

# Distinction between Color Photoreceptor Cell Fates Is Controlled by Prospero in *Drosophila*

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## Summary

The *Drosophila* compound eye consists of ~750 independently functioning ommatidia, each containing two photoreceptor subpopulations. The outer photoreceptors participate in motion detection, while the inner photoreceptors contribute to color vision. Although the inner photoreceptors, R7 and R8, terminally differentiate into functionally related cells, they differ in their molecular and morphological makeup. Our data indicates that several aspects of R7 versus R8 cell fate determination are regulated by the transcription factor Prospero (Pros). *pros* is specifically expressed in R7 cells, and R7 cells mutant for *pros* derepress R8 *rhodopsins*, lose R7 *rhodopsins* and acquire an R8-like morphology. This suggests that R7 inner photoreceptor cell fate is acquired from a default R8-like fate that is regulated, in part, via the direct transcriptional repression of R8 *rhodopsins* in R7 cells. Furthermore, this study provides transcriptional targets for *pros* that may lend insight into its role in regulating neuronal development in flies and vertebrates.

## Introduction

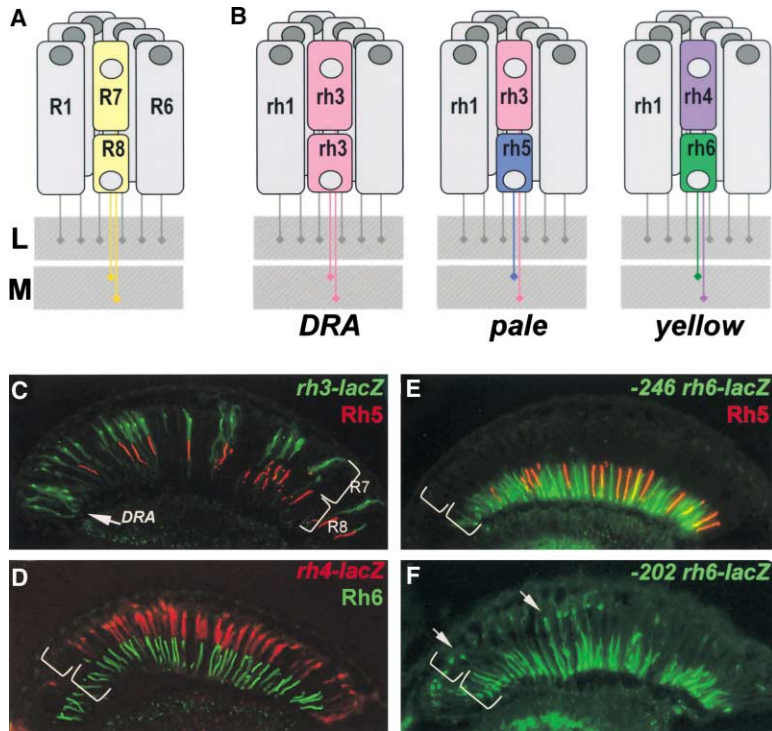
The fly visual system provides a powerful paradigm for understanding cell type specification. In 5 days, a relatively flat epithelial disc develops into a complex compound eye comprised of ~750 repeating ommatidial units containing cone, pigment, bristle, and photoreceptors cells (Wolff and Ready, 1993). The eight light-receiving photoreceptors (PRs), R1–R8, fall into two general subtypes: outer (R1–R6) and inner (R7 and R8) PRs (Figure 1A). Outer PRs, much like vertebrate rod PRs, participate in motion detection, while inner PRs are analogous to vertebrate cone PRs in that they are thought to function in color discrimination (Cook and Desplan, 2001). Several morphological characteristics help to distinguish between outer and inner subtypes, including the shape and position of their light-gathering apical membrane (the rhabdomere) and the location of their axonal terminations (Hardie, 1985). R1–R6 each possess a large diameter rhabdomere that spans the entire width of the retina, and together, form a trapezoidal array within an ommatidium; R7 and R8 are positioned at the center of the trapezoid, with their rhabdomeres spanning only half the thickness of the retina, and R7 sitting on top of R8. In addition, the axons of the outer PRs all terminate in

the first optic tectum in the brain, the lamina, while both inner PR axons project to a deeper optic tectum, the medulla, with R7 cells projecting to a slightly deeper layer than R8 cells (Figure 1B).

Work on eye development in the larval imaginal disc has demonstrated that PRs are recruited from equipotent epithelial cells in a stereotyped order: R8 first, then R2/R5, R3/R4, R1/R6, and finally R7 (see Kumar and Moses, 1997 for review). Each recruitment event leads to a unique configuration of signaling and transcriptional activity that aids in specifying these cells and establishing their correct axonal projections to the optic lobes. During the next ~100 hr, R1–R8 continue their differentiation program, acquiring the features described above that allow these cells to function properly. For instance, recent work on the *spalt* (*sal*) gene complex, *spalt major* and *spalt-related*, has revealed a role for these related zinc finger transcription factors in distinguishing between outer and inner PR fate decisions. The *sal* genes are specifically expressed in the inner PRs of the adult, and in the absence of the *sal* locus, R7 and R8 cells transform into outer PRs (Mollereau et al., 2001). These data revealed a new genetic switch between inner and outer PRs, and suggest that additional factors may be required downstream to further distinguish between R7 and R8 cells.

Photoreceptors absorb light with Rhodopsins (Rhs), the pigment proteins that are present in the stack of membranes that form the rhabdomeres. Several *rhodopsin* gene (*rh*) products exist with varying absorption spectra to ensure a broad range of light sensitivity. Outer PRs all express Rh1, while R7 cells express either Rh3 or Rh4 (UV-sensitive opsins), and R8 cells primarily express blue-sensitive Rh5 or green-sensitive Rh6 (Chou et al., 1996, 1999; Fortini and Rubin, 1990; Huber et al., 1997; Montell et al., 1987; Papatsenko et al., 1997) (Figures 1B–1E). Inner PR *rh* expression is coordinately regulated within an ommatidium (Figure 1B) with Rh3-expressing R7 cells associated with underlying Rh5-expressing R8 cells (termed “pale” ommatidia) (Figures 1B and 1C), and Rh4-expressing R7 cells associated with underlying Rh6-expressing R8 cells (“yellow” ommatidia) (Figures 1B and 1D) (Chou et al., 1996, 1999; Papatsenko et al., 1997). One exception to this is found in the two dorsal-most rows of ommatidia, the dorsal rim area (DRA), in which Rh3 is expressed in both R7 and R8 cells (Fortini and Rubin, 1990) (Figures 1B and 1C). The study of *rh* regulation has revealed that promoters of less than 300 bp can recapitulate endogenous *rh* expression in vivo (Fortini and Rubin, 1987, 1990; Mismar et al., 1988; Papatsenko et al., 2001) (Figures 1C–1E), and are organized in a simple bipartite structure: a conserved proximal element (RCSI, rhodopsin conserved sequence I) common to all *rh* promoters, and upstream *rh*-specific sequences (RUS, rhodopsin upstream sequences) unique to each *rh* promoter (Fortini and Rubin, 1990; Papatsenko et al., 2001). This organization predicts that common as well as unique factors function together to dictate *rh* gene expression, and consequently, to properly specify different ommatidial subtypes.

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**Figure 1. Subtype-Specific Expression of rhodopsins in the Adult *Drosophila* Retina: Presence of a Subtype-Specific Element in R8 *rh* genes, seq56**

(A) Diagram representing the position and morphological characteristics of outer (R1–R6) and inner (R7 and R8) photoreceptors. (B) Diagram representing the different subtypes of ommatidia. Rh3 is present in both the R7 and R8 cells of the dorsal rim area (DRA), whereas coupled expression of Rh3/Rh5 and Rh4/Rh6 is observed in the pale and yellow subsets of ommatidia, respectively. (C–E) Immunofluorescent staining for *rhodopsin-lacZ* reporters and Rhodopsin proteins in the adult eye. All images are oriented with dorsal left, and distal up. Brackets represent the different R7 and R8 layers within the retina. (C) Costaining for the rhodopsins, the minimal (–160/+18) *rh3-lacZ* reporter (green) in R7 and Rh5 protein (red) in R8 cells. (D) Costaining for the yellow rhodopsins, a –160/+85 *rh4-lacZ* construct (red) in R7, and Rh6 protein (green) in R8 cells. (E) Costaining for Rh5 (red) and a –246/+121 *rh6-lacZ* reporter shows the exclusion between the two R8 *rh* genes. (F) Deletion of the *rh6* promoter (–202/+121) results in *lacZ* misexpression in the R7 layer (arrowheads).

We have taken advantage of the subtype-specific expression of the inner PR *rh* genes as a tool for understanding later events in PR development. Here, we report the identification of a conserved element that is specifically present in the R8 *rh* promoters, *rh5* and *rh6*, which is responsible for repressing these genes in R7 cells. A yeast one-hybrid screen revealed that this element provides a binding site for Prospero (Pros), an atypical homeodomain transcription factor important for neuronal specification in the developing *Drosophila* embryo. We find that Pros is specifically expressed in R7 photoreceptors in the adult. Furthermore, genetic studies demonstrate that *prospero* (*pros*) is both necessary and sufficient for the repression of R8 *rh* genes in R7 cells. *pros* mutant R7 cells not only gain expression of R8 rhodopsins, but also acquire R8-like morphological features and lose R7-specific markers such as Rh3 and Rh4. These findings suggest that once inner PRs are coordinately distinguished from outer PRs through Sal, Pros then helps to further distinguish R7 from R8, with R8 being the ground state of differentiation for inner PRs. As the vertebrate homolog of *prospero*, *prox1*, is also expressed in subsets of retinal cell populations, we propose that a role for this transcription factor is evolutionarily conserved to provide cell type diversification in the eye.

## Results

### Identification of Seq56, an R7 Repression Element in R8 rhodopsin Promoters

We have found that, in addition to common RCSI sequences and unique RUS elements within the minimal *rh* promoters, some sequences are also shared between

subsets of *rh* genes (Figure 2A) (A. Tahayato et al., submitted). Here, we describe the characterization of an 11 bp sequence that is shared between the R8-specific *rh5* and *rh6* promoters (Figure 2B), a sequence we refer to as seq56. Seq56 is evolutionarily conserved within the *rh5* and *rh6* promoters of *D. melanogaster*, *D. pseudoobscura*, and *D. virilis*, both in sequence and relative position to other conserved elements (Figure 2B) (A. Tahayato et al., submitted), but is absent in the *rh1-rh4* promoters (see Experimental Procedures). Deletion mapping of the *rh6* promoter revealed that while the promoter  $-246/+121$  could drive wild-type expression (Figure 1E), a deletion construct that removed a portion of seq56 ( $-202/+121$ ) led to inappropriate reporter expression in R7 cells (Figure 1F, arrowheads; Figure 2A). These data suggest that the presence of seq56 in both *rh5* and *rh6* is important for their correct expression.

Seq56 constitutes an imperfect palindrome of the pentameric sequence  $AG^4/C_0CG$ , and several copies of this half-site are distributed throughout the *rh5* and *rh6* promoters (Figures 2A and 2B and <http://homepages.nyu.edu/~dap5/rh/rhodopsins.html>). To examine the in vivo function of seq56, we generated site-directed mutations that disrupted the A (5') or B (3') half site of the palindrome (a/B and A/b, Figure 2A) within the *rh6* promoter. Neither mutation affected reporter expression in R8 cells, but led to strong derepression in R7 cells (compare Figure 2C with 2D and 2E). Derepression into R7 cells was determined based on *lacZ* reporter expression in rhabdomeres that spanned only the distal half of the retina (Figures 2C–2E, brackets) and projections that terminated in the medulla (data not shown). Similar mutagenesis studies within the context of the *rh5* promoter did not affect reporter activity, even when combined with mutation of a perfect seq56<sub>B</sub> site located

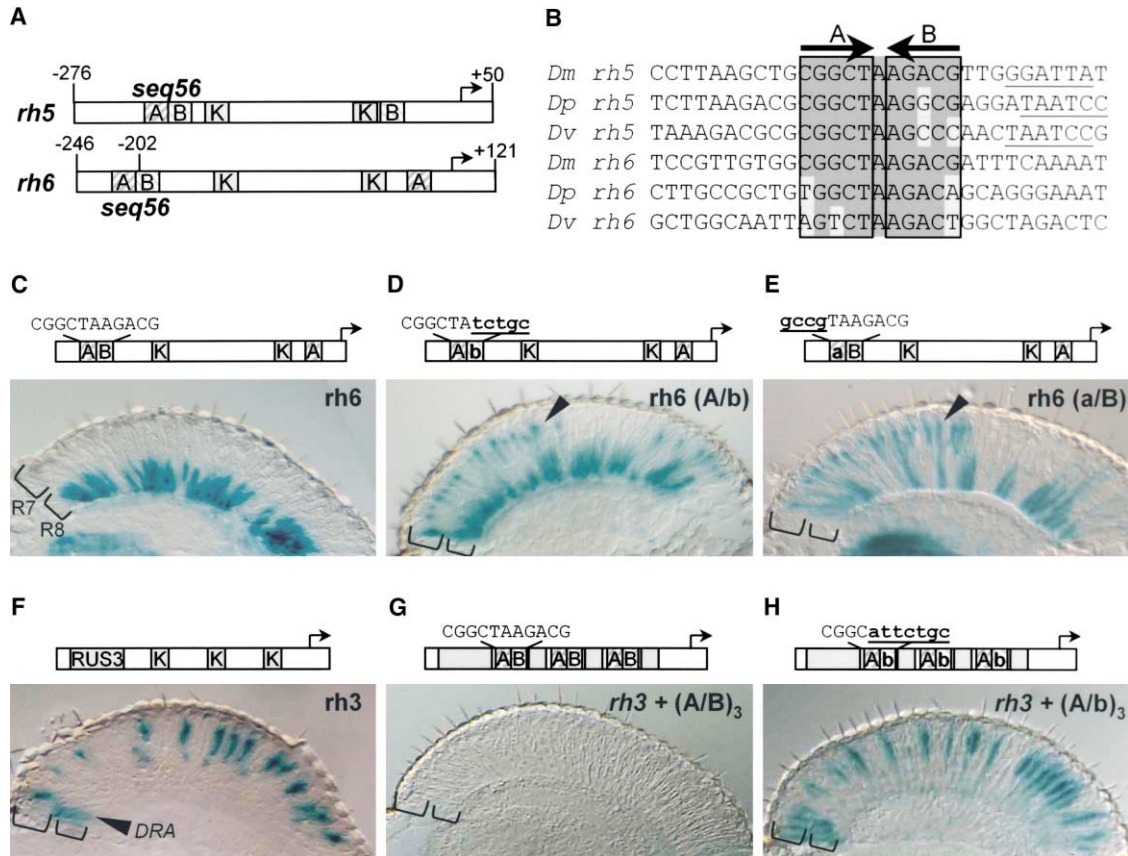


Figure 2. Seq56 as an R7-Specific Repressor Element for R8 Rhodopsins

(A) Diagram of the minimal *rh5* and *rh6* promoters (A. Tahayato et al., submitted). "K" boxes represent binding sites for  $K_{50}$  homeodomain proteins, and "A" and "B" boxes represent the seq56 half sites. Arrows represent the start of transcription.

(B) Alignment of seq56 sequences from *Drosophila melanogaster* (*Dm*), *D. pseudoobscura* (*Dp*), and *D. virilis* (*Dv*).  $K_{50}$  binding sites in close proximity to the seq56 palindrome in the *rh5* promoters are underlined.

(C–E) X-gal staining of wild-type and site-directed mutants of the minimal *rh6* promoter (–246/+121) (C–E) or minimal *rh3* promoter (–159/+18) (F–H) driving Gal4 lines and a UAS-*lacZ* reporter gene. Wild-type seq56 sites and mutants in the "A" or "B" boxes are indicated, with mutated nucleotides underlined, bold, and lowercased. RUS3 represents an evolutionarily conserved element present within the *rh3* promoter.

(C–E) Mutation of either side of the seq56 imperfect palindrome causes ectopic expression of *lacZ* in the R7 layer (D and E, arrowheads).

(F–H) Wild-type *rh3* expression (F) is lost by the insertion of three wild-type seq56 sequences (A/B)<sub>3</sub>, but is maintained with the introduction of three mutant seq56 elements (A/b)<sub>3</sub> (H).

upstream of its RCS1 (see Figure 2A) (data not shown). However, both loss- and gain-of-function experiments indicate that *rh5*, like *rh6*, is regulated by a seq56-mediated process (see below), suggesting that other elements are also involved in *rh5* repression.

To test whether seq56 was sufficient to repress R7-specific expression of a heterologous promoter, we placed wild-type or mutant versions of seq56 within the *rh3* minimal promoter. The addition of wild-type seq56 elements (A/B)<sub>3</sub> abolished *rh3* reporter activity in R7 cells (compare Figures 2F and 2G), whereas the addition of a mutant seq56 element (A/b)<sub>3</sub> at the same locations allowed normal *rh3* expression (Figure 2H). Together, these data indicate that seq56 is both necessary and sufficient to repress *rh* expression in R7 photoreceptors.

#### Identification of Prospero as a Seq56-Interacting Protein

To identify potential seq56 *trans*-acting factors, we screened a *Drosophila* adult head cDNA library with

this element using the yeast one-hybrid approach. The strongest *his3/lacZ* positives from this screen (five out of nine) encoded the DNA binding domain and C terminus of the homeodomain transcription factor, Pros. Immunostaining of head cryosections revealed nuclear expression of Pros in all adult R7 PRs (Figures 3A–3C). Furthermore, we observed weaker, cytoplasmic expression in cone cells (Figure 3D). During eye development, R7 and cone cells arise from the same R7 equivalence group of cells (Dickson et al., 1992). Previous studies have demonstrated that Pros is expressed in the developing eye disc at low levels in the nucleus of all five equivalence group cells, and later increases specifically in the R7 cell upon its initial specification (Kauffmann et al., 1996). R7-specific expression of Pros continues to increase through 48 hr after puparium formation (APF), remaining low in cone cells; however, by 56–64 hr APF, Pros immunoreactivity is no longer detected (Kauffmann et al., 1996). As *rh* gene expression is established at approximately 70 hr of pupation (Kumar et al., 1997;

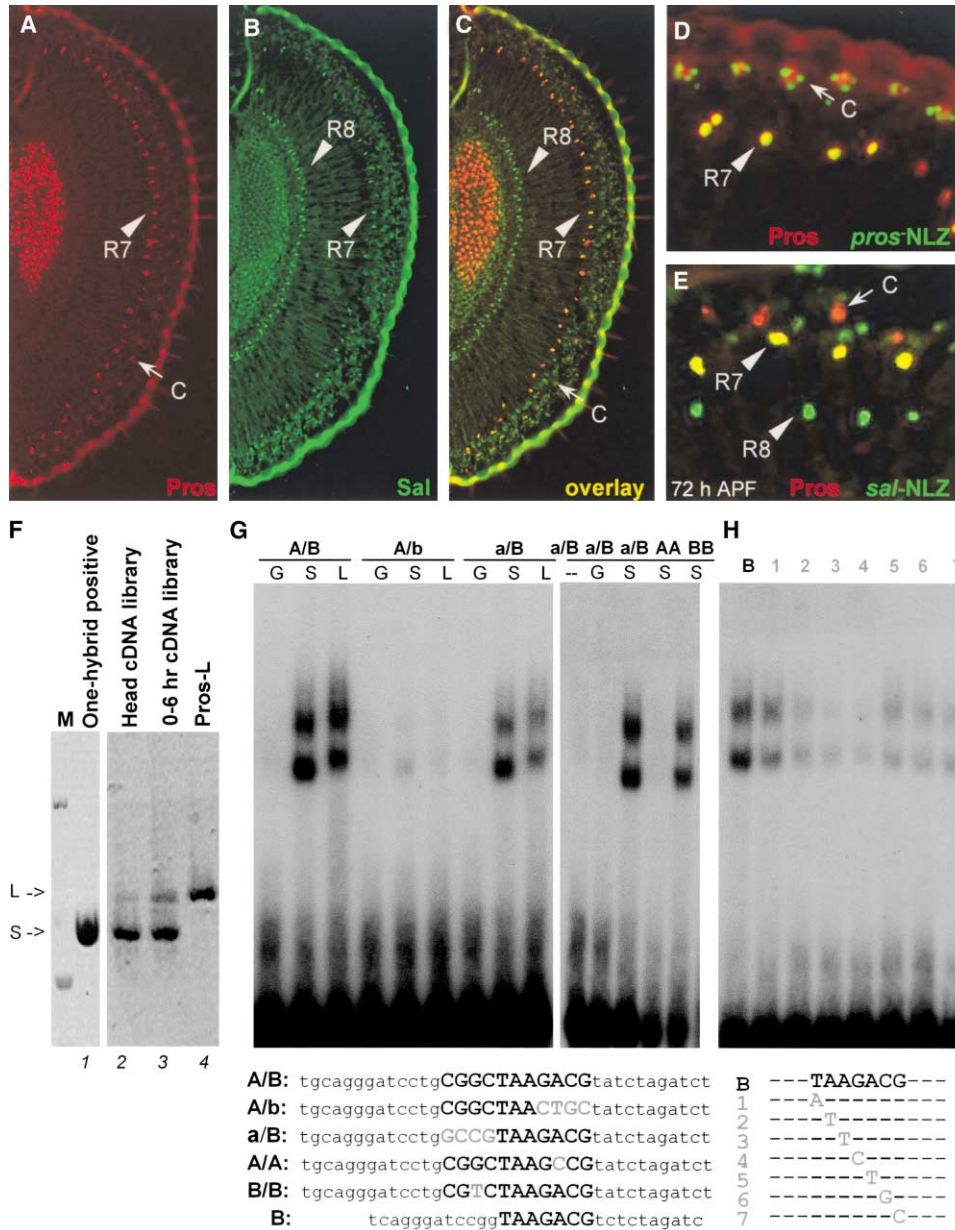


Figure 3. Prospero as an R7-Specific Factor that Binds seq56 In Vitro

(A–E) Expression pattern of Prospero in the adult retina (A–D) or pupal retina 72 hr APF (E) (dorsal up, distal right). Costaining of adult retinas with anti-Pros (red, [A]) or anti-Sal (green, [B]) antibodies (B). R7 nuclei are distally placed within the cells, whereas R8 nuclei are proximally located ([B], arrowheads). Overlapping these images revealed that Pros is present in all R7 nuclei (C). Also note that weak Pros immunoreactivity is present in the cone cells of the retina ([A and C], arrow “C”). (D) Costaining Pros (red) with a *pros* nuclear-lacZ enhancer trap, *pros*<sup>10419</sup> (green) revealed that Pros expression in R7 cells is nuclear (arrowhead), whereas cone cell expression is cytoplasmic (arrow). (E) 72 hr pupal retinas were stained for the *sal* nuclear lacZ enhancer trap, *sal*<sup>03602</sup> (green, sal-NLZ) and Pros (red) expression. Similar expression as that observed in adult retina (C) was observed.

(F) PCR amplification of *pros*-S (“S”) and *pros*-L (“L”). Lane 1, DNA from a *pros*-encoding one-hybrid clone; lane 2, adult *Drosophila* head cDNA library used for our screen; lane 3, 0–6 hr *Drosophila* embryonic cDNA library; lane 4, genomic DNA isolated from UAS-*pros*-L flies.

(G) Gel shift assays of GST (“G”), GST-ProsS (“S”), and GST-ProsL (“L”) fusion proteins purified from *E. coli*. Seq56-based oligonucleotides are aligned, with gray nucleotides representing those that differ from the control sequence. GST shows no specific interaction.

(H) Pros-S binding to seq56<sub>B</sub>. Probes represent variations of the core sequence from the oligonucleotide, tcagggatccggTAAGACGtctctagatct.

Sheng et al., 1997), we analyzed the expression of Pros in retinas at this stage of development. Both at 72 and 96 hr APF, we detected Pros expression in a similar pattern as what we observed in the adult (Figure 3E and data not shown). These data, in conjunction with those

of Kauffmann et al. (1996), suggest that *pros* is only very transiently turned off in R7 cells during midpupation, and its expression is reestablished in the same cell populations. The finding that Pros is expressed in R7 cells at the time when *rh* genes are expressed is consistent

with a role for Pros in mediating seq56-directed repression.

#### Binding Specificity of Prospero

The region of *pros* identified in our screen is alternatively spliced in *Drosophila* embryos, resulting in proteins that differ by the absence (Pros-S) or presence (Pros-L) of 27 aa directly upstream and including a portion of the homeodomain (Chu-Lagraff et al., 1991) (Figure 3F, lane 3). All five of the clones we identified represented *pros-S* (Figure 3F, lane 1). PCR from a 0–6 hr embryonic cDNA library, as well as the head cDNA library used in our screen, revealed that the *pros-S* isoform was more abundant than *pros-L* (Figure 3F, lanes 2 and 3); RT-PCR from head mRNA revealed similar findings (data not shown). Since these proteins differ within their DNA binding domains, we tested both Pros-S and Pros-L fusion proteins in DNA binding assays with seq56. Both Pros-S (S) and Pros-L (L) bound strongly to the wild-type imperfect palindromic seq56 (CGGCTAAGACG) (Figure 3G). As both half sites of the palindrome were required for *rh6* repression in R7 cells (Figures 2D and 2E), we also tested the ability of Pros to bind either the A or B site alone. While mutation of the B site (A/b) abolished Pros binding, mutation of the A site (a/B) had no effect (Figure 3G). Additionally, a perfect A/A palindrome provided no binding, indicating that Pros does not recognize seq56 as a palindrome, but instead, binds to the sequence present in the a/B sequence TAAGACG. Two complexes were observed in these experiments; however, the slower migrating band is likely to represent a GST-induced dimer, as identical complexes were formed with probes carrying A/B, B/B, or B sites alone (Figures 3G and 3H), and the slower migrating complex was not observed under different buffer conditions (data not shown). Regardless of the experimental conditions, however, no significant differences in binding strength or specificity were observed between Pros-S and Pros-L (Figure 3 and data not shown). We also created site-directed mutants within the minimal binding sequence, TAAGACG. While mutation in any position disrupted Pros binding to some extent, mutation of the T at position 1 resulted in the weakest disruption, while mutation at positions 3 and 4 drastically decreased binding (Figure 3H). Together, these data indicate that Prospero preferentially recognizes the seq56<sub>B</sub> element, AAGACG.

#### Prospero Is Necessary and Sufficient to Repress R8 Rhodopsins In Vivo

To test whether Pros binds seq56 to repress R8 *rhodopsins* in R7 cells, we generated flies entirely null for *pros* function in the eye (see Experimental Procedures) (Stowers and Schwarz, 1999). Immunostaining of cryosections of *pros*<sup>17</sup> null mutant eyes demonstrated that both *rh5* and *rh6* expression was expanded into the R7 layer of the retina, while their expression in R8 cells was maintained (Figures 4A–4D). In contrast, the misexpression of *pros-L* in differentiated cells of the eye using the GMR-GAL4 driver significantly repressed both Rh5 and Rh6 expression in R8 cells (Figures 4E–4H), but had no effect on the expression of Rh1 in outer PRs, Rh3 and

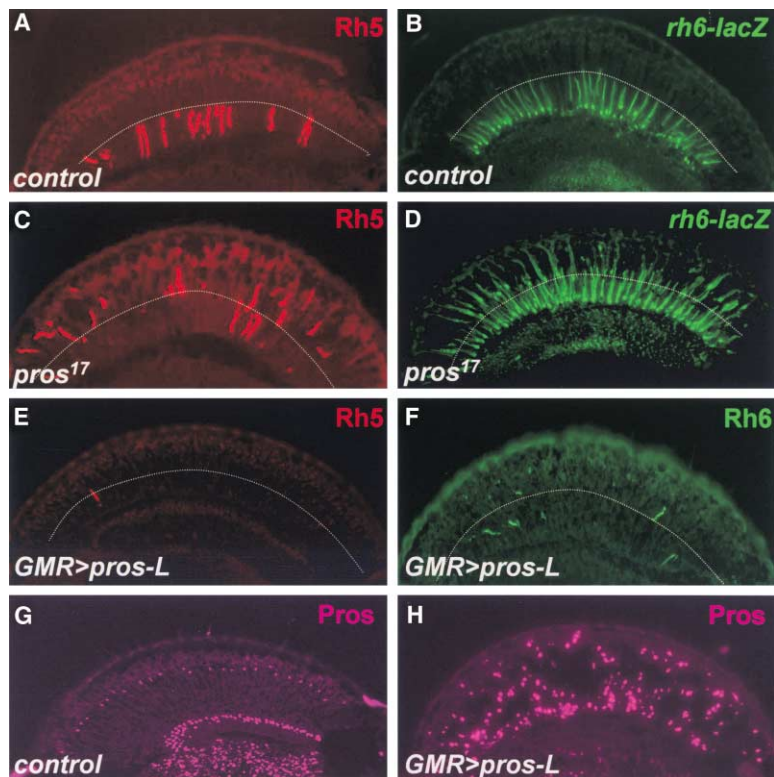
Rh4 in R7 cells, or Cut, a cone cell marker expressed in the adult (data not shown). These results support the idea that *rh5* and *rh6* expression is directly regulated by *pros*, and that Pros does not require an R7-specific factor for repression.

#### Derepression of R8 Rhodopsins Results in Loss of R7 Rhodopsins

Most sensory systems have developed a mechanism of mutual exclusion of their various receptor proteins to prevent the overlap of inputs into the brain (Pilpel et al., 1998). To test whether this occurs in the context of *pros* mutant eyes, where R8 rhodopsins are inappropriately present in R7 cells, we next examined the expression of Rh3 and Rh4 in *pros*<sup>17</sup> mutants. Rh3 expression was maintained in the dorsal rim area of the retina, where it is normally present in both R7 and R8 cells; however, in the remaining portion of the retina, Rh3 expression was significantly reduced or abolished (Figure 5A versus 5B). The concomitant gain of Rh5 and loss of Rh3 in pale R7 cells suggest that an exclusion mechanism occurs in this subset of R7 cells. As seq56 elements are absent in the *rh3* promoter (Figure 2), and misexpression of *pros* was not sufficient to activate *rh3* expression (see above), the loss of *rh3* expression is likely indirectly due to the misexpression of Rh5 in R7 cells. This interpretation also helps to explain why *rh3* was not lost in the dorsal rim area (DRA), where *rh5* is normally absent. Exclusion also can occur between Rh4 and Rh6, and requires the presence of a functional Rh6 protein. For instance, in eyes derived from the *pros*<sup>17</sup> allele, *rh6-lacZ* was significantly derepressed in R7 cells and overlapped with Rh4 expression (Figure 5E); in contrast, flies carrying the *pros*<sup>01</sup> allele showed much fewer Rh6-positive R7 cells, and no Rh4/Rh6 overlap was observed (Figure 5J). This difference was due to the fact that the chromosomal arm carrying the FRT82B-*pros*<sup>17</sup> allele also contained a nonsense mutation within the *rh6* gene, *rh6*<sup>(1)</sup> (see Experimental Procedures): recombination of a wild-type *rh6* allele onto the *pros*<sup>17</sup> arm led to a phenotype similar to that observed with *pros*<sup>01</sup>. Flies homozygous for the *rh6*<sup>(1)</sup> allele, but otherwise wild-type, showed no effect on the expression of the other *rh* genes (data not shown). Together, these data are consistent with the hypothesis that, in addition to a role for *pros* in preventing R8 *rh* gene expression in R7 cells, the regulation of *rh* expression also includes exclusion pathways involving the Rhodopsin molecules themselves. Indeed, recent findings from our laboratory suggest that certain Rhodopsin proteins exclude the expression other *rh* genes and will be addressed in a future publication (E. Mazzoni, P. Beaufils, F.P., T.C., and C.D., unpublished data).

#### Pros Affects R7 versus R8 Cell Fate Decisions

The above results describing the misexpression of R8 *rh* genes and loss of R7 *rh* genes in *pros* mutant eyes suggest that R7 cells lacking *pros* transform toward an R8-like state. As few other cell-specific markers are known in adult PRs, we examined morphological characteristics that are unique to R7 and R8 cells: rhabdome position, axonal projections, and localization of the nucleus. For these experiments, all *pros* alleles gave



**Figure 4. Pros Is Necessary and Sufficient to Repress R8 *rh* Expression**

Adult retinal cryosections from control heads (A, C, E, and G), eyes homozygous for the *pros*<sup>17</sup> null allele (B and D), or eyes misexpressing *pros-L* with the GMR-GAL4 driver (F and H). All images are oriented with dorsal left, and distal up, and R7 and R8 layers are separated by a dotted line.

(A–D) Wild-type expression of Rh5 (A) and –555/+121 *rh6-lacZ* (C) expression in heterozygous *pros*<sup>17</sup> retina (A and C) and derepression of Rh5 (B) and *rh6-lacZ* (D) in homozygous *pros*<sup>17</sup> mutant eyes.

(E–H) Misexpression of *pros-L* results in significant repression of Rh5 (F) and Rh6 (H) expression in R8 cells, while flies carrying only UAS-*pros-L* or GMR-GAL4 appear wild-type (data not shown). The eye is slightly disrupted by the misexpression of Pros, indicated by the misplaced photoreceptor nuclei and rhabdomeres (F and H).

consistent results, independent of the presence or absence of the *rh6*<sup>11</sup> allele.

As mentioned earlier, R7 and R8 cells both project to the medulla lobe, with R7 cells projecting slightly deeper than R8 cells. Labeling whole-mounted brain preparations with the PR-specific chaoptin antibody 24B10, we found that although the retinotopic map was slightly disturbed (data not shown), R7 and R8 terminations formed two obvious layers in the medulla of *pros* mutant eyes (Figures 6A and 6B). Staining for the *rh6-lacZ* reporter also revealed that, while *rh6* expression was restricted to the R8 layer in controls, *pros* mutants led to *lacZ*-positive terminations in both the R7 and R8 layer (Figures 6C and 6D). While the results shown were acquired from *pros*<sup>17</sup>, *rh6*<sup>11</sup> double mutants, *rh6-lacZ*-positive R7 terminations were also observed with *pros* alleles carrying a wild-type *rh6* gene, albeit fewer in number. R7- and R8-positive terminations were also observed in *pros* mutants carrying the *rh5-lacZ* reporter (data not shown). These data are consistent with findings by Kauffmann et al. (1996) who detected no changes in early PR differentiation in *pros* mutant cells, and indicate that neuronal specification and terminal PR differentiation can be uncoupled in R7 cells lacking *pros*.

Two additional characteristics that distinguish R7 from R8 cells are the distal versus proximal positions of their rhabdomeres and nuclei. As indicated in Figures 4 and 5, distal rhabdomere staining for Rh5 and Rh6 was observed in *pros* mutant eyes. These are not R8 rhabdomeres that have escaped into the R7 layer, a feature observed with certain R7-less mutants (e.g., *sev*, *boss*) (Chou et al., 1999), as we could observe both R7

and R8 rhabdomeres positive for Rh5 or Rh6 within a single ommatidium (Figures 6G–6J). Staining for *rh6-lacZ* and the DNA marker, Hoechst 33258, however, revealed that the position of the R7 nuclei most often adopted a proximal localization (Figures 6L and 6N), while wild-type R7 nuclei were distal (Figures 6K and 6M). These results indicate that while the rhabdomeres of *pros* mutant R7 cells are able to properly assemble within an ommatidium, their nuclei remain proximal, giving the impression that two “R8-like” inner PRs are present on top of each other. This phenotype was observed even in cells which maintained Rh4 expression (data not shown), indicating that this event is due to the loss of *pros* function and not from the acquisition of R8 rhodopsins. Because nuclear position is associated with the developmental state of the cell (Tomlinson, 1985), these data indicate that *pros* mutant R7 cells not only acquire final R8-specific differentiation markers, but also additional R8-specific features.

We next examined non-*rh* molecular markers that are expressed in adult inner PRs to ascertain the degree of R7-to-R8 transformation that occurs in *pros* mutant R7 cells. Two such markers, Spalt and Senseless (Sens), are specifically expressed in inner PRs (Mollereau et al., 2001) and R8 cells (Figure 6Q) (Frankfort et al., 2001), respectively, in the adult retina. Spalt is expressed in R7 and R8 cells from larval to adult stages and is concomitant with Pros activation in the imaginal disc (M. Wernet, F.P. and C.D., unpublished data). Previous experiments aimed at characterizing the *sal* mutant phenotype indicated that *pros* expression remained in R7 cells lacking *sal* (Mollereau et al., 2001), despite the fact that

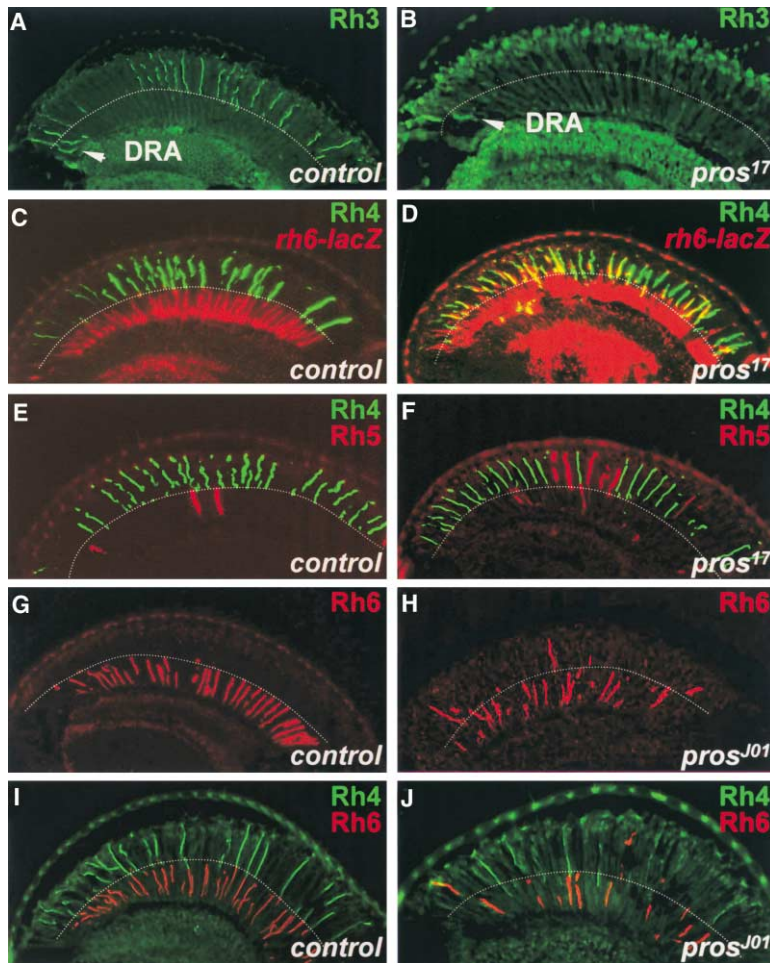


Figure 5. R8 versus R7 *rh* Expression in *pros* Mutant Eyes

Adult cryosections from control heterozygous (A, C, E, G, and I), *pros<sup>17</sup> rh6<sup>11</sup>* homozygous (B, D, and F), or *pros<sup>01</sup>* homozygous (H and J) eyes immunostained with antibodies to Rh3 (A and B), Rh4 (C–F, I, and J), Rh5 (E and F), or Rh6 (G–J). *pros<sup>17</sup>* mutant flies also carried a  $-555/+121$  *rh6-lacZ* reporter detected using anti- $\beta$ -galactosidase (C and D). Rh3 expression is lost in the majority of the retina in *pros<sup>17</sup>* mutant eyes (A versus B), although its expression in the DRA is maintained ([B], arrowhead). (C and D) Rh4 expression (green) remains essentially unchanged and overlaps significantly with *rh6-lacZ* (red) in *pros<sup>17</sup>* mutant eyes. (E and F) Costaining for Rh4 (green) and Rh5 (red) demonstrates that, in *pros<sup>17</sup>* mutants (F), Rh5 is expressed in R7 cells not expressing Rh4. (G and H) Homozygous *pros<sup>01</sup>* eyes exhibit derepression of Rh6 (H) compared to control flies (G), but the extent of derepression is less than that observed with *rh6-lacZ* with the *pros<sup>17</sup>* allele (compare H and D) due to the presence of the *rh6<sup>11</sup>* mutation on the *pros<sup>17</sup>* chromosome (see text for details). (I and J) Costaining for Rh4 (green) and Rh6 (red) reveals little to no Rh6/Rh4 overlap in *FRT-pros<sup>01</sup>* mutant eyes.

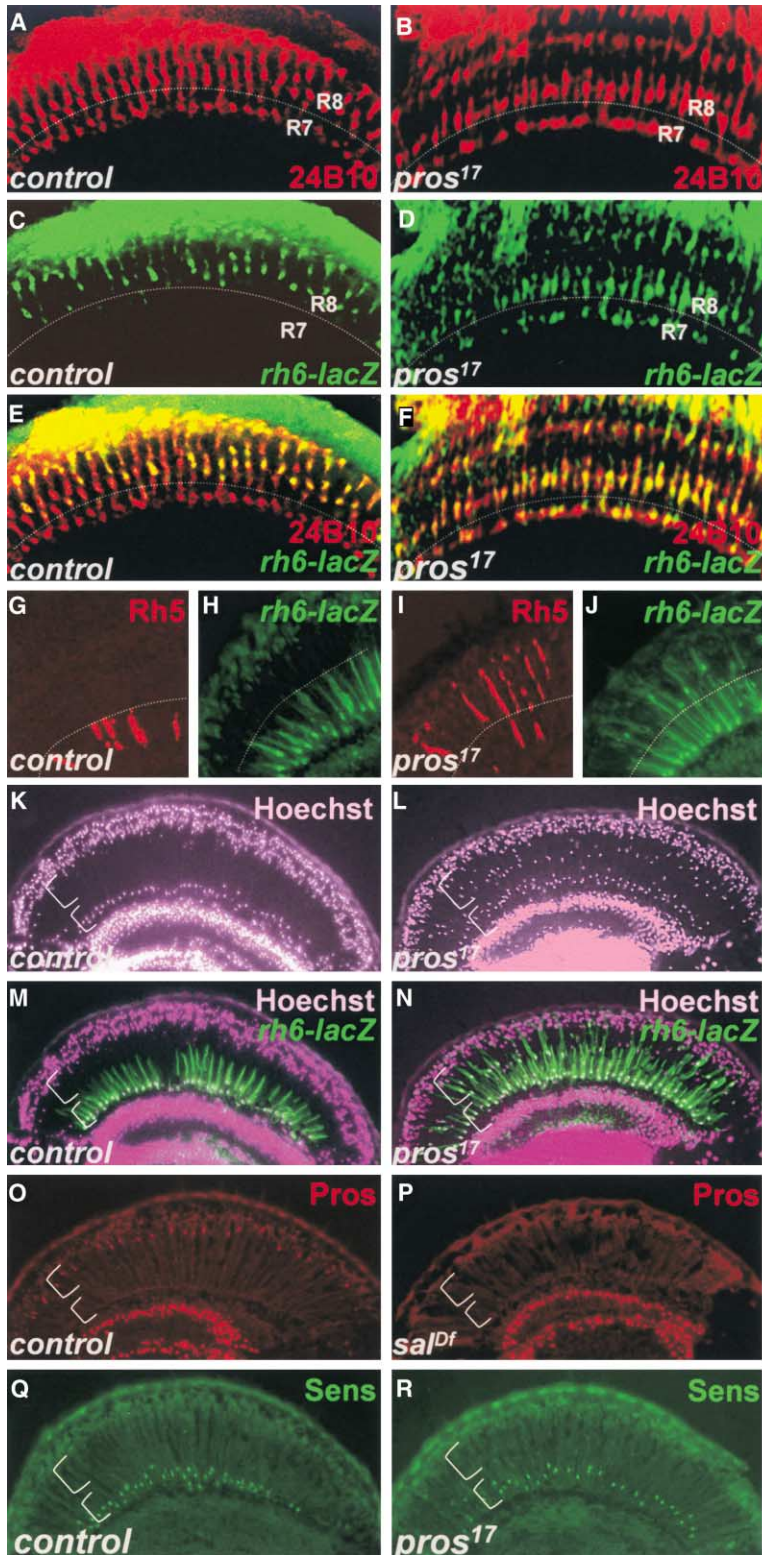
R7 cells resembled outer PRs in all other later aspects of development. Contrary to this earlier report, we observed that eyes homozygous mutant for both *sal* genes no longer expressed *pros* (Figures 6O and 6P). Furthermore, *sal* expression was maintained in *pros* mutant eyes (data not shown). These data strongly suggest that *pros* functions downstream of *sal*-mediated inner PR specification. We have also recently found that Senseless (Sens), a transcription factor important for R8 specification in the imaginal disc (Frankfort et al., 2001), remains restricted to R8 cells in the adult retina (Figure 6Q). Sens was not expanded in *pros* mutants (Figure 6R) and was maintained in eyes misexpressing *pros* with GMR-GAL4 (data not shown). These data confirm our earlier observations that *pros* is necessary for preventing R7 cells from adopting many, but not all, R8 characteristics and suggest that inner PRs develop through several genetically distinct stages (see Figure 7).

## Discussion

### Regulation of R7 Photoreceptor Differentiation

During eye disc development, R7 cells are specified by three different signaling cascades: Sevenless (Sev),

EGFR, and Notch (Cooper and Bray, 2000; Freeman, 1996; Hafen and Basler, 1991; Tomlinson and Struhl, 2001). Previous studies have demonstrated that normal levels of *pros* expression in R7 cells require Ras pathway activation via EGFR and Sev signaling (Kauffmann et al., 1996; Xu et al., 2000), as well as Notch activation (R. Carthew, personal communication). These data suggest that *pros* could be a critical target for inducing R7 differentiation. However, no changes in early R7-specific markers in *pros* mutant eye discs could be detected (Kauffmann et al., 1996), and morphological characteristics such as correct projections to the medulla and rhabdomere positioning on top of the R8 rhabdomere within the ommatidial center were largely unaffected even in the adult retina (Kauffmann et al., 1996) (Figure 6). The findings reported here, however, indicate that while *pros* mutant R7 cells maintain some R7-specific gene products (e.g., Rh4) and lack R8-specific markers such as Sens, other aspects of R7/R8 differentiation involving R7 nuclear positioning and correct *rh* gene expression are dramatically affected (Figure 7A). We also found that *pros* expression was lost in *sal* mutants, while *sal* expression remained in *pros* mutants. These findings suggest that R7 cells acquire their functional identity through several distinct stages (Figure 7A). Initially, all



**Figure 6. R7 versus R8 Features of *pros* Mutant Photoreceptors**

Analysis of PR projections (A–F), rhabdomere localization (G–J), nuclear position (K–N), and Sens immunostaining (Q and R) in control heterozygous (A, C, E, G, H, K, M, and Q) or *pros*<sup>17</sup> homozygous (B, D, F, I, J, L, N, and R) eyes. Projections are labeled with 24B10/anti-chaoptin (red, [A, B, E, and F]), *rh6-lacZ*-positive cells with anti- $\beta$ -galactosidase (green, [C, D, H, J, M, and N]), and nuclei with Hoechst 33258 (K and N).

(A–F) Control stainings demonstrate that *rh6-lacZ* is only expressed in the R8 layer of the medulla (yellow, [A, C, and E]), whereas in *pros* mutant eyes, *lacZ* expression is also observed in the deeper R7 layer (yellow, [B, D, and F]).

(G–J) Confocal imaging of Rh5 (G and I) and *rh6-lacZ* (H and J) in control (G and H) or *pros*<sup>17</sup> mutant (I and J) eyes. Positive rhabdomere staining is only present in the R8 layer of controls (G and H), but extends to both the R7 and R8 layer of *pros* mutant eyes (I and J). (K–N) Hoechst staining of control retinas reveals distally located R7 nuclei (bracketed) in control retinas (K and M), slightly lower than the outer photoreceptor, pigment cell, and cone cell nuclei at the most external edge of the eye. R8 nuclei, in contrast, are proximally located (K and M). Costaining with *rh6-lacZ* reveals mispositioned nuclei in *pros* mutant eyes (L and N) that correspond to R7 cells (N). (K) and (M), as well as (L) and (N), are from the same cryosections to directly compare R7 and R8 nuclei position.

(O and P) Pros immunostaining in control (Q) versus *salm/salr* deficiency mutant (R) eyes reveals that Pros expression is lost in the absence of *sal* function. The presence of R7 and R8 nuclei in each retinal cryosection (M–R) was confirmed with Hoechst staining, and the loss of *sal* function was confirmed by lack of Sal and Rh6 expression (data not shown).

(Q and R) Immunostaining for Sens in the adult retina reveals that adult R8 cells express Sens (Q). Sens expression remains wild-type in *pros* mutant eyes (R).

eight PRs are recruited at distinct times within the imaginal disc, allowing each to be influenced by a unique cell signaling environment. This recruitment/specification results in the proper establishment of projections to

the optic lobe. At some point afterwards, a common pathway involving *sal* converges onto both the R7 and R8 cells that allows them to continue to develop as inner PRs, rather than adopting an outer PR state. These cells,



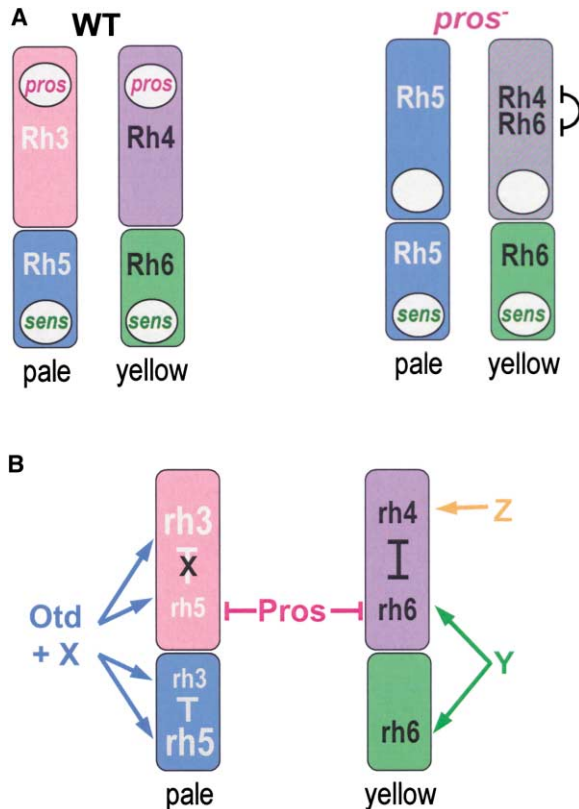


Figure 7. Model of Inner Photoreceptor Cell Fate Decisions

(A) Diagram representing inner PR characteristics in wild-type (WT) or *pros* mutant eyes. These cells differ by their nuclear position in the cell, as well as the expression of the transcription factors Pros and Sens. Under wild-type conditions, Rh3 in R7 cells is coupled with Rh5 in R8 cells in pale ommatidia, whereas Rh4 in R7 cells is coupled with Rh6 in R8 cells in yellow ommatidia. In the absence of *pros*, the nuclei of R7 cells become distally localized in the cell, pale R7 cells express Rh5 and lose Rh3 expression, and yellow R7 cells express either Rh4 or Rh6, depending on the presence or absence of a functional Rh6 molecule.

(B) Model for the regulation of *rh* genes in the adult retina. Pros represses the expression of R8 rhodopsin, *rh5* and *rh6*, in R7 cells. In pale ommatidia, Otd, probably in conjunction with a subset-specific factor (X), activates both *rh3* and *rh5* in both R7 and R8 cells (T.C. and C.D., unpublished data). We also have genetic evidence that factors Y and Z help to establish the expression of the yellow rhodopsins *rh6* and *rh4*, respectively. In the absence of Pros in yellow R7 cells, *rh6* expression is derepressed. The ectopic presence of the Rh6 protein induces an exclusion pathway that leads to the elimination of either *rh4* or *rh6*. By extrapolation, we propose that in pale ommatidia, Rh5 is capable of excluding Rh3 expression.

based on our *pros* loss-of-function experiments, are likely to adopt an R8-like morphology in the absence of additional signals, but must also remain distinct in their expression of cell-specific markers such as *pros* and *sens*. In R7 cells, *pros* helps to promote additional R7-specific characteristics, including the direct repression of R8 rhodopsins and nuclear positioning. Other factors must participate in events leading to their distal positioning in the retina as well as the subtype-specific expression of *rh3* and *rh4* (see Figure 7B). Similarly, additional factors such as Sens are likely to contribute to equivalent aspects of R8 development. The findings reported

here for *pros*, however, are particularly exciting as they provide a genetic inroad for blocking PR differentiation at an intermediate step. Future studies aimed at investigating these later events should be useful for understanding the pathways that transform eight unique cell types into the two functional visual systems in the adult.

#### Prospero and the Regulation of Gene Expression

*prospero* is critical for neuronal cell specification in the developing central and peripheral nervous system, and is transiently expressed in the nucleus of neuronal precursor cells (Knoblich et al., 1995; Reddy and Rodrigues, 1999; Spana and Doe, 1995). Despite extensive work aimed at understanding Pros function, little is known regarding its direct molecular targets. As exit from the cell cycle often precedes terminal differentiation, several studies have correlated the expression of cell-cycle regulators with Pros function. Indeed, the expression of genes such as *decapo*, *string*, *cyclin E*, *E2F*, and *cyclin A* is reduced during *pros*-mediated differentiation, and increased in the absence of *pros* (Li and Vaessin, 2000; Liu et al., 2002). However, it has been difficult to assess which genes are directly controlled by Pros, and those whose expression changes as an indirect requirement to exit the cell cycle, as the Pros target sites within these genes are not known (Myster and Duronio, 2000).

Here, we have identified a functional Prospero target sequence, (T)AAGACG. The only other known Pros binding site,  $C^A/T^C/T^T NNC^C/T^T$  was identified with a site selection assay (SELEX) (Hassan et al., 1997). We could not detect significant similarity between the minimal seq56<sub>B</sub> and the SELEX consensus, but did find a loose SELEX consensus within the full seq56 element, CGGCTAAGAC (underlined, reverse complement of  $C^A/T^C/T^T NNC^C/T^T$ ). However, gel shifts with the GGCTAAG sequence showed no binding to Pros-S or Pros-L, consistent with our findings that the 3' end of seq56 is critical for Pros binding; additional gel shifts revealed that both Pros-S and Pros-L could bind weakly to individual SELEX sites, but only when these sites were multimerized was binding similar to that observed with seq56 (data not shown). Thus, it is likely that Pros exhibits some flexibility in its binding specificity, and as additional Pros target sites are identified, a clearer consensus will develop.

One observation that is supported by both this study and that of Hassan et al. (1997) is that Pros is likely to regulate transcription in combination with other factors. For instance, our findings that seq56 is a conserved imperfect palindrome, and that mutations that do not disrupt Pros binding still lead to R7 derepression (e.g., seq56<sub>A</sub> mutants), imply that the entire seq56 element is necessary to mediate repression. Indeed, although a perfect seq56<sub>B</sub> binding site was found within the *rh4* promoter, no significant changes in Rh4 expression were observed in *pros*<sup>17</sup>, *rh6*<sup>11</sup> mutant eyes (Figure 5), nor in flies misexpressing *pros-L* in all photoreceptors (data not shown); furthermore, mutation of this site within the *rh4* minimal promoter did not abolish reporter expression in R7 cells (data not shown). Thus, these data suggest an important role for the 5' end of the seq56 element in mediating repression.

### Potential for an Evolutionarily Conserved Role of Pros in Rhodopsin Repression and Photoreceptor Development

Similar to the *Drosophila* visual system, fate mapping in vertebrates has revealed that all retinal cell types are derived from the same precursor cell population, and that differentiation of these cell types occurs in a stereotyped order (Cepko et al., 1996). For instance, cone, horizontal, and amacrine cell genesis occurs prior to rod photoreceptor formation, followed by bipolar and Müller glia genesis. Recent findings have demonstrated that Prox1, the vertebrate counterpart of Pros, is embryonically expressed in dividing retinal progenitor cells, and postnatally expressed in differentiating horizontal cells, A<sub>11</sub> amacrine cells, and at lower levels in bipolar cells (Belecky-Adams et al., 1997; Tomarev et al., 1996; 1998; Dyer et al., 2003). Thus, Prox1 expression in horizontal/amacrine precursors may serve to drive these cells toward terminal differentiation and prevent them from dividing to become later cell types such as PRs. Indeed, in the *prox1* knockout mouse, horizontal cells fail to form, while the rod PR number is increased 50%–70% (Dyer et al., 2003). Therefore, it is likely that Prox1/Pros plays an evolutionarily conserved role in specifying unique neuronal cell types in the eye. While a more precise comparison between vertebrate and invertebrate PRs is difficult, *opsin* gene expression represents a late differentiation step in all PR development. Thus, it will be interesting to explore the possibility that Prox1/Pros regulate *opsin* expression throughout evolution. Indeed, preliminary analyses have revealed the presence of putative Pros binding sites within the minimal promoter of the *Xenopus rhodopsin* promoter (T.C., unpublished data; data not shown). Furthermore, these sites are located precisely in a region demonstrated to function as a repressor element both in cultured cells and in vivo (Mani et al., 2001). Thus, we believe that Pros/Prox1 is a key regulator of both early and late stages of PR development, not only in insects but also in vertebrates.

### Experimental Procedures

#### Identification of Common Promoter Elements in the Rhodopsin Genes

The six *rh* gene promoters from several *Drosophila* species was aligned using Megalign software (DNASStar, Madison, WI). Evolutionarily conserved elements (strings) were extracted and analyzed further using motif extraction software (e.g., Gibbs sampler). This revealed the specific presence of the motif CGGCTAAGACG in the two R8 *rh* promoters, *rh5* and *rh6* (data available from our web resource <http://homepages.nyu.edu/~dap5/rh/rhodopsins.html>).

#### One-Hybrid Screen

Bait plasmids carrying four concatamerized copies of 5'-gacctgCGGCTAAGACGctgctgCGGCTAAGACGctgctgtagatct-3' (capitalized letters representing seq56) were cloned into pHis1 and pLacZi (ClonTECH) and stably transformed into the yeast strain YPH499 (*MATa ade2-101<sup>o</sup> lys2-801a ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1*, kindly provided by Isabelle Brun). These yeast were then transformed with a transactivation fused *Drosophila* head-specific cDNA library (kindly provided by Michael Rosbash, Brandeis University). ~5 × 10<sup>6</sup> CFU were amplified on SD/His<sup>+</sup>/Ura<sup>-</sup>/Trp<sup>-</sup> media, and selected on the same media containing 40 mM 3-amino-1,2,4-triazole (Sigma), 2% galactose, 1% raffinose. Of ~5 × 10<sup>8</sup> amplified CFU, 9 *HIS3<sup>+</sup>/lacZ<sup>+</sup>* clones were identified: four that weakly activated *lacZ* expression, and five that strongly activated. The cDNAs from all

positives were PCR-amplified and sequenced. The weak positives represented CG1883 twice (ribosomal protein S7), CG7726 (ribosomal protein DL11), and CG8621 (function unknown). All five strong *lacZ<sup>+</sup>* colonies corresponded to aa 1112–1371 of Pros-S (GenBank #P29617).

#### Characterization of Pros-S and Pros-L

0.5 μg DNA from a 0–6 hr *Drosophila* embryonic cDNA library (kindly provided by Isabelle Brun), the adult *Drosophila* head cDNA library used for the screen, one-hybrid positives, or genomic DNA from UAS-*pros-L* flies was used to amplify the homeodomain and C terminus of Pros using the primers tacgggatccATCACGGCGGATCGCCGGACTAC (5') and cgtactcggagTCGGCAGGCCAACTCCTCCACT (3') (capital letters representing *pros*, restriction sites underlined). Both isoforms from the embryonic cDNA library were purified, digested with BamHI/XbaI (sites included in the primers), cloned in-frame into the GST fusion vector pGEX-5X-1 (Pharmacia), and verified by sequencing. GST protein purification and gel shift assays were performed as described (Cook et al., 1998). Gel shift probes were comprised of the sequences tgcagggatcctgCGGCTAAGACGtatctagatct (for A/B variants) or tcagggatccggTAAAGACGtctctagatct (for B box variants) (seq56-derived sequence in capitals).

#### Transgenic Constructs and Genetic Crosses

*rh* promoter mutants were generated by PCR and verified by sequencing. A pCasper GAL4 driver was generated by replacing the BamHI-XbaI *lacZ* gene fragment in pCHABΔSal (Wimmer et al., 1997) with a BamHI-SpeI GAL4 coding sequence from pGaTB. Transformants were generated in a *yw* background as previously described (Wimmer et al., 1997), and at least five different lines were generated for each construct. Whole mutant *pros* eyes were generated using the EGUF system, a method which kills wild-type homozygous and heterozygous eye tissue by virtue of a GMR-Hid transgene and a cell-lethal mutation (l(3)CL-R<sup>1</sup>) on the FRT82B chromosome (Stowers and Schwarz, 1999). FRT-induced mitotic recombination was catalyzed by *ey-flip*, an eye-specific source of the FLIP recombinase. *ey-FLP*; Sp/CyO; FRT82B-GMR-Hid/TM6b flies were crossed to *yw*; -;FRT82B-*pros*<sup>17</sup>/TM6b or *yw*; -;FRT82B-*pros*<sup>101</sup>/TM6b. *ey-FLP*; +/Sp,CyO; FRT82B-GMR-Hid/FRT82B-*pros* flies were analyzed as *pros*<sup>-/-</sup>, and *ey-FLP*; +/Sp,CyO; FRT-*pros*/TM6b were used as controls. *ey-FLP*, GMR-GAL4, and UAS-*lacZ* flies were kindly provided from J. Treisman (Skirball, NYU), UAS-*pros-L* and FRT82B-*pros*<sup>17</sup> flies from C. Doe (University of Oregon), and FRT82B-*pros*<sup>101</sup> flies from R. Carthew (Northwestern University). *salm*<sup>03602</sup>, *pros*<sup>10419</sup>, FRT82B-GMR-Hid, l(3)CL-R<sup>1</sup> and FRT40-GMR-Hid, l(2)CL-L/CyO flies were supplied by the Bloomington Stock Center. Flies carrying the FRT82B-*pros*<sup>17</sup> allele also carried the *rh6*<sup>11</sup> mutation (see below). Thus, we recombined wild-type *rh6* from *yw*<sup>122</sup> flies onto the FRT82B-*pros*<sup>17</sup> chromosome. Wild-type *rh6* and null *pros* expression of newly established FRT82B-*pros*<sup>17</sup> alleles were analyzed by immunostaining. Two stocks, named *pros*<sup>17,15</sup> and *pros*<sup>17,17</sup>, were retained. Minimal *rh-lacZ* transgenes were as described (Papatsenko et al., 2001) and introduced on the second chromosome. Flies were raised at 25°C, and pupal staging was according to Bainbridge and Bownes (1981).

#### β-Galactosidase Stainings and Immunostainings

X-gal staining of adult head cryosections was performed as previously described (Papatsenko et al., 2001). Immunostaining of adult head cryosections was as follows: 8–10 μm sections were fixed in 4% paraformaldehyde for 15 min, washed thrice with PBX (PBS, 0.3% Triton-X100), and incubated with primary antibodies diluted in BNT (PBS, 0.1% BSA, 0.1% Tween 20, 250 mM NaCl). The antibodies and dilutions used were: rabbit anti-Rh4 (gift from C. Zuker, University of California at San Diego) 1:300, chicken anti-Rh3 (generated against the peptide sequence LALNEKAPESSAVA) 1:20, mouse anti-Rh4 1:10 and mouse anti-Rh5 1:50 (both gifts from S. Britt, University of Colorado), rabbit anti-Rh6 (A. Tahayato et al., submitted) 1:5000, rabbit anti-β-galactosidase (Cappel) 1:5000, mouse anti-β-galactosidase (Promega) 1:500; guinea pig anti-Sens (H. Bellen, Baylor College of Medicine) 1:750, mouse anti-Pros (C. Doe, University of Oregon) 1:10, rabbit anti-Sal (B. Mollereau, Rockefeller University) 1:100, and mouse anti-chaoptin, 24B10 (Developmental

Studies Hybridoma Bank, University of Iowa) 1:10. Samples were then washed with PBX and incubated with appropriate secondary antibodies (AlexaFluor488, Cy3 or Cy5 conjugated) from Molecular Probes (Portland, OR) and Jackson ImmunoResearch Laboratories (West Grove, PA) at manufacturer's suggestions. Nuclei were stained 2 min with 1  $\mu$ g/ml Hoechst 33258 (Sigma) prior to mounting. Samples were mounted with Aquamount (Lerner Laboratories) and analyzed with a Leica TCS S2 confocal system or a Nikon Microphot-SA microscope. Digital images were obtained using SPOT software.

#### Rh6 Mutant Analysis

The 3R chromosomal arm carrying the FRT82B-*pros*<sup>17</sup> allele contained a naturally occurring 19 bp deletion in the *rh6* gene (the existence of this mutation in common stocks was originally pointed out to us by S. Britt). This mutation, *rh6*<sup>11</sup>, includes a stop codon that leads to truncation of the Rh6 protein within the 5<sup>th</sup> transmembrane domain and thus is not recognized by anti-Rh6 antibodies. It should be noted that this mutant sequence, and not that originally described (Huber et al., 1997), is currently deposited in the Berkeley *Drosophila* Genome Project, and thus is likely to be present in a large number of available fly stocks. Specifically, it is present in *w*<sup>1118</sup> flies from the Bloomington Stock Center.

#### Acknowledgments

Thanks go to the many members of the fly community who have provided us with reagents and helpful comments, and Terry Turner-Blackman for her assistance in the creation of transgenic flies. We also appreciate S. Britt, R. Carthew, C. Cepko, M. Dyer, and G. Olivier for discussions and for allowing us to cite their unpublished results. Finally, we thank members of the Desplan lab, J. Blau, B. Gebelein, and R. Mann for invaluable comments to the manuscript. T.C. was supported by NIH fellowship F32-EY07054, and F.P. by a fellowship from HFSP. This work was supported by NIH/NEI grant R01-EY13010 to C.D.

Received: February 4, 2003

Revised: April 1, 2003

Accepted: April 14, 2003

Published: June 2, 2003

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