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Senseless functions as a molecular switch for color photoreceptor differentiation in *Drosophila*

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A major question in development is how different specialized cell types arise from a common progenitor. In the adult *Drosophila* compound eye, color discrimination is achieved by UV-, blue- and green-sensitive photoreceptors (PRs). These different PR subsets arise from neuronal precursors called R7 and R8 cells. Recent studies have demonstrated that R7-based UV-sensitive PRs require the repression of R8-based blue/green-sensitive PR characteristics to properly develop. This repression is mediated by the transcription factor Prospero (Pros). Here, we report that Senseless (Sens), a *Drosophila* ortholog of the vertebrate Gfi1 transcription factor, plays an opposing role to Pros by both negatively regulating R7-based features and positively enforcing R8-based features during terminal differentiation. In addition, we demonstrate that Pros and Sens function together with the transcription factor Orthodenticle (Otd) to oppositely regulate R7 and R8 PR Rhodopsin gene expression in vitro. These data show that *sens*, previously shown to be essential for neuronal specification, also controls differentiation of specific neuronal subtypes in the retina. Interestingly, Pros has recently been shown to function as a tumor suppressor, whereas Gfi1 is a well-characterized oncogene. Thus, we propose that *sens*/*pros* antagonism is important for regulating many biological processes.

KEY WORDS: Prox1, Gfi1, Otx2, Opsin, Cell-specific gene expression, Photoreceptor cell

INTRODUCTION

Drosophila photoreceptor cells (PRs) constitute a unique system for studying how neuronal diversity is achieved during sensory system development. The fly compound eye comprises ~750 individual eye units, or ommatidia. Initial ommatidial formation requires conserved Notch and bHLH-dependent events important for the development of many sensory organs (Bertrand et al., 2002; Frankfort and Mardon, 2002; Lai and Orgogozo, 2004; Pi and Chien, 2007). The first ommatidial cell to undergo neurogenesis is called the presumptive R8 cell, the specification of which during late larval development requires expression of the bHLH transcription factor Atonal (Ato) and the zinc-finger transcription factor Senseless (Sens) (Frankfort and Mardon, 2002; Frankfort et al., 2001; Jarman et al., 1994; Lage et al., 1997; Nolo et al., 2000; White and Jarman, 2000). Next, seven additional PR neurons (R1-R7) and accessory cells are recruited to each ommatidium through a reiterative cascade of EGF and Notch signaling (see Doroquez and Rebay, 2006). During late pupal development, all PRs complete differentiation, generating several subtypes required for shape, motion, color and polarized light perception (Fig. 1A-C) (Cook and Desplan, 2001; Wernet and Desplan, 2004). Although the events that initiate the recruitment of the R1-R8 precursors have been much studied, far less is known regarding how these precursors later diversify into specialized sensory neurons in the adult eye.

During terminal differentiation, functionally distinct PRs develop different morphologies, locations within the retina and photopigment (opsin) expression (Cook and Desplan, 2001; Hardie, 1985). R1-R6-derived cells form adult 'outer PRs' (OPRs), which function much like vertebrate rod PRs for image formation, motion detection and vision under dim light conditions. OPRs express the broad wavelength-sensitive Rhodopsin (Rh) protein, Rh1 (also known as NinaE – FlyBase), develop rhabdomeres (apical light-gathering surfaces) that extend the full depth of the retina, and form a trapezoidal array within each ommatidium (Fig. 1A,C). R7- and R8-derived cells, called 'inner PRs' (IPRs), are genetically distinguishable from OPRs (Mollereau et al., 2001) and, like vertebrate cone PRs, they discriminate color (Cook and Desplan, 2001; Hardie, 1985). In the adult retina, R7s sit atop R8s in the center of the OPR trapezoid (Fig. 1A) and they differentiate into distinct cell populations: mature R7s express one of two different UV-sensitive opsins, Rh3 or Rh4, whereas R8s primarily express blue (Rh5) or green (Rh6)-sensitive opsins (Fig. 1C) (Chou et al., 1996; Huber et al., 1997; Montell et al., 1987; Papatsenko et al., 1997).

The opsins expressed within the inner PRs define three different subtypes of adult ommatidia: pale (p), yellow (y) and dorsal rim area (DRA) (see Wernet and Desplan, 2004). p ommatidia couple Rh3 and Rh5 expression in the R7 and R8, respectively, whereas y ommatidia couple Rh4 and Rh6 expression (Fig. 1C) (Chou et al., 1996; Chou et al., 1999; Papatsenko et al., 1997). p/y subsets comprise the majority of ommatidia and are randomly distributed throughout the retina in a 30:70 (p:y) ratio (Bell et al., 2007; Kirschfeld and Franceschini, 1977; Stark and Thomas, 2004). DRA ommatidia are two specialized rows of ommatidia that express the same opsin, Rh3, in both R7 and R8 PRs, form distinct polarizing rhabdomeres, and interpret the e-vector of polarized light (Fig. 1B,C) (Fortini and Rubin, 1990; Labhart and Meyer, 1999; Wernet et al., 2003). Thus, at least six different adult IPRs exist: R7 and R8 cells of the p, y and DRA subtypes.

IPRs arise through continual restriction in cell fate (for reviews, see Bateman and McNeill, 2005; Freeman, 2005; Mollereau and Domingos, 2005; Wernet et al., 2006). The first restriction involves members of the Spalt (Sal) family of transcription factor-encoding genes, *salm* and *salr* (Mollereau et al., 2001). After PR recruitment is complete during larval development, Sal genes are specifically expressed in R7 and R8 precursors (Domingos et al., 2004). This expression is essential for IPR differentiation as loss of Sal gene function causes R7 and R8 precursors to develop as OPRs

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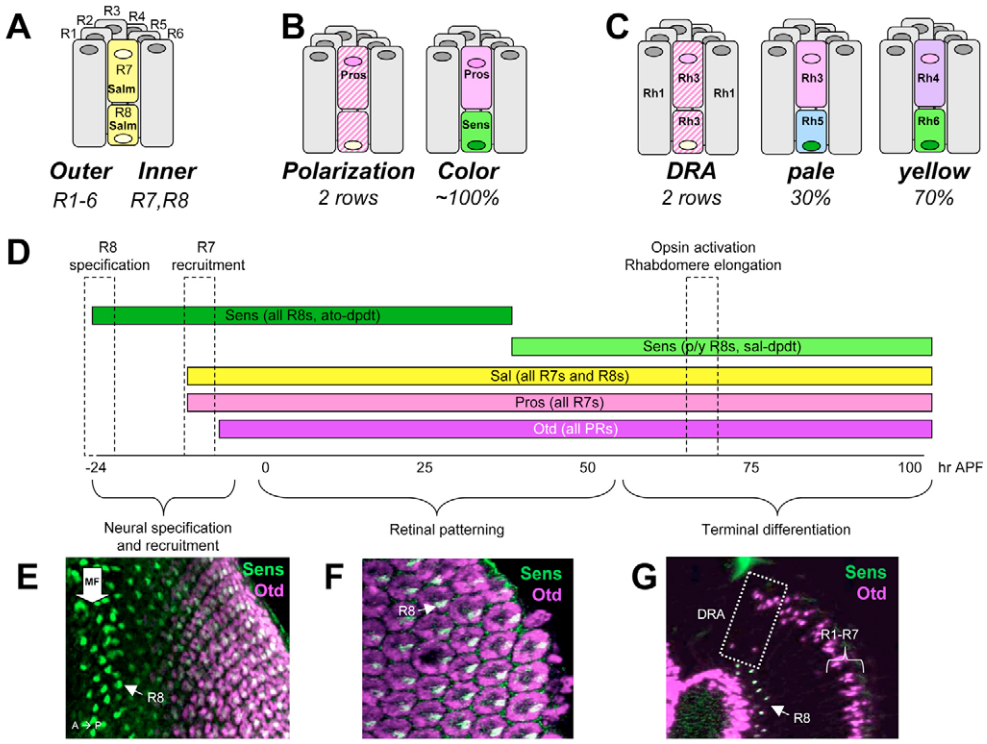

Fig. 1. Inner photoreceptors exhibit differential gene expression during *Drosophila* eye development. (**A-C**) Adult photoreceptor (PR) subtypes and relevant cell-specific factors. (A) Inner photoreceptor cells (IPRs) versus outer photoreceptor cells (OPRs): R7 and R8 IPRs express Salm and are surrounded by R1-R6 OPRs. (B) Polarization versus color-sensitive IPRs: all R7s express Pros (pink nucleus), whereas only pale/yellow R8 PRs express Sens (green nucleus). (C) Ommatidial subtypes: DRA ommatidia (two dorsal rows) express Rh3 in both R7 and R8, 30% ommatidia (termed pale) express Rh3 and Rh5 in R7 and R8, respectively, and 70% ommatidia (termed yellow) express Rh4 and Rh6 in R7 and R8, respectively. (**D**) Transcription factor expression timeline during PR development. From R8 specification at the morphogenetic furrow (MF) onward, Sens expression is R8-specific. Early *sens* expression is *ato*-dependent, whereas later expression is Sal gene-dependent. R7 recruitment occurs more posteriorly than R8 specification and coincides with Pros and Salm expression (Domingos et al., 2004; Kauffmann et al., 1996). Otd expression begins at the end of PR recruitment and is expressed in all PRs throughout development (Vandendries et al., 1996). (**E-G**) Larval (E), 50% pupal (F) and adult (G) eye co-staining with Otd (purple) and Sens (green). E is oriented anterior to posterior, F is oriented dorsal right and includes the DRA (not indicated), and G is oriented dorsal up, distal right, with the DRA boxed. From R8 specification at the MF onward, Sens expression is R8-specific. Sens is expressed in all R8 cells during larval and ~50% pupation (E,F), but is absent from adult DRA (G). It should be noted that Sens and Hth are coexpressed in DRA R8 cells from ~15-50% pupation (data not shown; and M. Wernet, personal communication) (Wernet et al., 2003).

(Mollereau et al., 2001). Downstream of the Sal genes, another transcription factor-encoding gene, *prospero* (*pros*), is selectively activated in R7 precursors. *pros* functions in R7s to block R8 characteristics such as blue/green-opsin expression and nuclear polarity, subsequently allowing UV-sensitive PR differentiation (Cook et al., 2003). Thus, in the absence of *pros*, both Sal-positive PRs differentiate molecularly and morphologically as R8-related PRs in the adult retina.

Based on the ability of *pros* to suppress R8-related characteristics in R7 IPRs, we asked whether similar restrictive processes also occur during R8 IPR differentiation or whether R8s represent the ground state for color-sensitive PRs. A good candidate to positively regulate R8 terminal differentiation is the transcription factor Senseless (Sens) (Frankfort et al., 2001; Jafar-Nejad et al., 2003; Nolo et al., 2000; Quan et al., 2004). In the developing *Drosophila* peripheral nervous system (PNS), Sens both positively and negatively feeds back on proneural bHLH gene expression to enhance the selection of single sensory organ precursors (SOPs) (Acar et al., 2006; Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006; Nolo et al., 2000). Similarly, Sens is required for the specification of the eye SOP-like cell, the R8 precursor (Frankfort and Mardon, 2002). Moreover, increasing evidence suggests that *sens* might participate downstream of neural selection to specify different neuronal subtypes (Domingos et al., 2004; Jafar-Nejad et al., 2006; Wallis et al., 2003). Unfortunately, owing to *sens* early requirements in neural selection and in inhibiting apoptosis, such roles have been difficult to analyze. In the eye, *sens* is expressed throughout R8 development and undergoes two stages of regulation (Cook et al., 2003; Domingos et al., 2004; Frankfort et al., 2001; Frankfort et al., 2004) (Fig. 1D). During R8 specification, *sens* is activated by the *ato* proneural gene (Frankfort et al., 2001); later, *sens* expression depends on the Sal genes. Since Sal genes are required for IPR formation (Domingos et al., 2004), these data suggest that *sens* plays two separable roles in R8 development: specification and differentiation. This latter function has been difficult to assess because removal of *sens* during neuronal recruitment transforms the pre-R8 cell into an R2/R5 OPR (Frankfort and Mardon, 2004; Frankfort et al., 2001). Also, because R7 cell recruitment requires an R8-specific signal, Boss, this loss of R8 identity causes additional non-cell-autonomous failure in R7 recruitment (Frankfort et al., 2001). Here, we rescue the early *sens* loss-of-function (LOF) phenotype and show that IPR development can be restored. LOF and gain-of-function (GOF) experiments in maturing PRs reveal that *sens* is both necessary and sufficient to induce R8-like characteristics and repress R7-related features in terminally differentiating IPRs. Moreover, *sens* partially recapitulates this process in vitro by differentially regulating R7 versus R8 opsin gene expression. These data reveal that IPRs require the opposing actions of *sens* and *pros* to form functionally distinct color-sensitive PRs. Because *pros* and *sens* are expressed in similar, yet distinct, cell types in many developing tissues, we propose that comparable antagonistic *pros/sens*-dependent regulation helps to create cellular diversity in many developmental contexts.

MATERIALS AND METHODS

Fly genetics

The following strains were used: *ey^{flp}*; GMRHid, *cl3L*, FRT79D/TM6B; *yw*; *sens^{E1}*-FRT79D/TM6B, *sens^{E2}*-FRT79D/TM6B (G. Mardon, Baylor College of Medicine, Houston, TX), *yw*; UAS-*sens* (C1) (H. Bellen, HHMI, Baylor College of Medicine, Houston, TX), *w*; *sca$^{109.68}$*-GAL4/CyO (J. Treisman, NYU Skirball Institute, New York, NY), pWIZ-wΔ13 (a white gene RNAi line) (R. Carthew, Northwestern University, Evanston, IL), *Rh6Δseq56B*-GAL4 (Cook et al., 2003). A mutant *Rh6* allele, *Rh6$^{[1]}$*, is present on both FRT79D-*sens^{E1}* and *sens^{E2}* chromosomes (data not shown). Thus, a wild-type *Rh6*, derived from Bloomington Stock #93, was recombined onto the FRT79D-*sens^{E2}* chromosome similarly to previously described (Cook et al., 2003). Two lines were maintained, *sens$^{E2.1}$* and *sens$^{E2.3}$*. Both behaved identically to *sens^{E2}*. *sens$^{E2.1}$* was used for all experiments reported here. For *sens* full LOF experiments, *ey^{flp}*; Sp/CyO; GMRHid, *cl3L*, FRT79D/TM6B flies were crossed with *yw^{67}*; Sp/CyO; *sens$^{E2.1}$* (or *sens^{E1}*)-FRT79D/TM6B. *ey^{flp}*; Sp/CyO; GMRHid, *cl3L*, FRT79D/*sens$^{E2.1}$*-FRT79D and *ey^{flp}*; Sp/CyO; *sens$^{E2.1}$*-FRT79D/TM6B were used for LOF and control sections, respectively. *sens* lateLOF eyes were generated from straight-winged offspring from the cross: *ey^{flp}*; UAS-*sens*/CyO; GMRHid, *cl3L*, FRT79D/TM6B × pWIZ-wΔ13; *sca$^{109.68}$*-GAL4/CyO; *sens$^{E2.1}$*, FRT79D/TM6B.

Antibody production

Full-length *sens* coding sequence, kindly provided by H. Bellen, was cloned into pET28b and transformed into BL21-CodonPlus (DE3)-RP (Stratagene). Protein expression was induced with 0.1 mM IPTG for 4 hours. Cells were lysed for 2 hours at room temperature (RT) in 8 M urea lysis buffer (ULB: 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8.0, 10 mM imidazole, 8 M urea, 10 mM β-mercaptoethanol, 0.5% NP40), centrifuged 30 minutes at 16,000 ***g***, and the supernatant mixed with Ni-NTA beads (Qiagen) for 4 hours at RT. Beads were washed five times with ULB + 250 mM NaCl, and protein was eluted with ULB + 300 mM imidazole. This was used to immunize rats (Cocalico Biologicals). Rt8 serum was pre-absorbed with 0- to 5-hour *Drosophila* embryos. Anti-Salm and anti-Otd polyclonal antibodies were generated using the same approach as above. Salm aa410-916 were used as antigen in rabbits, whereas full-length Otd protein was used to immunize guinea pigs. Antibody specificity was tested in the appropriate mutant backgrounds.

Immunofluorescence, plastic sectioning and imaging

For cryosections, fly heads were embedded and frozen in OCT, sectioned (10 μm), processed and stained as previously described (Cook et al., 2003). For plastic sections, retinas were dissected in PBT (PBS + 0.1% Triton X-100, pH 7.2), fixed in 2% glutaraldehyde in 0.2 M PBS and post-fixed in 2% OsO_4 in PBS. Tissue was serially dehydrated with ethanol and washed twice, 10 minutes each, with propylene oxide (Ted Pella). 1:1 propylene oxide:Durcapan resin (Sigma) was applied overnight, and replaced with pure Durcapan for 4 hours at RT. Resin-embedded retinas were transferred to plastic molds, baked 70°C overnight and then sectioned on a Reichert OmU3 ultramicrotome. Sections (1 μm) were stained with 1% toluidine blue/borax for 10 minutes, mounted in 50:50 PBS:glycerol and imaged. Whole-mounted retinas were dissected at RT in PBT, fixed in PLP (PBS, 4% paraformaldehyde, 0.075 M lysine, 0.01 M sodium periodate, 0.05% saponin) (McLean and Nakane, 1974), and washed three times, 10 minutes each, with PBT. Retinas were transferred to Signal-iT FX (Invitrogen) for 30 minutes at RT, before incubation with primary antibodies overnight at 4°C in BNTS (PBT, 1.5 M NaCl, 0.1% BSA, 0.05% saponin). Samples were washed three times, 20 minutes each, with PBT, and incubated 90 minutes at RT with secondary antibodies diluted in BNTS and, when used, Alexa Fluor 488-conjugated phalloidin (1:40) (Invitrogen). After washing three times, 20 minutes each, with PBT, retinas were mounted in Prolong Gold antifade-reagent (Invitrogen) and imaged 24 hours later. Antibody dilutions were: guinea pig anti-Sens (1:800; H. Bellen) and Otd (1:750); rat anti-Sens (1:100) and Elav (1:200; DHSB); mouse anti-Pros (1:10; DHSB), Rh3 (1:10; S. Britt, University of Colorado, Aurora, CO) and Rh5 (1:1000; S. Britt); rabbit anti-Salm (1:500; B. Mollereau, Ecole Normale Supérieure, Lyon, France) or Salm (as described above, 1:150), Rh4 (1:150; C. Zuker, University of California San Diego, La Jolla, CA), Rh6 [1:2000; C. Desplan (Tahayato et al., 2003)], GFP (1:500; Abcam) and β-gal (1:1000; Cappel); chicken anti-Rh3 [1:40 (Cook et al., 2003)] and β-gal (1:1000; Abcam). Alexa Fluor 488, 555 and 655-conjugated secondary antibodies (1:1500; Invitrogen) were used. Digital images were obtained with the Apotome deconvolution system (Zeiss) and processed with Axiovision 4.5 (Zeiss) and Adobe Photoshop 7.0 software.

In vitro reporter assays

Luciferase reporter constructs were generated by subcloning minimal promoters for *Rh3* (–247 to +18), *Rh4* (–159 to +85), *Rh5* (–236 to +50) and *Rh6* (–555 to +121) (Cook et al., 2003; Papatsenko et al., 2001; Tahayato et al., 2003) into promoterless pGL3 (Promega). Full-length *otd* (E. Wimmer, University of Göttingen, Germany), *prosS* [M. Mortin (NICHD/NIH, Bethesda, MD) and C. Doe (University of Oregon, Eugene, OR)] and *sens* (H. Bellen) cDNAs were subcloned into pAc5.1 (Invitrogen) (details available upon request). pAc-LacZ (J. Culi and R. Mann, Columbia University, New York, NY) was used for transfection controls. *Drosophila* S2 cells (Invitrogen) were maintained in HyQ SFX-Insect media (Hyclone) at RT. 1×10^6 cells were plated in 6-well tissue culture dishes (Corning) 48 hours prior to transfection with 3 μL Fugene HD (Roche) and 250 ng pGL3 reporter, 250 ng pAc-LacZ and 500 ng total pAc-expressing vectors (250 ng of any one transcription factor). 48 hours post-transfection, cells were lysed in 70 μL Passive Lysis Buffer (Promega). Luciferase activity was measured using Luciferase Assay Reagent (Promega) and a Veritas Microplate luminometer (Turner Biosystems). β-galactosidase activity was measured with ONPG substrate using a μQuant Microplate spectrophotometer (Bio-Tek). Luciferase values were normalized to β-galactosidase activity, Rh-specific activity was normalized to the pGL3 control, and factor-specific activity was normalized with the pAc control. Samples were transfected in triplicate for each experiment, and each experiment was performed at least three independent times. Data from single representative experiments are shown. Statistical analysis was performed using SPSS.

Electromobility gel shift assays (EMSAs)

An *Eco*RI fragment encoding the four zinc fingers of Sens (amino acids 348-541; sensZF) was subcloned into pET-28a (Novagen) and transformed into BL21-CodonPlus-RP cells (Stratagene). Protein induction with 0.1 mM IPTG was performed overnight at 16°C. Protein purification and EMSAs were performed as previously described (Gebelein et al., 2004). *Rh3*, *Rh4*, *Rh5* and *Rh6* promoters were PCR-amplified from pGL3 reporters (above), gel purified (Qiagen) and end-labeled with [γ-^{32}P]ATP. Purified His-SensZF protein (50 or 500 ng) and approximately 30 ng probe was used for EMSAs.

Sens binding site mutagenesis and in vivo *lacZ* reporter assays

The core AATC Sens-binding sequence was mutated to GGTC within the *Rh3* and *Rh4* promoters by PCR (details available upon request). Sens failed to bind to these sites in vitro (data not shown). Mutant promoters were subcloned into pGL3 or pCHABΔSal (Wimmer et al., 1997). pCHAB reporter constructs were injected into *yw^{67}* flies, and at least three independent insertions were tested for expression. X-Gal staining was performed as previously described (Tahayato et al., 2003).

RESULTS

Transient *sens* expression rescues inner photoreceptor development

To determine if *sens* participates in late aspects of R8 cell differentiation, we first investigated whether transient *sens* expression during early R8 selection could rescue R8 specification and subsequent R7 recruitment. *scabrous* (*sca*) lies upstream of *sens* during SOP development and is expressed transiently in R8 precursors (Lebestky et al., 2000; Maurel-Zaffran et al., 2001). We observed *sca$^{109.68}$*-GAL4-dependent expression of a nuclear-localized GFP reporter (*sca>nGFP*) in R8s from early specification through ~55% pupation; however, by 70% pupation, when PRs begin to terminally differentiate (Earl and Britt, 2006; Kumar and

Ready, 1995), reporter expression was no longer observed (Fig. 2A). Reporter expression was also absent in adult PRs (data not shown). Thus, we tested whether, in an eye-specific *sens* mutant background (see Materials and methods), *sca*$^{109.68}$-GAL4 driving UAS-*sens* expression (*sca>sens*) during PR recruitment would rescue inner PR specification and allow subsequent *sens*-negative IPRs to terminally differentiate.

To analyze IPR formation, we monitored Spalt major (Salm) protein expression in adult retinas. In wild-type or *sens* heterozygous control eyes, two rows of Salm-positive nuclei were observed: R8 nuclei at the base (the proximal region) of the retina, and R7 nuclei, positioned distally, slightly below OPR nuclei (Fig. 1A; Fig. 2C,F). As previously reported, full *sens* LOF eyes largely fail to develop IPRs (Frankfort and Mardon, 2004; Frankfort et al., 2001). Occasional Salm-positive cells were observed and these primarily expressed the R7-specific marker Pros (Fig. 2D,G,G′, arrowheads), suggesting that few *sens* mutant R8 cells persist long enough to allow R7 recruitment before being transformed into an OPR. In contrast to *sens* full LOF eyes, we observed two discrete layers of Salm-positive nuclei in *sens* mutants rescued with *sca>sens* (Fig. 2E,H) (henceforth called *sens* late LOF). Pros is expressed in all distal-most Salm-positive nuclei, whereas the proximal Salm-positive nuclei lack both Pros and Sens expression. These findings indicate that transient *sens* expression is able to restore IPR development.

Sens is necessary for proper R8 terminal differentiation

To address whether *sens* functions in R8 terminal differentiation, we analyzed three IPR-specific features: Rhodopsin gene expression, intracellular nuclear polarity and rhabdomere position (see Fig. 1C). Wild-type R7s express Rh3 and Rh4, have distal nuclei, and rhabdomeres that lie within the distal part of the retina (Fig. 3A-B,G,I). By contrast, wild-type R8s primarily express Rh5 and Rh6, have proximal nuclei, and have rhabdomeres that occupy the proximal third of the retina directly beneath the R7 rhabdomere (Fig. 3B,C,G,I). In *sens* late LOF eyes, the distal-most IPRs maintained all three characteristics of 'R7-ness' (Fig. 3D,F,H,J), consistent with their maintained Pros expression (Fig. 2H′). However, numerous features within the R8 layer differed from that of wild-type eyes. First, the numbers of PRs expressing R8 opsins were significantly reduced, with only occasional Rh5- or Rh6-positive cells observed (compare Fig. 3B,C with E,F). Instead, the R7 opsin, Rh3, was expressed in the majority of cells within the proximal 'R8' layer (Fig. 3D,E,H,J). Rh4 was also occasionally observed proximally (Fig. 3D,H, arrowheads). Second, the R8 nuclear layer largely localized at the center of the retina (compare Fig. 2C with E, Fig. 3I with J), rather than at the base of the retina. Finally, the R8 rhabdomeres no longer underlay R7 rhabdomeres, but instead inappropriately extended into the R7 layer (Fig. 3H). Thin plastic sections of these eyes confirmed the presence of two narrow rhabdomeres in the center of many ommatidia (characteristic of IPRs) at the plane of the R7 layer (Fig. 3L), whereas in wild-type retina, only one IPR rhabdomere was present (Fig. 3K). Whole-mount retinal staining also revealed that Rh3 and Rh4 were restricted to the single R7 cell in control eyes (Fig. 3M), but were present in both the R7 and R8 cells within individual *sens* late LOF ommatidia (Fig. 3N). Together, these data suggest that *sens* mutant R8s share aspects of both R7 and R8 PRs: the cells reside at the R7 layer of the retina, lose R8-based opsins and acquire R7 opsin expression, yet maintain an R8-specific proximal nuclear position.

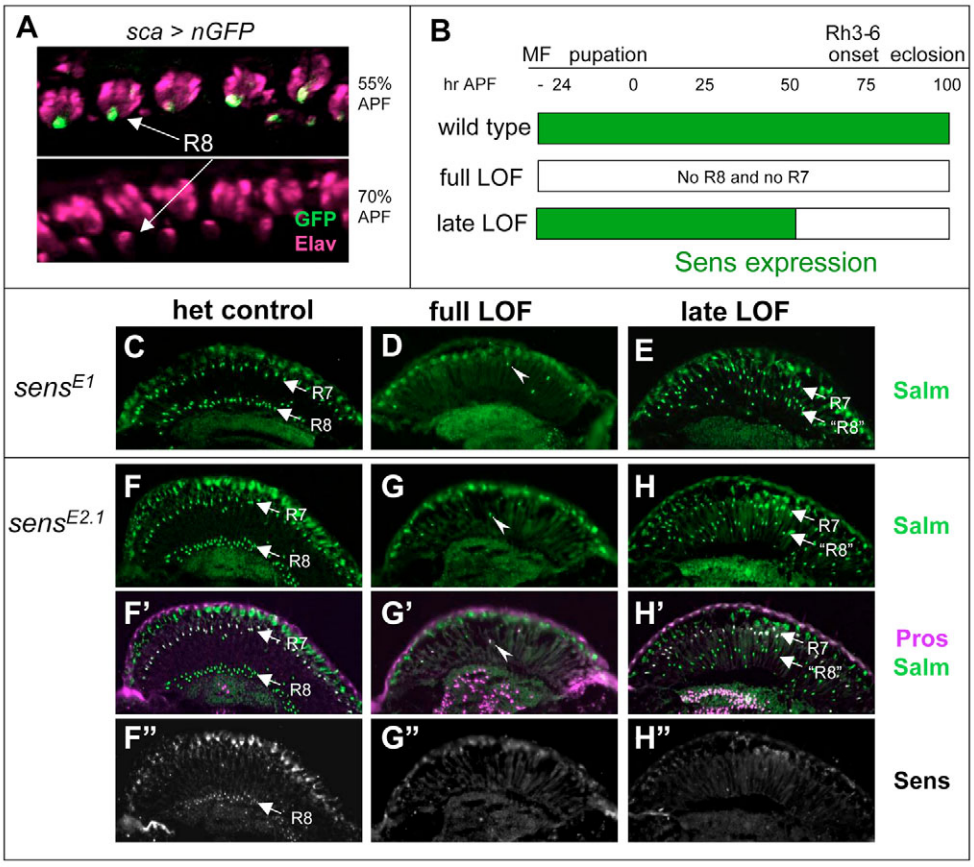

Fig. 2. Transient *sens* expression rescues inner photoreceptor specification. (**A**) Coronal section of 55% pupal and 70% pupal heads from *sca*$^{109.68}$-GAL4, UAS-nuclear GFP (*sca>nGFP*) *Drosophila*. All PRs are marked with Elav (purple). GFP (green) is present in R8 cells at 55% APF, but not after 70% APF. (**B**) Summary of *sens* expression in full LOF and late LOF experiments. (**C-E**) Adult retinal sections stained for Salm (green); dorsal left, distal up. (C) UAS-*sens*/+, *sens*^{E1}/TM6B: the R7 and R8 layers of Salm-positive nuclei are labeled. (D) *sens*^{E1} full LOF eyes (see Materials and methods) rarely develop IPRs (arrowhead). (E) *sens*^{E1} mutant eyes with *sca*$^{109.68}$*>sens* (*sens* late LOF) develop two Salm-positive layers of IPRs (arrows). (**F-H″**) Adult cryosections of control and *sens*$^{E2.1}$ late LOF retinas stained for Salm (green, F-H,F′-H′), Pros (purple, F′-H′), or Sens (white, F″-H″). F-F″, G-G″, and H-H″ represent eyes co-stained for all three factors. Pros stains distal Salm-positive cells in both control and *sens*$^{E2.1}$ late LOF retinas, and the majority of Salm-positive cells in *sens*$^{E2.1}$ full LOF retinas. Sens is expressed in R8 cells only in control retinas (F″).

Sens is sufficient to repress R7 features and activate R8 features in adult inner PRs

We next hypothesized that Sens misexpression in terminally differentiating R7s would promote R8-based characteristics. Previous studies have demonstrated that *sens* misexpression during early neuronal recruitment is sufficient to convert non-R8 precursors into R8 cells (Frankfort et al., 2001), consistent with its requirement in R8 specification. Not surprisingly, misexpression of *sens* using an early panPR GAL4 driver, GMR-GAL4, leads to a severely disrupted retina that primarily expresses the R8 opsin, *Rh6* (B.X. and T.C., unpublished; M. Wernet, personal communication). Misexpression of *sens* in maturing OPRs, by contrast, can only weakly activate *Rh6* (Domingos et al., 2004) while other aspects of OPR differentiation appear unaffected, even with *salm* co-expression (B.X., unpublished). This suggests that differentiated OPRs are no longer sensitive to *sens*-dependent R8 transformation. To specifically address whether *sens* affects IPR terminal differentiation, we expressed *sens* in R7 and R8 cells during late pupation. Currently, the only GAL4 drivers restricted in expression to maturing inner PRs involve Rhodopsin promoter/GAL4 fusions. Hypothesizing that *sens* might repress R7-specific Rh genes, we chose to misexpress *sens* using a modified *Rh6*-based driver that is expressed in R7 and R8 cells during their terminal differentiation (Cook et al., 2003) (Fig. 4A). Henceforth, this driver will be called '*i*nner *p*hotoreceptor' IP-GAL4. Note that IP-GAL4 drives high levels of gene expression in all dorsal R7s but not in all ventral R7s (Fig. 4A), allowing us to compare wild-type and *sens*-misexpressing cells in the same retina.

We examined multiple aspects of R7 versus R8 differentiation in *IP>sens* (*sens* GOF) eyes. As shown in Fig. 4, R7s inappropriately expressing high levels of Sens (i.e. dorsal R7s) expressed high levels of the R8 opsin, Rh6, and lost the R7 opsins, Rh3 and Rh4 (compare Fig. 4B-D with F-H). The other R8 opsin, Rh5, was also weakly detected in some Sens-expressing R7 cells (Fig. 4H, arrows). Occasional weak co-expression of Rh3 and Rh6 in R7 cells was also observed, suggesting a partial transformation (data not shown). In addition to opsin changes, we detected a distal-to-proximal change in the nuclear position of many *sens*-misexpressing R7s (compare Fig. 4I with E,J-L). Since R8s are the only cells within the eye with a proximal nucleus, this indicates that R7s misexpressing *sens* acquire an R8-specific nuclear polarity. Finally, expression of R7-specific Pros was significantly reduced in *sens*-misexpressing R7s

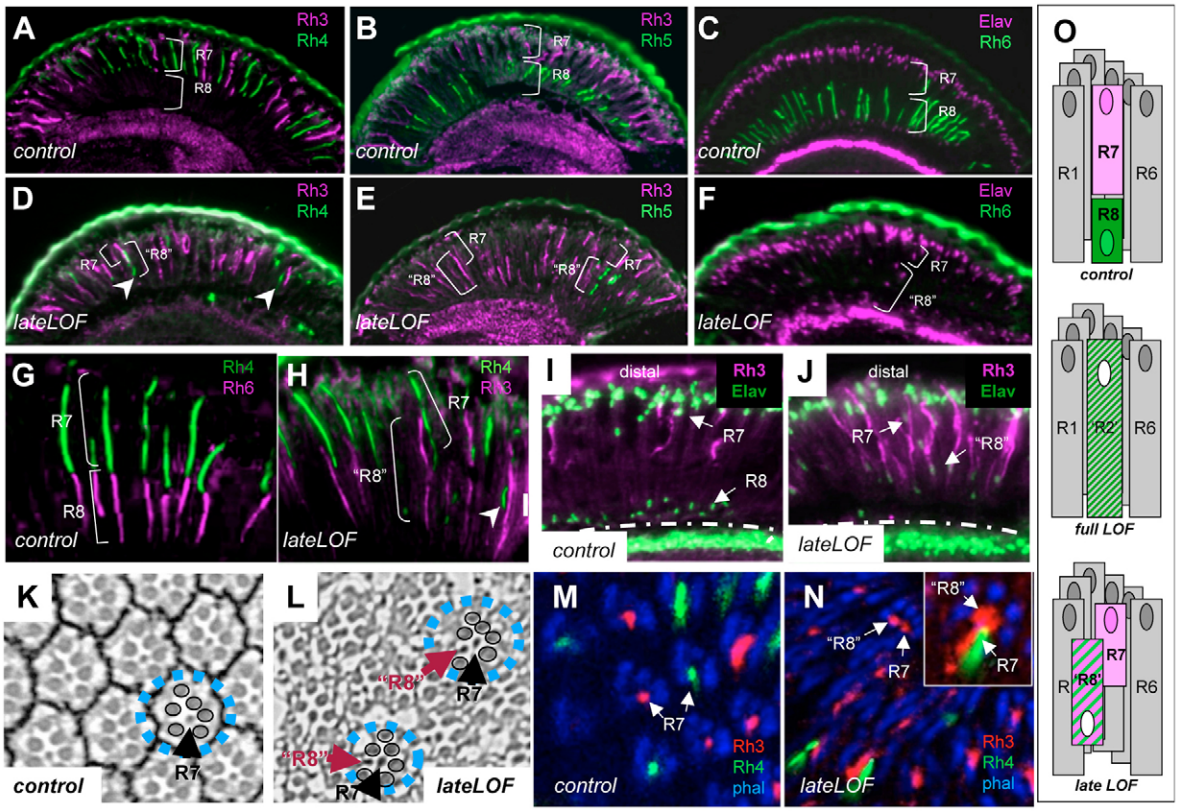

Fig. 3. Sens is required for inner photoreceptor differentiation. Adult retinas from wild-type (**A-C**,**G**,**I**,**K**,**M**) or $sens^{E2.1}$ late LOF (**D-F**,**H**,**J**,**L**,**N**) *Drosophila*. (A-J) Cryosections (10 μm); dorsal left, distal up. (K,L) Plastic sections (1 μm). (M,N) Optical sections from retinal whole-mounts. Samples were stained for Rh3 (purple, A,B,D,E,H-J; red, M,N), Rh4 (green, A,D,G,H,M,N), Rh5 (green, B,E), Rh6 (green, C,F; purple, G), Elav (purple, C,F; green, I,J), or the actin/rhabdomere marker, phalloidin (blue, M,N). R7 and R8 layers of WT rhabdomeres are clearly separated within the retina (A-C,G,I), but are ambiguously positioned in *sens* late LOF eyes (D,E,H,J). Wild-type control R8 nuclei (I, Elav, green) lie along the base of the retina (dotted white line), whereas *sens* late LOF R8 nuclei (F,J) are often located midway in the retina (purple, F or green, J). Wild-type control plastic sections (K) show a single small IPR rhabdomere (arrow); OPR rhabdomeres are circled in black; single ommatidia are outlined with a dotted blue line. In *sens* late LOF eyes (L), two small rhabdomeres are observed in the R7 and R8 positions (arrows). Rh3 and Rh4 staining reveals a single opsin-expressing cell (the R7) in wild-type ommatidia (M), whereas two opsin-positive cells (R7 and sens-negative 'R8') are observed in *sens* late LOF ommatidia (N). Phalloidin marks OPR rhabdomeres. Inset in N is higher magnification of a single ommatidium with Rh4 expression in R7, and Rh3 expression in R8. (**O**) Summary of control, full *sens* LOF, or late *sens* LOF phenotypes. 'R2' represents the pre-R8 cell that transforms into an R2/R5 OPR; 'R8' represents *sens*-negative R8s. See Fig. 1 for color code.

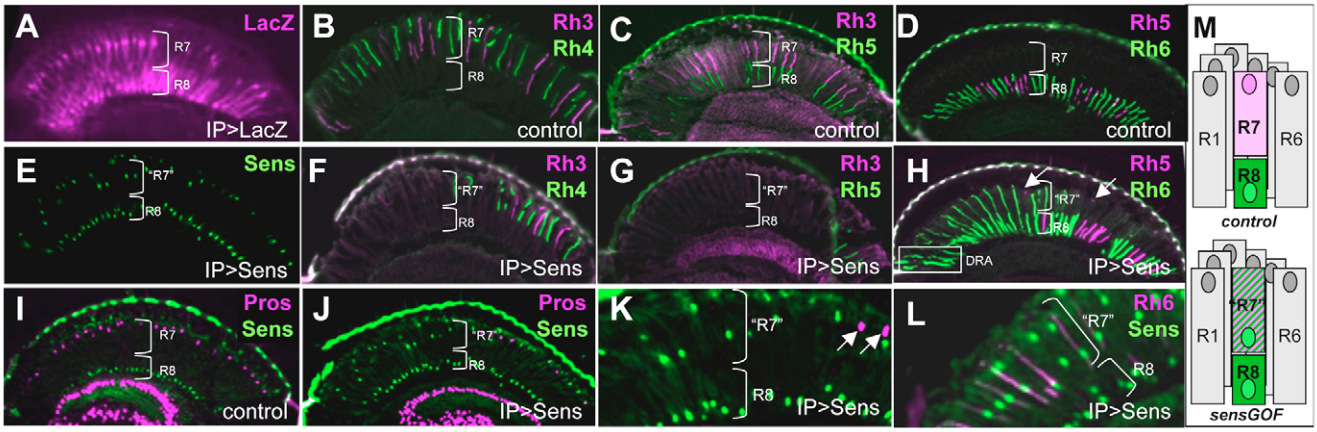

Fig. 4. Sens alone can both repress R7 and activate R8 features. Adult cryosections from control and *sens* GOF *Drosophila*. (**A**) IP-GAL4; UAS-lacZ; (**B**,**D**) *yw*^{67}; UAS-*sens*/CyO; TM2/TM6B; (**C**,**I**) IP-GAL4; Sp/CyO; TM2/TM6B; (**E-G**,**J-L**) IP-GAL4; UAS-*sens* (**H**) IP-GAL4; UAS-*sens*/CyO; TM2/TM6B. All retinas oriented dorsal left, distal up; R7 and R8 layers are bracketed. Sections were stained for β-gal (purple, A), Rh3 (purple, B,C,F,G), Rh4 (green, B,F), Rh5 (green, C,G; purple, D,H), Rh6 (green, D,H; purple, L), Sens (green, E,I-L) and Pros (purple, I-K). Note that many *sens*-misexpressing R7 cells exhibit an R8-like proximal nucleus (E,J-L). (**M**) Summary of control or *sens* GOF phenotypes. 'R7' represents *sens*-misexpressing R7 cells in *sens* GOF eyes.

(Fig. 4I versus J,K), but was maintained in R7s expressing little or no Sens (Fig. 4K, arrows). We noted that Rh5 expression was significantly reduced in *sens* GOF R8s, and this effect was recapitulated when *sens* was misexpressed with an R7-specific driver (data not shown). Since Rh5 expression requires an active signal from Rh3-expressing R7s (Chou et al., 1999; Mikeladze-Dvali et al., 2005; Wernet et al., 2006), we take this as further evidence that R7s misexpressing *sens* lose Rh3 and possibly other important R7-specific features. Each *sens*-dependent change becomes more evident with age (data not shown), indicating that multiple aspects of IPR differentiation remain plastic throughout development. No changes in rhabdomere positioning were observed in *sens* GOF eyes. Together, these data suggest that *sens* is sufficient to mis-specify several aspects of R7 IPR differentiation into R8-related characteristics, including opsin expression, reduction in Pros expression and changes in nuclear position.

Sens is sufficient to repress R7 opsins in vitro

In vivo, *sens* activates R8 opsins and represses R7 opsins. Because Sens is a transcription factor, we tested whether Sens directly regulates Rh gene expression in vitro. First, we analyzed the Rh promoters for the presence of Sens binding sites. Previous work delineated a core binding sequence of AATC for Sens and its vertebrate ortholog, Gfi1 (growth factor independent factor 1) (Nolo et al., 2000; Zweidler-Mckay et al., 1996) (Fig. 5A). Using a position-weighted matrix (PWM) of Sens/Gfi binding sites [(Sandelin et al., 2004) jasper.genereg.net], we found that the optimal Gfi/Sens binding site, R21 (Jafar-Nejad et al., 2003), achieves a score of 12.2, and a bona fide Sens binding site (S-box) within the *achaete* (*ac*) promoter (Jafar-Nejad et al., 2003) scores 9.0. Consistent with the idea that PWM scores correlate with binding affinity, the purified DNA-binding domain of Sens binds with ~10-fold higher affinity to R21 than the S-box sequence in electrophoretic mobility shift assays (EMSAs) (Jafar-Nejad et al., 2003) (B.G., unpublished). Identical results were obtained using purified full-length Sens protein (data not shown).

Using the Sens/Gfi1 PWM, we identified several high-scoring (>8) Sens sites within the *Rh3* and *Rh4* promoters. However, only low-scoring sites (<6.5) were found within the *Rh5* and *Rh6* promoters. We next performed in vitro EMSAs using purified Sens protein and minimal, in vivo functional, Rh promoters (Fortini and Rubin, 1990; Papatsenko et al., 2001; Tahayato et al., 2003). These showed that multiple complexes of Sens formed on the *Rh3* and *Rh4* promoters (Fig. 5B,C), whereas little to no binding was observed on the *Rh5* and *Rh6* promoters (Fig. 6A,B). Individual Sens binding sites from all four promoters were also tested using short oligonucleotide sequences, and only sites from the *Rh3* and *Rh4* promoters showed significant binding (data not shown). One site within the *Rh5* promoter (the d site in Fig. 6A) bound Sens weakly, consistent with the observation of a single shift complex with the full-length *Rh5* promoter (Fig. 6B, arrow). These data suggest that R7 opsin genes are direct *sens* targets, whereas R8 opsin genes are weak or indirect *sens* targets.

We next measured the ability of Sens to regulate Rh promoter-luciferase reporter expression in the non-neuronal *Drosophila* S2 cell line. Sens was sufficient to repress the promoter activity of both R7 opsins, *Rh3* and *Rh4*, in vitro (Fig. 5C); however, we observed no change in R8-based *Rh5* or *Rh6* expression (Fig. 5C). Mutations of site A, the highest scoring site within the *Rh3* and *Rh4* promoters, were sufficient to prevent Sens-dependent repression in vitro (Fig. 5D, rh3ΔA, rh3ΔAC, rh3ΔACD, rh4ΔA, rh4ΔAB). Mutation of the *Rh4* B site also led to a significant loss in repression. To test whether Sens mediates direct *Rh3* and/or *Rh4* transcriptional repression in vivo, we analyzed *Rh3* and *Rh4* Sens-binding-mutant promoter expression in adult eyes. An *Rh3* reporter carrying a promoter that is unresponsive to Sens ex vivo (rh3ΔAC) was expanded into the majority of R8s in vivo (Fig. 5E), whereas an in vitro Sens-responsive rh3ΔC promoter, rh3ΔC, remained restricted to R7 cells (Fig. 5E). We detected few, if any, R8 cells expressing the mutated *Rh4* promoters (Fig. 5F). This is not surprising, as *sens* late LOF R8s predominantly express Rh3 and only a few express Rh4 (Fig. 3D,H). Lack of *Rh4* expansion in R8s is also parsimonious with recent studies indicating that *Rh4* induction requires the yR7-specific factor Spineless (Ss) in vivo, and that without Ss, *Rh3* is expressed in R7s by default (Wernet et al., 2006) (see Discussion). Together, our in vitro and in vivo studies demonstrate that *sens* is important for actively repressing R7 opsins, particularly *Rh3*.

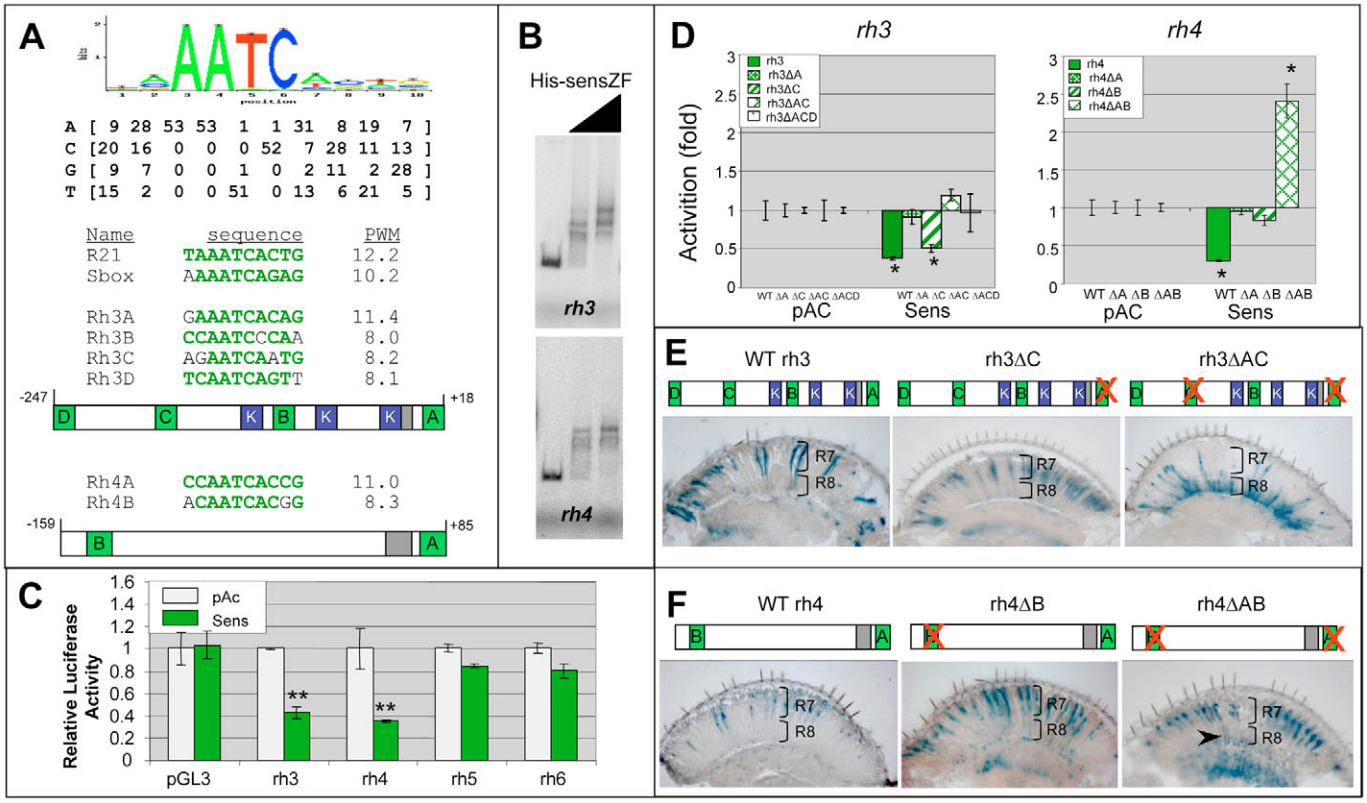

Fig. 5. Sens directly represses *Rh3* and *Rh4* promoter activity. (**A**) Position-weighted matrix (PWM) of Gfi1 binding sites (top), table of two known Sens binding sites, R21 and S-box (Jafar-Nejad et al., 2003), and sites within the *Rh3* and *Rh4* promoters with >70% homology to the consensus. Corresponding PWM scores are listed. Nucleotides that are present >20% in the PWM data set are highlighted green. *Rh3* and *Rh4* promoter diagrams represent the conserved RCSI/Pax6 binding site present in all Rh promoters (Papatsenko et al., 2001; Sheng et al., 1997) (gray), K50 Otd-binding sites (Tahayato et al., 2003) (blue) and potential Sens binding sites (green). (**B**) EMSAs with *Rh3* (–247 to +18) and *Rh4* (–159 to +85) promoters and 0, 50 or 500 ng His-SensZF. (**C**) Relative luciferase activity in *Drosophila* S2 cells transfected with pAc5.1 or pAc-Sens and pGL3 with or without *Rh3* (–247 to +18), *Rh4* (–159 to +85), *Rh5* (–236 to +50) or *Rh6* (–555 to +121) promoters. **, $P<0.01$ compared with pAc alone. (**D**) Relative luciferase activity of *Rh3* or *Rh4*-containing pGL3 reporters with mutated Sens binding sites (AATC core → GGTC). Sites correspond to those in A. *, $P<0.05$ compared with pAc alone. (**E**,**F**) X-Gal staining of cryosections from transgenic *lacZ* reporter lines carrying wild-type or Sens mutant binding sites in the *Rh3* (E) or *Rh4* (F) promoters. R7 and R8 layers are bracketed.

Sens activates R8 opsin promoter expression in an Otd-dependent manner

Although *sens* activates *Rh5* and *Rh6* in vivo, *sens* is not sufficient to regulate these promoters in vitro (Fig. 4H). Two likely possibilities explain this result: (1) *sens* indirectly regulates R8 opsins by affecting cell fate decisions; and/or (2) Sens functions in conjunction with other factors to regulate *Rh5* and *Rh6* gene expression. One candidate for such a factor is the transcription factor Orthodenticle (Otd; also known as Ocelliless – FlyBase). In the adult retina, Otd is expressed in all PRs (Fig. 1G) and regulates the *Rh3*, *Rh5* and *Rh6* promoters by binding K50 sites (Tahayato et al., 2003). Similarly to in vivo (Tahayato et al., 2003), we found that Otd activates *Rh3* and *Rh5*, but not *Rh1* or *Rh4*, in vitro (Fig. 6C and data not shown). We also observed Otd-dependent *Rh6* activation in S2 cells (Fig. 6C), although in vivo, Otd is best characterized for *Rh6* repression in OPRs. *Rh6* activation in vitro required intact K50 sites (Fig. 6E), indicating that this Otd-dependent activation is specific.

To test whether *sens* and *otd* function together to regulate Rh gene expression, we co-expressed these factors in S2 cells. Sens could still repress *Rh3* expression even in the presence of Otd; however, Sens specifically increased Otd-mediated *Rh5* and *Rh6* activation (Fig. 6D). Modest, but significant increases were observed for *Rh5* activation (~0.5X), whereas a strong synergistic effect was observed for *Rh6* activation (50-100X). An Otd binding site mutation within the *Rh6* promoter significantly reduced this Otd/Sens-dependent synergism (Fig. 6E), supporting an essential role for Otd in mediating this activity. Together, these data demonstrate that in vitro, Sens can recapitulate its in vivo ability to regulate R7 and R8 opsins. Repression of R7 opsins involves direct DNA-binding, whereas activation of R8 opsins requires cooperation with Otd. These findings correlate with recent data showing that Sens functions as a site-specific repressor and a DNA-binding-independent co-activator during proneural gene regulation (Acar et al., 2006).

Sens and Pros reciprocally regulate opsin genes in vitro

Combined, the results here and our previous results on R7 differentiation (Cook et al., 2003) suggest that Sal-restricted IPRs can adopt either R7 or R8 characteristics, and that *sens* in R8s, or *pros* in R7s, is important to functionally distinguish these PRs. In vivo, *pros* represses R8 opsins, but does not affect R7-based opsins. To test whether we could recapitulate this regulation in vitro, we also

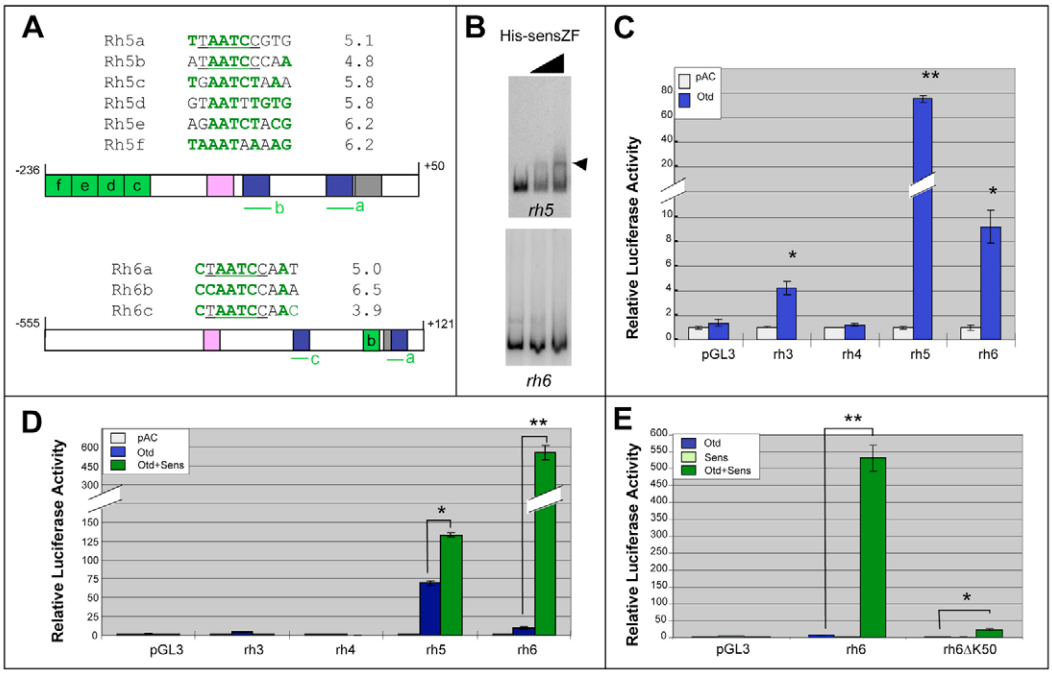

Fig. 6. Sens and Otd regulate R8 opsin genes in vitro. (**A**) Table of potential Sens binding sites in *Rh5* (–239 to +50) and *Rh6* (–555 to +121) promoters. Underlined sequences represent overlapping Otd-binding K50 sites (Tahayato et al., 2003). Promoter diagrams represent binding sites for Otd (blue), Pros (pink) (Cook et al., 2003), or Pax6 (gray) (Papatsenko et al., 2001). (**B**) EMSA with *Rh5* (–239 to +50) and *Rh6* (–555 to +121) promoters and 0, 50 and 500 ng His-SensZF. (**C-E**) Relative luciferase activity of pGL3 or pGL3 with *Rh3*, *Rh4*, *Rh5* or *Rh6* promoters in S2 cells transfected with pAc, pAc-Otd and/or pAc-Sens. (C) *, $P<0.01$ and **, $P<0.001$, compared with pAc alone. (D) *, $P<0.01$ and **, $P<0.001$, compared with Otd alone. (E) *, $P<0.005$ and **, $P<0.001$, compared with Otd alone.

performed Rh reporter assays with Pros. As shown in Fig. 7A, Rh promoter activity was unaffected by *pros* alone. However, Pros specifically repressed Otd-mediated *Rh5* and *Rh6* activation by approximately 75% and 50%, respectively (Fig. 7A). Pros binding sites were required for this repression (data not shown). Together, these studies suggest that Pros and Sens both require Otd to regulate the R8-based opsins: Sens activates, whereas Pros represses, with Otd (Fig. 7B). Because Otd is found in all PRs, this combinatorial regulation is consistent with the expression of these factors in vivo.

DISCUSSION

Sens is necessary for multiple aspects of color visual system development in *Drosophila*

During PNS development, *sens* regulates the selection of a single SOP within a field of equipotent proneural cells (Jafar-Nejad et al., 2003; Nolo et al., 2000). In *sens* mutants, neuronal precursors either undergo apoptosis or, as in the case of R8 cells, inappropriately develop into another neuronal population (for a review, see Jafar-Nejad and Bellen, 2004). *sens/Gfi1*-dependent specification of distinct neuronal populations also occurs in mouse inner ear cells and fly mechanosensory bristles (Frankfort et al., 2001; Jafar-Nejad et al., 2006; Wallis et al., 2003). However, it remains unclear what fate these neurons take in the absence of *sens/Gfi1*. Here, we demonstrate that in the *Drosophila* visual system, *sens* not only controls R8 specification and R7 recruitment, but is also essential for distinguishing blue/green-sensitive from UV-sensitive PRs.

A multistep pathway necessary for achieving cellular diversity

This study, together with previous work, indicates that a multistep process generates functionally distinct neuronal cell types during *Drosophila* eye development (Cook et al., 2003; Mikeladze-Dvali et al., 2005; Mollereau et al., 2001; Tahayato et al., 2003; Wernet et al., 2003; Wernet et al., 2006). Once a full complement of PR neurons

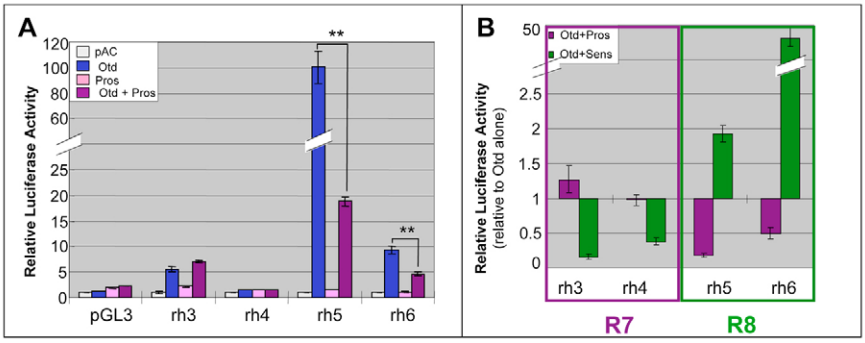

Fig. 7. Sens and Pros oppositely regulate R8 opsin genes in vitro. (**A**) Relative luciferase activity of pGL3 or pGL3 with *Rh3*, *Rh4*, *Rh5* or *Rh6* promoters in S2 cells transfected with pAc, pAc-Otd and/or pAc-Pros. (**B**) Otd+Sens (green) versus Otd+Pros (purple) regulation of IPR opsin expression in S2 cells. Values are normalized to cells transfected with Otd alone, and represent the average of two experiments.

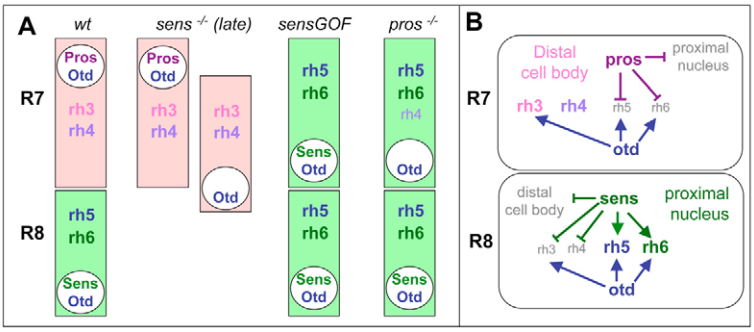

Fig. 8. Model for R7 versus R8 color PR differentiation in *Drosophila*. (**A**) Summary of phenotypes observed in inner PRs from wild type, late *sens* LOF (see Figs 2, 3), *sens* GOF (see Fig. 4) and *pros* LOF (Cook et al., 2003) eye-specific mutants. (**B**) Model for *pros*, *sens* and *otd* function in wild-type R7s and R8s.

is recruited during late larval development, the Sal gene complex genetically distinguishes IPRs from OPRs (Mollereau et al., 2001). Based on our *pros* and *sens* LOF experiments, such Sal-specified IPRs would adopt the following characteristics: a shortened rhabdomere that projects upwards from a proximally localized nucleus, a cell body that resides at the R7/R8 interface, and expression of Rh3 (Fig. 8A) (Cook et al., 2003). From this 'generic' IPR fate, various aspects of R7 versus R8 differentiation are promoted or repressed by the mutually exclusive expression of *pros* or *sens*, respectively (Fig. 8B): *pros* functions in R7s to prevent R8 opsin expression and to cause distal positioning of the R7 nucleus, whereas *sens* functions in R8s to prevent R7 opsin expression, promote R8 opsin expression and cause cells to develop proximally in the retina. Neither *sens* nor *pros* seems required to suppress each other's expression (Fig. 2) (Cook et al., 2003). However, misexpression of *sens* is sufficient to suppress *pros* expression in our GOF experiments. We predict that this is owing to two independent events: (1) *sens* indirectly inhibits *pros* expression via its ability to alter R7 differentiation; and (2) *sens* suppresses *pros* expression by preventing Pointed (Pnt) transcription factor function. This latter prediction is based on previous findings that *pros* is a direct transcriptional target of the MAPK-dependent Pnt transcriptional activator (Xu et al., 2000), and that *sens* suppresses Pnt-dependent transcription during R8 selection (Frankfort and Mardon, 2004). Future experiments will be important to directly link cell signaling with *sens* and *pros* antagonism during IPR development.

During early development, R8 cells are indistinguishable; however, three distinct subsets are present in the adult retina: pR8, yR8 and DRA R8s (Fig. 1C) (Wernet and Desplan, 2004). Interestingly, *ato*-dependent Sens expression occurs in all R8 cells, but its later Sal-dependent expression is restricted to p/y ommatidia (Fig. 1F,G and data not shown) (Wernet et al., 2003). Since DRA R8s are unique in expressing the typically R7-specific Rh3, absence of *sens* in these cells is consistent with its importance in repressing *Rh3*. Indeed, ectopic *sens* expression in the DRA is sufficient to inhibit *Rh3* expression and activate *Rh6* expression (Fig. 4H). The transcription factor Hth is both necessary and sufficient for DRA development: misexpression of Hth expands Rh3 to all inner PRs and represses Sens expression, and dominant-negative Hth leads to DRA-specific expansion of Rh6 and Sens and loss of Rh3 (Wernet et al., 2003) (data not shown). We observe no change in Hth expression in *sens* late LOF or *sens* GOF eyes, and Hth and Sens co-expression causes *sens*-dependent *Rh6* activation and *Rh3* repression (B.X., unpublished). Together, these data indicate that Hth-dependent repression of *sens* is necessary for DRA development.

Once polarization versus color decisions are made, p versus y ommatidial subsets develop. Spineless was recently shown to be crucial for this decision (Wernet et al., 2006), being expressed transiently and specifically in yR7 cells to induce Rh4 (versus Rh3) expression (Wernet et al., 2006). That we rarely detect Rh4 in *sens*-negative R8s supports the importance of R7-induction of this gene, and further indicates that late *sens* LOF R8s do not fully transform into R7 cells. After p versus y fate is established in R7 cells, Rh3-expressing pR7s induce Rh5 in underlying R8 cells via the signaling molecule Melt (Mikeladze-Dvali et al., 2005). In the absence of pR8 *melt* induction, all R8 cells express Rh6 (Chou et al., 1996; Chou et al., 1999; Papatsenko et al., 1997). Our finding that *sens* more robustly activates *Rh6* than it does *Rh5* both in vivo and in vitro (Fig. 4H and Fig. 6D) suggests that *sens* is not sufficient to induce *melt* expression and/or activity. Thus, current data suggest that *Rh3* represents the default opsin for all IPRs. Consistent with this, misexpression of Sal genes during early PR development transforms OPRs into IPRs and these all express *Rh3* (Domingos et al., 2004). Together, our ability to genetically isolate the stepwise events of PR differentiation will allow us to uncover new mechanisms important for achieving neuronal diversity.

Are similar restrictions important for vertebrate retinogenesis? Interestingly, recent studies have revealed the unexpected finding that vertebrate cone and rod PRs develop from a common precursor (Mears et al., 2001; Oh et al., 2007) (e.g. akin to OPRs versus IPRs), and that medium/long wavelength-sensitive cone PRs develop at least in part by suppressing short wavelength-sensitive cone PR development (Applebury et al., 2007; Deeb, 2006; Ng et al., 2001; Roberts et al., 2006) (e.g. akin to R8 versus R7 decisions). Whether these developmental relationships are indeed homologous remains unexplored. However, recent data have shown that the thyroid hormone receptor is important in cone PR cell fate decisions, and Lim et al. have reported that the thyroid hormone receptor associated proteins Trap230/240 (Kohtalo/Skuld – FlyBase) are important for inducing *sens* in the eye (Lim et al., 2007). Moreover, the factors important for *Drosophila* PR differentiation have vertebrate orthologs expressed in distinct mouse retinal cell populations [e.g. Sal3 (Bpnt1), Prox1, Gfi1, Meis1/Hth and Otx2] (Blackshaw et al., 2001; Dyer et al., 2003; Hisa et al., 2004; Nishida et al., 2003; Yang et al., 2003). Although the interactions of these factors have not yet been explored, such studies are likely to uncover conserved genetic cascades necessary to generate functionally distinct PR neurons.

Gene regulation by Gfi1 and Sens

Vertebrate Gfi1 and *Drosophila* Sens share striking homology within the DNA-binding four zinc-finger domains (Jafar-Nejad and Bellen, 2004). Gfi1, but not Sens, also contains an N-terminal SNAG domain that recruits multiple co-repressor complexes (McGhee et al., 2003; Zweidler-Mckay et al., 1996). Gfi1 SNAG domain mutants behave similarly to Gfi1 DNA-binding mutants (Grimes et al., 1996), suggesting that Gfi1 functions primarily as a repressor. Although Sens lacks a SNAG domain, recent studies show that, at low concentrations, Sens functions as a site-specific transcriptional

repressor of the *achaete* gene. However, at high concentrations, Sens functions as a DNA-binding-independent co-activator (Acar et al., 2006). Both activation and repression depend on the zinc-finger domain that is conserved with Gfi1; thus, such regulation might also be important for Gfi1 function. Here, we similarly find that Sens represses *Rh3* and *Rh4* expression through direct DNA binding, whereas activation of *Rh5* and *Rh6* appears to occur through an Otd-binding-dependent mechanism. However, we find that Sens regulation of Rh genes is context-dependent, but not dose-dependent (B.X. and T.C., unpublished). Future studies aimed at comparing Ac/Sens- versus Otd/Sens-dependent gene regulation will be important to better understand how Sens controls such diverse aspects of PNS development.

Conserved antagonism between Sens- and Pros-related factors?

Gfi1 is best characterized as an oncoprotein in lymphoid leukemias (see reviews, see Moroy, 2005; Duan and Horwitz, 2005; Jafar-Nejad and Bellen, 2004). Gfi1 positively influences lymphoid lineage development in part by suppressing the differentiation of the myeloid lineage. Gfi1 is also necessary for hematopoietic stem cell maintenance, indicating an important role for this gene in both proliferation and differentiation (Cellot and Sauvageau, 2005; Duan and Horwitz, 2005; Hock et al., 2004). Interestingly, Pros has recently been shown to repress neural stem cell proliferation and induce differentiation, and both Pros and Prox1 have been proposed to function as tumor suppressors (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Nagai et al., 2003; Shimoda et al., 2006). Although Prox1 is not associated with the hematopoiesis system under wild-type conditions, recent studies have shown that its expression is associated with several leukemias (Nagai et al., 2003; Shimoda et al., 2006). Together, we suggest that *pros*/*Prox1* and *sens*/*Gfi1* factors share an evolutionarily conserved antagonism in regulating a number of developing organ systems, ranging from stem cell growth to neuronal and hematopoietic lineage specification.

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