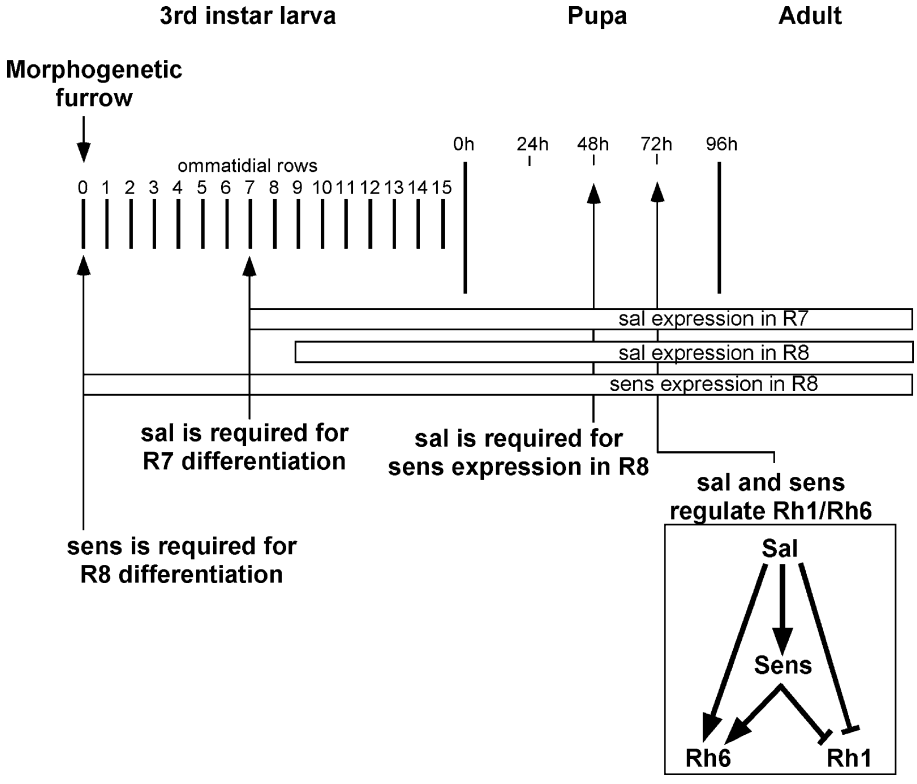

Fig. 8. Timeline for the requirement of *sal* and *sens* during R7 and R8 differentiation. During larval stages, *sal* expression in R7 starts 7 rows after the morphogenetic furrow where it is required for R7 differentiation. *sal* expression in R8 starts nine rows after the morphogenetic furrow but is not required for R8 differentiation in the larval eye. From 48 h pupation onwards, *sal* is required for *sens* expression in R8. Around 72 h pupation, *sal* represses *rh1* and activates *rh6* expression both via *sens*-dependent and -independent mechanisms. *sens* expression in R8 starts in the morphogenetic furrow and is maintained through adulthood. In addition to the previously described role of *sens* in early R8 differentiation (Frankfort et al., 2001), *sens* regulates *rh1* and *rh6* expression during R8 terminal differentiation.

Alternatively, cofactor(s) required for *sens* induction by *sal* in R1–R6 may be absent in R7. Further investigations are necessary to validate these hypotheses and to determine if the regulation of *rh1* and *rh6* by *sal* and *sens* occurs in a direct or indirect manner.

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