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Feedback from Rhodopsin controls *rhodopsin* exclusion in *Drosophila* photoreceptors

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Abstract

Sensory systems with high discriminatory power employ neurons that express only one of several alternative sensory receptor proteins. This exclusive receptor gene expression restricts the sensitivity spectrum of neurons and is coordinated with the choice of their synaptic targets[1-3]. However, little is known about how it is maintained throughout the life of a neuron. Here we show that the green-light sensing receptor Rhodopsin 6 (Rh6) acts to exclude an alternative blue-sensitive Rhodopsin 5 (Rh5) from a subset of *Drosophila* R8 photoreceptor neurons[4]. Loss of Rh6 leads to a gradual expansion of Rh5 expression into all R8 photoreceptors of the aging adult retina. The Rh6 feedback signal results in repression of the *rh5* promoter and can be mimicked by other *Drosophila* Rhodopsins; it is partially dependent on activation of Rhodopsin by light, and relies on $G_{\alpha q}$ activity, but not on the subsequent steps of the phototransduction cascade[5]. Our observations reveal a thus far unappreciated spectral plasticity of R8 photoreceptors, and identify Rhodopsin feedback as an exclusion mechanism.

In the *Drosophila* visual system, Rhodopsins (Rh), G-protein coupled receptors, detect light and initiate the phototransduction cascade leading to depolarization of photoreceptor neurons[5] (PR). Each ommatidium, the unit eye of the adult retina, contains eight PRs. Six outer PRs, R1-R6, express Rh1, and are involved in motion detection and dim light vision (reviewed in ref. 4). Inner PRs R7 and R8 mediate color vision and define two main

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ommatidial subtypes based on the Rh they express: In pale (p) ommatidia, pR7 expresses UV-sensitive Rh3 while pR8 expresses Rh5; in yellow (y) ommatidia, yR7 expresses a distinct UV-sensitive Rh4 while yR8 expresses Rh6[4]. p and y subtypes are distributed stochastically throughout the main part of the retina with an approximate 30:70 ratio (Fig. 1c)[6]. An exception to the exclusive Rh expression exists in the medio-dorsal area of the eye, where although the p and y subsets are correctly specified, Rh3/Rh4 are co-expressed in yR7s[7]. This Rh expression pattern is established by a well understood developmental program executed during pupal stages[4,8,9] (Supplementary Fig. 1a, b). It is unknown, however, how p and y PR subtypes are maintained in the adult fly. The example of vertebrate olfaction, where sensory receptors act to repress expression of alternative receptor genes[10-14], led us to ask whether Rhs themselves participate in maintaining their mutual exclusion by analyzing Rh expression in various *rh* mutants. We found that in *rh6*^{1} mutants (Fig. 1a), the number of R8 cells expressing Rh5 increases dramatically and that this expansion of Rh5 is age-dependent (Fig. 1b,d,e, Supplementary Table 1). In one-day-old *rh6*^{1} mutant flies, Rh5 expression appears normal with ~38% of R8s expressing uniformly high levels of Rh5 protein. In three-day-old flies, additional R8s begin to express low levels of Rh5. By 14 days, nearly all (95%) R8s express Rh5. The levels of ectopic Rh5 in individual yR8s also increase over time, but remain variable and often are lower than in pR8 (Fig. 1e). In control flies, the number of Rh5-expressing R8s and the levels of expression remain stable as the flies age (36%, Fig. 1b,c, Supplementary Table 1).

We next asked if other Rhs are controlled by Rh-mediated repression. We examined whether Rh6 expression is de-repressed in *rh5* mutants, but did not detect any Rh6 protein in pR8 in 3 week old *rh5* mutants (Supplementary Fig. 2f). Expression of the non-R8 Rhodopsins Rh1, Rh3 and Rh4 also remains normal in *rh5* or *rh6* mutants older than 3 weeks as well as in *rh5; rh6* double mutants (Supplementary Fig. 2a-e,g,h). Nonsense mutations in *rh3* or *rh4* genes do not affect expression of the remaining Rhs in R7s in either young or old (over 3 weeks) flies (Supplementary Fig. 3, 4, data not shown). Thus, a Rh-dependent mechanism for controlling Rh expression occurs only in yR8s. Moreover, Rh5 is the only Rh that is actively repressed by Rh6. In the *rh6*^{1} allele, commonly found in lab stocks, a short deletion that spans the first exon-intron junction leads to a truncation of the protein after its 5th transmembrane domain[15] (Fig. 1a). The levels of *rh6* mRNA measured by qRT-PCR are more than tenfold lower in *rh6*^{1} mutants than in wild-type flies (Supplementary Fig. 9a), likely due to nonsense mediated decay. The Rh5 de-repression phenotype does not become more severe when *rh6*^{1} is placed over a deficiency (Supplementary Fig. 9b), suggesting that *rh6*^{1} is a null allele. And, *rh6*^{1} can be rescued by a 2,575bp genomic fragment encompassing the *rh6* locus (Supplementary Fig. 7a, 9b). Hereafter we refer to both *rh6*^{1} homozygotes, and *rh6*^{1} trans-heterozygotes over a deficiency as *rh6* mutants and, unless otherwise noted, all phenotypes described are in “old” flies two weeks post-eclosion or older.

We identified a second *rh6* allele, also in a lab stock, which we named *frank sinatra* (*rh6*^{fs}) after the singer known as “Ol’ blue eyes” (Fig. 1a). This mutation removes 58bp of the *rh6* regulatory region without affecting the coding sequence. In *rh6*^{fs} mutants, Rh6 protein is detectable only in a few R8s in retinas of young flies (6.5% ± 4.4 SD, Supplementary Fig. 9b) where it is expressed at levels generally lower than normal (Fig. 1f,g). As in *rh6*^{1}

mutants, Rh5 is initially expressed normally in 41% of R8 in *rh6*^{fs} flies (Fig. 1f), leaving most yR8s devoid of Rh expression. However, Rh5 becomes broadly de-repressed in R8s of old flies (Fig. 1g, Supplementary Fig. 9b). Rh5 is rarely expressed in the few Rh6-positive R8s of *rh6*^{fs} mutants and co-expression only occurs in cells with low Rh6 levels (not shown). We also used a *rh6* promoter-based driver (*rh6-Gal4*) to express a UAS-RNAi construct targeting *rh6* in differentiated yR8s. Although this does not completely abolish Rh6 in yR8 rhabdomeres, it leads to de-repression of Rh5 in old flies (Supplementary Fig. 5a). These results support the idea that reducing the levels of normal Rh6 activity leads over time to de-repression of Rh5 expression in yR8s.

Repression of Rh5 by Rh6 in wild-type yR8 could occur transcriptionally, or post-transcriptionally. We thus asked whether *rh5* mRNA expression is de-repressed in *rh6* mutants by performing *in situ* hybridization. *rh5* mRNA is present in many more R8s in old *rh6* mutants than in age-matched wild-type flies (Fig. 2a,b). To more clearly visualize this phenotype, we repeated the experiment in a *sevenless* (*sev*) mutant background in which R7 PRs are absent[16]. Because specification of *rh5*-expressing pR8s depends on the overlying pR7s (Supplementary Fig. 1a), most cells become yR8 and express Rh6 in *sev* flies while Rh5 is only expressed in a few R8 PRs[17-19] (~3%, Fig. 2c). However, in old *sev; rh6* double mutants, *rh5* mRNA is de-repressed in most R8s (Fig. 2d). We also quantified changes in *rh5* mRNA expression using qRT-PCR: in 2 week old *rh6* mutants, *rh5* mRNA more than doubles over normal levels (Supplementary Fig. 9a). To ask whether this occurs through repression of the *rh5* promoter rather than by affecting mRNA stability, we analyzed the expression of a *rh5* reporter (*rh5*>GFP) containing a −690 to +50 *rh5* promoter fragment[20]. In control flies, *rh5*>GFP is co-expressed with Rh5 protein in pR8s (Fig. 2e). In *rh6* mutants *rh5*>GFP expression begins normally but with age expands to most yR8s (Fig. 2f, Supplementary Fig. 9f). This supports the model that Rh6 generates a feedback signal that acts to repress transcription from the *rh5* promoter and that the relevant regulatory sites are contained within the short promoter fragment of the *rh5*>GFP transgene.

Expression of *rh5* in yR8s of *rh6* mutants could be due to a change in yR8 cell identity, either during specification or in adults. To test this, we first asked whether a reporter for *rh6* expression (*rh6-lacZ*) is correctly activated in *rh6* mutant flies. In young *rh6* mutants, *rh6-lacZ* is robustly expressed in R8s in a pattern complementary to Rh5 expression (Fig. 3a, Supplementary Fig. 9c) suggesting correct specification of the yR8 subtype. As the fly ages, these cells de-repress Rh5 but remain positive for βGal (Fig. 3b, Supplementary Fig. 9c). We also tested for a possible yR8-to-pR8 fate transition using the marker genes that specify these cells. The p *vs*. y subtype specification of R8 cells depends on an R8-intrinsic bistable switch involving mutual transcriptional repression between *warts (wts*) and *melted* (*melt*) (Supplementary Fig. 1b). During pupal development, Wts represses *melt* to specify yR8 PRs. In response to an extrinsic signal originating in pR7, *melt* is up-regulated in pR8, leading to repression of *wts* transcription and expression of Rh5[8]. Thus, Melt marks pR8 and Wts marks yR8 cells (Fig. 3c,e). In old *rh6* mutant flies, a *melt* reporter (*melt-nlacZ*) remains restricted to a subset of R8 cells, while Rh5 expression expands broadly to cells that do not express *melt-nlacZ* (Fig. 3d, Supplementary Fig. 9c). In addition, we do not observe down-regulation of a *wts* reporter (*wts-nlacZ*) in yR8s of old *rh6* mutants, leading to co-expression

of *wts* with ectopic Rh5 (Fig. 3f, Supplementary Fig. 9d). While maintenance of *rh6-lacZ* and *wts-nlacZ* could potentially be due to perdurance of βGal protein, lack of de-repression of *melt-lacZ* argues that loss of *rh6* function does not affect the identity of yR8 in old flies. Moreover, it shows that *melt* is not involved in Rh5 de-repression. Thus, in *rh6* mutants, the yR8 fate is specified normally and remains stable. This indicates that yR8 produces positive transcriptional regulatory inputs to which the *rh5* promoter can respond and which must be actively repressed by the presence of Rh6. In contrast to the way pR8 *rh5*-expressing PR fate is established, these inputs do not depend on extrinsic signals from R7 cells since, as described earlier, the absence of R7s in *sev* mutants does not suppress the *rh6* mutant phenotype.

yR8 cells are not the only PRs expressing Rh6. The larval eye, Bolwig's organ, is composed of about twelve PRs[21,22]. Four primary PRs express Rh5 while the eight secondary PRs express Rh6 (Supplementary Fig. 6a). During metamorphosis, secondary PRs die while the primary PRs down-regulate Rh5 and up-regulate Rh6[23]. The newly Rh6-expressing cells form the eyelet, an adult extra-retinal visual organ[24,25] (Sup. Fig. 6c). In *rh6* mutants, neither the secondary Bolwig PRs nor the eyelet PRs ever express Rh5 and are thus devoid of any Rh (Supplementary Fig. 6b, d). Therefore, in contrast to the retina, Rh6 is not necessary for exclusion of Rh5 expression in the eyelet, consistent with the view that expression of Rh5 and Rh6 is under distinct control mechanisms in the Bolwig's organ/eyelet and in the adult retina[22]. This result, together with the absence of Rh5 de-repression in R7s of *rh3* and *rh4* mutants, argues that, in the absence of a Rh signal, de-repression of Rh5 can only occur in yR8 PRs.

Because Rh5 is only de-repressed in yR8s of *rh6* mutants, it is possible that the repressive signal is generated uniquely by Rh6. Therefore, we tested whether the *rh6* mutant phenotype in yR8s could be rescued by Rhs other than Rh6. We used *rh6*-Gal4 to drive expression of UAS-Rh1, -Rh3, -Rh4 or -Rh6 in *rh6* mutants. In every case, the de-repression was rescued and little or no Rh5 expression was detectable in yR8 PRs (Fig. 4 a,b, Supplementary Fig. 7b-e, 9e). Expression of UAS-Rh5, as with Rh1, Rh3 and Rh6, also largely blocked de-repression of the *rh5*>GFP reporter in *rh6* mutants (Fig. 4c, Supplementary Fig. 7f-i, 9f), suggesting that a generic *Drosophila* Rh signal is sufficient to maintain exclusion of Rh5 in yR8 cells. Since these transgenes are controlled by the *rh6* promoter, they are expressed only after specification of the yR8 subtype, further arguing that the signal is only required for the maintenance of the exclusion of Rh5, and not for yR8 subtype specification. In addition, negative regulation by Rh5 of its own expression in yR8 could provide an explanation for why the levels of Rh5 expression in yR8 of *rh6* mutants are generally lower than in wild-type pR8 cells.

The requirement for a Rh-dependent signal to maintain repression of *rh5* in yR8s led us to ask whether activation of Rh6 by light is involved in this process. We maintained wild-type flies in complete darkness for more than 2 weeks starting at mid-pupal stages. In these flies, a significant proportion (~12%, Supplementary Table 2) of the Rh6-expressing yR8s also express low levels of Rh5 (Fig. 4d,e, Supplementary Fig. 9g), which is not observed in old wild-type flies reared in the light. Interestingly, this de-repression of Rh5 occurs predominantly in the dorsal retina (Supplementary Table 2, Supplementary Fig. 9g)

indicating an underlying spatial variation in Rh5 de-repression. In contrast, Rh6 is not de-repressed in pR8s of dark-reared flies. Thus, it appears that adult yR8 PRs remain plastic with respect to Rh exclusion and that simply preventing activation of Rh6 by light can evoke Rh5 expression in yR8s. This derepression of Rh5, however, is substantially weaker than in *rh6* mutants. This could indicate that either activated Rh6 somehow accumulates in the dark and is able to partially repress *rh5*, or that Rh6 retains a residual ability to repress *rh5* without being activated by light. These alternatives are consistent with the observation that partial reduction of Rh6 protein through RNA interference can lead to de-repression of *rh5* (Supplementary Fig. 5). Hence, *rh5* repression is sensitive to the level/activity of Rh6.

The role of light and interchangeability of Rhs in controlling expression of *rh5* raised the possibility that components of the phototransduction cascade (reviewed in ref. 5) might play a role in repression of *rh5*. In flies, activated Rh converts the $G_{\alpha q}$ subunit of a heterotrimeric G-protein to a GTP-bound form which dissociates from the $G_{\beta\gamma}$ dimer and activates Phospholipase C (PLC) encoded by the *norpA* gene. PLC then catalyzes hydrolysis of PIP_2 which leads to the activation of TRPC channels[26], inflow of Ca^{2+}, and depolarization of the PRs. We asked whether components of this phototransduction cascade mediate the *rh5*-repressive signal. In $G_{\alpha q}{}^{1}$ hypomorphic mutants, Rh5 is expressed normally in young flies but becomes de-repressed in yR8 as the flies age (Fig. 4f,g, Supplementary Fig. 9h), a phenotype similar to that of *rh6* mutants. This results in the co-expression of Rh5 and Rh6 in yR8 cells. However, neither removal of PLC (in $norpA^{36}$ mutants) nor of TRPC channels (in $trpl^{302}$; trp^{301} double mutants) leads to de-repression of Rh5 in yR8s of old flies (Supplementary Fig. 8, 9h). The observation that $G_{\alpha q}$, but not the rest of the phototransduction cascade is important for the *rh5*-repressive signal indicates a bifurcation of the phototransduction and *rh5*-repression pathways downstream of $G_{\alpha q}$. Alternatively, $G_{\alpha q}$ might function genetically upstream of Rh6, for example, by stabilizing the Rh6 protein. In either case, Rh6 uses a pathway distinct from phototransduction to repress *rh5*. Importantly, the $G_{\alpha q}$ mutant phenotype and de-repression of Rh5 in dark-raised wild-type flies further support the idea that maintenance of *rh5* repression requires the activity of the Rh6 protein.

Rhs canonically act as sensory receptor proteins. However, Rh1 also has non-visual functions; it is required for the proper formation and maintenance of the rhabdomeres of R1-R6 PRs[27,28] and has recently been shown to be involved in thermotactic discrimination[29]. We showed here a new and surprising role for Rh6: it represses transcription of an alternative receptor gene, *rh5*, and thereby maintains the sensory specificity of yR8. This mechanism prevents Rh5/Rh6 co-expression, which would broaden the sensitivity spectrum of yR8s PRs[30], limiting the ability of the visual system to discriminate colors. Furthermore, change in the yR8 spectrum could lead to sensory confusion if the downstream neuronal circuits misinterpret the information they receive. The repression of *rh5* by Rh6 also illustrates a so far unappreciated plasticity of yR8 PRs, as revealed by de-repression of Rh5 in wild-type flies reared in darkness. Constant darkness could mimic special environmental conditions, natural for the fly, under which lowered Rh6 activity evokes expression of Rh5 in yR8 PRs to change spectral properties of the eye, or simply to boost the overall light

response. Finally, the fact that we found two different *rh6* mutations in laboratory stocks raises a possibility that mutations in the *rh6* gene are also frequent in the natural population.

Repression of *rh5* by Rh6 is reminiscent of the control of olfactory receptor (OR) genes in vertebrate olfactory neurons (OSN)[14], which encode G protein-coupled receptors (GPCR) similar to Rhs. With rare exception, each OSN expresses only one allele of one OR gene. This exclusion mechanism is not well understood, but requires an active OR to generate a feedback signal for repression of other OR genes[10-14]. There, however, the feedback control of exclusion appears to be a common mechanism in all OSNs, in contrast to the fly retina where only *rh5* is regulated by another Rh, and only in the yR8 PR subtype.

Our findings show that cross-repression of sensory receptors is not unique to vertebrate chemosensory systems, but could be a more widely implemented mechanism by which mature sensory neurons, or other GPCR-expressing cells, maintain their functional specificity. The relative simplicity of yR8 photoreceptors as a system should allow us to uncover the molecular details by which a GPCR can exclude expression of other seven TM receptors in the same cell.

METHODS SUMMARY

Flies were raised on standard corn meal-molasses-agar medium at room temperature (24°C) in ambient laboratory light except for RNA interference experiments (at 29°C) and dark isolation experiments (in complete darkness). Dissected adult retinas were stained whole-mount with specific primary antibodies and then with Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Larval eyes were stained as in ref. 22. *In situ* hybridization on cryo-sectioned adult retinas was performed with DIG-labeled antisense probe transcribed from *rh5* 3′UTR region as described in ref. 7. Samples were imaged using Leica TCS SP2 and SP5 confocal microscopes. Images were processed and counts performed using Leica Confocal Software, Adobe Photoshop and Fiji software. For real time PCR RNA was purified from 20 flies/sample and cDNA amplified using SYBR-Green PCR Mix (Stratagene).

METHODS

Flies were raised on standard cornmeal/molasses/agar medium at room temperature (24°C) in ambient laboratory light unless otherwise noted. RNA interference experiments were performed at 29°C. For dark isolation experiments, flies were reared in a light-proof box, and for aging transferred between vials in complete darkness starting at mid-pupal stages (prior to Rh expression[31]).

Drosophila strains

For ***wt*** controls we used y^1 w^{67}; *Sp/CyO;* wt^{isoB} flies. "*isoB*" represents an isogenized *wt* 3rd chromosome.

rh6 alleles—The ***rh6*^{1}** allele[15] is found in many commonly used laboratory fly strains. The existence of this mutation in common stocks was originally pointed out to us by S. Britt.

This mutation is present on some TM6B balancer chromosomes and in the reference fly strain sequenced for the published fly genome[15] (BDGP Release 5.29). The mutation replaces 21 bases (lowercase in TGACCATCATCTTCTcctactggcacatcatgaaggTATGACATTCGTTA) at the end of the 1st exon with two As, removing a splice donor site and introducing a stop codon immediately afterwards. This results in the truncation of the ORF within the 5th trans-membrane domain of the presumptive protein. The original allele was backcrossed into *wt* ^{isoB} background (see above) four times. We identified ***rh6*^{fs}** as a mutation in a stock from the Bloomington stock center (Stock 1385, named genotype z^{v77h} w^{67c23}) which mapped to the 3rd chromosome. Sequencing of *rh6* locus revealed a 58 bp deletion upstream of the *rh6* transcription start site, which removes sequence AGCGGCAATCGAAAGCCCAATTCGAACGGTTAGCTTTGGATTGGCCAAGTGCCGGCTA within the *rh6* promoter. We named this mutation after the singer Frank Sinatra, for his nick name “Ol’ blue eyes”, since eyes of old *rh6*^{fs} mutant flies broadly express the blue-sensitive Rhodopsin, Rh5. The deficiency used in this study that covers *rh6* gene, ***Df(3R)Exel6174***, was generated by Exelexis Inc. and spans 3R: 11154443-11154444..11363188 [32].

To generate flies with **a *rh6* genomic rescue fragment, *C{rh6*$^{+}$*}***, the *rh6* sequence was PCR-amplified from genomic DNA of y^{1} w^{67}*; Sp/CyO; wt* ^{isoB} flies with dv173 (acaagcttacctacaagagcaccagtcc) and dv174 (acgaattcacctcggcctgaacacctac) primers to produce a 2575bp genomic fragment (ACCTACAAGAGCACCAGTCC… GTAGGTGTTCAGGCCGAGGT) with HindIII and EcoRI flanking sites. PCR product was ligated into HindIII, EcoRI sites of pBS-loxP-w-lox2272 vector [33]. Cre-recombinase mediated integration was used to insert this construct into lox landing site A11 (on 2nd chromosome, S. Small, personal communication). A single integration occurred without replacement of y^{+} marker of the landing site. Successful transformation was confirmed with antibody stain for Rh6 protein in whole mount retinas: normal Rh6 expression was detected in *rh6* mutant background.

UAS-rh6RNAi (Transformant #102152) was obtained from Vienna Drosophila RNAi Center (VDRC)[34].

Other mutants generated for this study—*rh3*^{1} mutant (a nucleotide change C278T resulting in Q46* truncation) was obtained by TILLING (Seattle TILLING Project) [35]. The mutation was back crossed into *wt* background four times (confirmed by genomic PCR and by stain of whole mount retinas with anti-Rh3 antibody). ***rh4*^{1}** mutant (a nucleotide change T727A resulting in Y203* truncation between 4th and 5th trans-membrane domains) was obtained by TILLING (Seattle TILLING Project) [35]. The mutation was back crossed into *wt* background four times (confirmed by genomic PCR and by stain of whole mount retinas with anti-Rh4 antibody).

Transgenes generated for this study—*rh5>GFP* flies carry two transgenes recombined on the 2nd chromosome: ***rh5-lexA*** and ***lexAop-GFP***.

rh5-lexA: *lexA* (from pBS-lexA SV40 3′ UTR [36]) was cloned into pBS-LoxP-white-Lox2272 [33] and named LexA-Lox. 740 bp fragment of *rh5* promoter which ends 23 bases upstream of ATG (TCGGAAAATGTCGTGCAAGTGTTC … AATGTCGACCTGCAAAGGAAACTA; Fly genome: 12007686..12008425) was PCR amplified from genomic DNA using oBJ109 (tcggaaaatgtcgtgcaagtg) and oBJ140 (tagtttcctttgcaggtcgac) and cloned into the PCRII-TOPO vector (Invitrogen). The *rh5* promoter was cut with ClaI, blunted, and subcloned into the LexA-Lox which was cut with SpeI and blunted. Cre-recombinase mediated cassette exchange was used to insert this construct onto the 2nd chromosome[33].

lexAop-GFP: GFP with SV40 3′UTR was PCR amplified from the pIRES2-eGFP vector (Clontech) with the primers oBJ78 (taatactagtatggtgagcaagggcgaggag) and oBJ79 (gtcaggatccaccacaactagaatgcagtg) and cloned into the PCRII-TOPO vector (Invitrogen). The GFP-SV40 3′UTR was subcloned into the pLOT vector (containing lexAop) [36] using the EcoRI site.

UAS-Rh1: EcoRI-KpnI fragment of *rh1* cDNA (containing sequence spanned by GGCAGGTTTCCAACGACCAATCGC … AAGGACAAAAAAAAAACTCAAC+15A) from rh1-pFLC-1 plasmid (Drosophila Genomics Resource Center (DGRC) clone RH01460[37]) was ligated into EcoRI-KpnI sites of pUASTattB vector[38] to produce pDV131 plasmid. ϕC31-mediated integration was used to insert this construct into 2nd chromosome landing sites *attP-51D, attP-58A* and *attP40* [38,39]. w^+ and *3xP3-RFP* markers of *attP-51D* and *attP-58A* landing sites were removed through lox-mediated recombination by crossing in Cre recombinase transgene [38].

UAS-Rh3: EcoRI-XhoI fragment of *rh3* cDNA (containing sequence spanned by CAGACCGGAGCATGGAGTCCGGTA … AATATAGTAAAATTACAGCAAGCT +19A) from rh3-pOT2 (Drosophila Genomics Resource Center (DGRC) clone GH02505 [37]) into EcoRI-XhoI sites of pUASTattB vector [38] to produce pDV133 plasmid. ϕC31-mediated integration was used to insert this construct into 2nd chromosome landing sites *attP-51D, attP-58A* and *attP40* [38,39]. w^+ and *3xP3-RFP* markers of *attP-51D* and *attP-58A* landing sites were removed through lox-mediated recombination by crossing in Cre recombinase transgene [38].

UAS-Rh4: Cloned EcoRI-KpnI fragment of *rh4* cDNA from rh4-pFLC-1 (Drosophila Genomics Resource Center (DGRC) clone RH33063[37]) into pUASTattB vector [38]. To correct a frameshift in the sequence, EcoRI-BglII fragment was replaced with cDNA fragment that had a longer 5′ UTR. The resulting pDV134 plasmid contained *rh4* cDNA sequence spanned by CAGAGCGAAACGGGTAGCGGT… AACTTATTGCAAACGAAGTAG+16A. ϕC31-mediated integration was used to insert this construct into 2nd chromosome landing sites *attP-51D* and *attP40* [38,39]. w^+ and *3xP3-RFP* markers of *attP-51D* landing site were removed through lox-mediated recombination by crossing in Cre recombinase transgene[38].

UAS-Rh5: EcoRI-XhoI fragment of *rh5* cDNA (containing sequence spanned by CGGAGGCCAGAATGTCGACCT … TACAAACCAAAAAAAGTTGGCATT +78A) from rh5-pOT2 (Drosophila Genomics Resource Center (DGRC) clone GH28578 [37]) into EcoRI-XhoI sites of pUASTattB vector[38] to produce pDV135 plasmid. ϕC31-mediated integration was used to insert this construct into 2nd chromosome landing site *attP40* [39].

UAS-Rh6: It has proven difficult to generate a *UAS-Rh6* cDNA construct expressing high levels of Rh6. Therefore, we cloned a PCR-amplified genomic (with introns) fragment of *rh6* gene downstream of transcriptional start site (containing sequence spanned by CAGGCATTGCCGCCGAGTTCGCGT … ACAGCAATTGATACAAAATC) into EcoRI-KpnI sites of pUASTattB vector [38] to produce pDV160 plasmid. ϕC31-mediated integration was used to insert this construct into 2nd chromosome landing site *attP40* [39].

Other strains—*G_{aq}^{1}* [40], *norpA*^{36} [41], *rh5*^{2} [42], *sev*^{14} [43], *trpl*^{302}*; trp*^{301} [44], *melt-nlacZ* [8], *rh6-Gal4* [20], *rh6-lacZ* [45], *wts-nlacZ* [46,47].

Antibodies

Antibodies and dilutions used were as follows: mouse anti-Rh1 (1:10) (DSHB, clone 4C5); mouse anti-Rh3 (1:10) and mouse anti-Rh5 (1:100) (gifts from S. Britt, University of Colorado); rabbit anti-Rh4 (1:100) (gift from C. Zuker, Columbia University); rabbit anti-Rh6 (1:2000) [20]; goat anti-βGal (1:5000) (Biogenesis); mouse anti-βGal (1:500) (Promega); rat anti-Elav (1:40) (DSHB, clone Rat-Elav-7E8A10); and sheep anti-GFP (1:500) (AbD Serotec); rabbit anti-GFP (1:800) (Biogenesis). Secondary antibodies raised in donkey and goat were Alexa Fluor-conjugated (Alexa Fluor 488 at 1:1000, Alexa Fluor 555 at 1:750, Alexa Fluor 647 at 1:500) (Molecular Probes). Alexa Fluor 488-conjugated phalloidin was used to visualize rhabdomeres (1:100, Molecular Probes).

Stains

Adult retinas were dissected out in phosphate buffered saline (PBS), fixed for 15 minutes with 4% formaldehyde at room temperature (RT), washed three times in PBS, and incubated with the primary antibodies diluted in Block (PBS, 0.1% Triton-X-100, 2% Horse Serum) overnight at 4°C. After two rinses and two 1 hour washes with PBT (PBS, 0.3% Triton-X-100), the retinas were incubated overnight at 4°C with secondary antibodies diluted in Block. Retinas were rinsed twice and after two 1 hour washes with PBT, were mounted in SlowFade Gold (Invitrogen). Antibody staining for larval eye was performed as described in ref. 22. *In situ* hybridization for cryo-sectioned adult retinas was performed as described in ref. 7 with DIG-labeled antisense probe transcribed from cloned rh5 3′UTR region (bp 900-1411). Samples were imaged using Leica TCS SP2 and SP5 confocal microscopes. Images were processed using Leica Confocal Software (LCS), Adobe Photoshop and Fiji software.

Counting

Optical sections were photographed approximately 10μm distal to R8 nuclei in the center of the retina. The portion of the image of the retina section containing R8 rhabdomeres was defined as area populated with Rh5 positive cells. The number of Rh5-expressing R8s and

the total number of R8s (represented by ommatidia visualized with phalloidin) in this area were counted using Fiji software with Cell Counter plug in.

RNA analysis

RNA was purified from each sample of about 20 flies with TRIzol (Invitrogen), RNeasy mini columns (Qiagen, Valencia, CA) and treated with DNAse1 (Qiagen). Three μg of total RNA was reverse transcribed with oligo(dT)20 and SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was amplified in duplicate reactions using SYBR-Green PCR Mix (Stratagene) by real time PCR. Primers used are listed in Supplementary Table 3. Target gene levels were normalized to levels of *rp49* mRNA[48] and expressed relative to levels in 0 day old wild-type flies. At least three independent replicates were averaged for each experimental condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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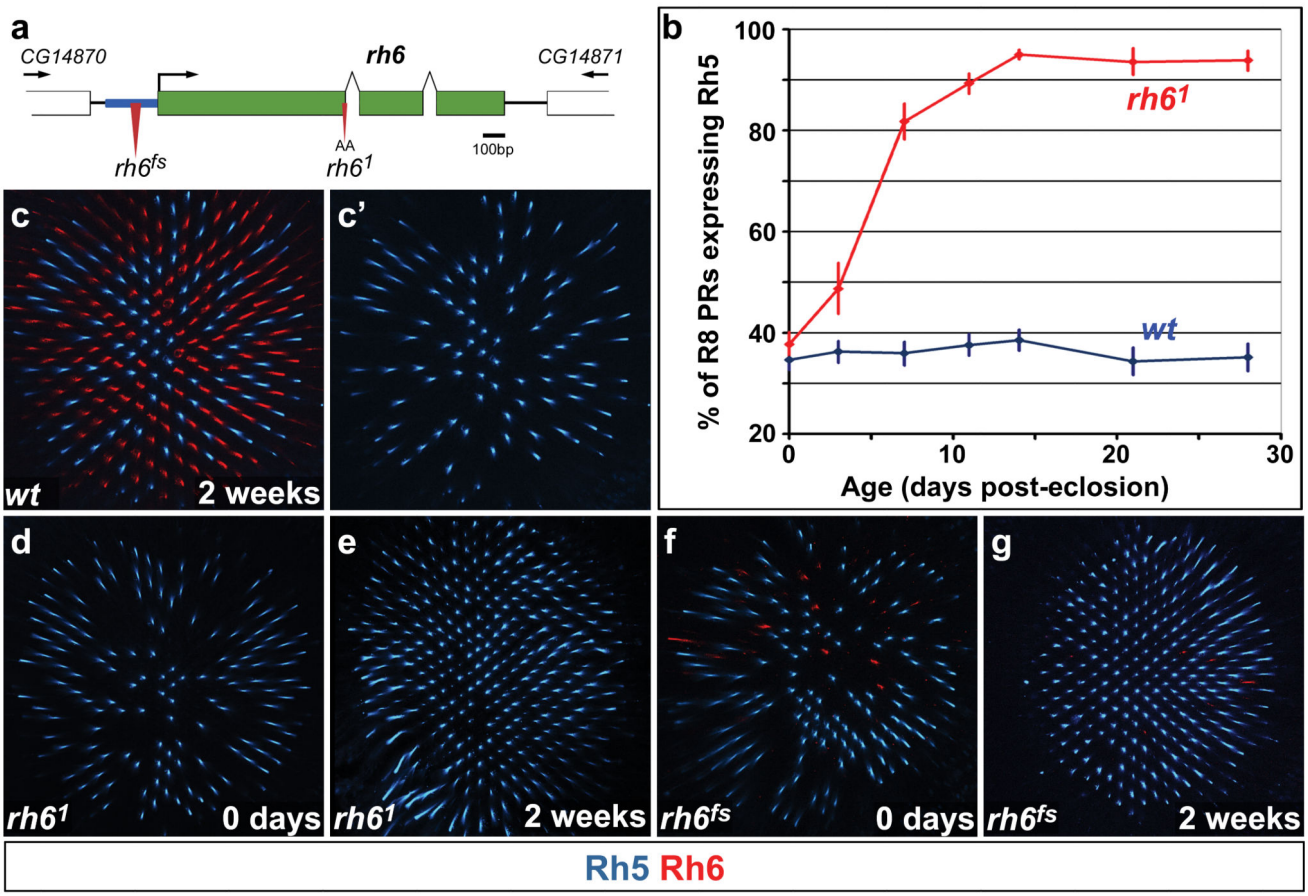

Figure 1. Rh6 acts to repress Rh5 expression in yR8 PRs

a: Genomic *rh6* locus. The promoter region sufficient to drive *rh6* expression in yR8 is in blue, exons are in green and mutations in red. In $rh6^{fs}$ mutants, 58bp of the promoter are deleted. In $rh6^{1}$ mutants, 21bp at the first exon-intron junction are replaced with AA, leading to an immediate truncation of the ORF.

b: Percentage of R8 PRs expressing Rh5 as a function of time (days post-eclosion) in wild-type (blue) and $rh6^{1}$ mutants (red). Error bars represent 84% Confidence Intervals.

c-g: Whole mount retina stained with specific antibodies for Rh5 (blue) and Rh6 (red).

c, c': Normal expression of Rh5 and Rh6 in 2 week old flies. **c'** Shows Rh5 alone.

d, e: In $rh6^{1}$ mutants, Rh5 is gradually de-repressed. At eclosion, retinas have a normal number of Rh5-expressing R8s (**d**). By 2 weeks post-eclosion, most R8s express Rh5 (**e**).

f, g: $rh6^{fs}$ promoter mutation leads to loss of detectable Rh6 expression in almost all yR8s. As in $rh6^{1}$ mutants (**d**,**e**), at eclosion $rh6^{fs}$ retinas have a normal number of Rh5-expressing R8s (**f**), but by 2 weeks post-eclosion, most R8 express Rh5 (**g**).

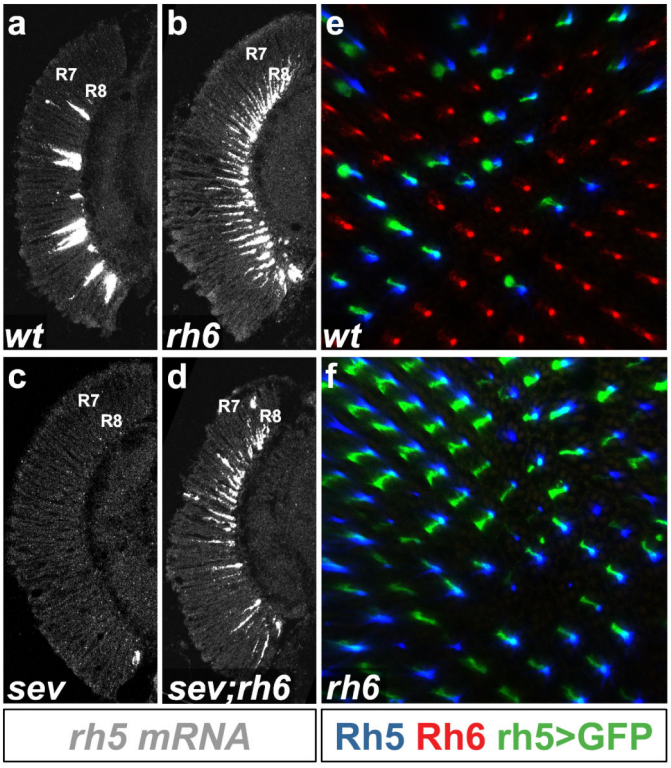

Figure 2. Rh6 represses transcription of the *rh5* gene

a-d: *rh5* mRNA, detected by *in situ* hybridization in transverse cryo-sections of 3 week old fly eyes. Many more cells are expressing *rh5* mRNA in the R8 layer of *rh6* mutants (**b**) as compared to wild-type flies (**a**). In *sev* mutants, very few cells express *rh5* (**c**). However, in *sev; rh6* double mutants, *rh5* is extensively de-repressed in R8 PRs (**d**).

e, f: In 3 week old control flies, a *rh5* reporter (*rh5>GFP*) (green) is expressed in pR8s that also express Rh5 protein (blue), but not in yR8 cells which express Rh6 (red) (**e**). In *rh6* mutants, *rh5>GFP* is de-repressed in most yR8 cells (**f**).

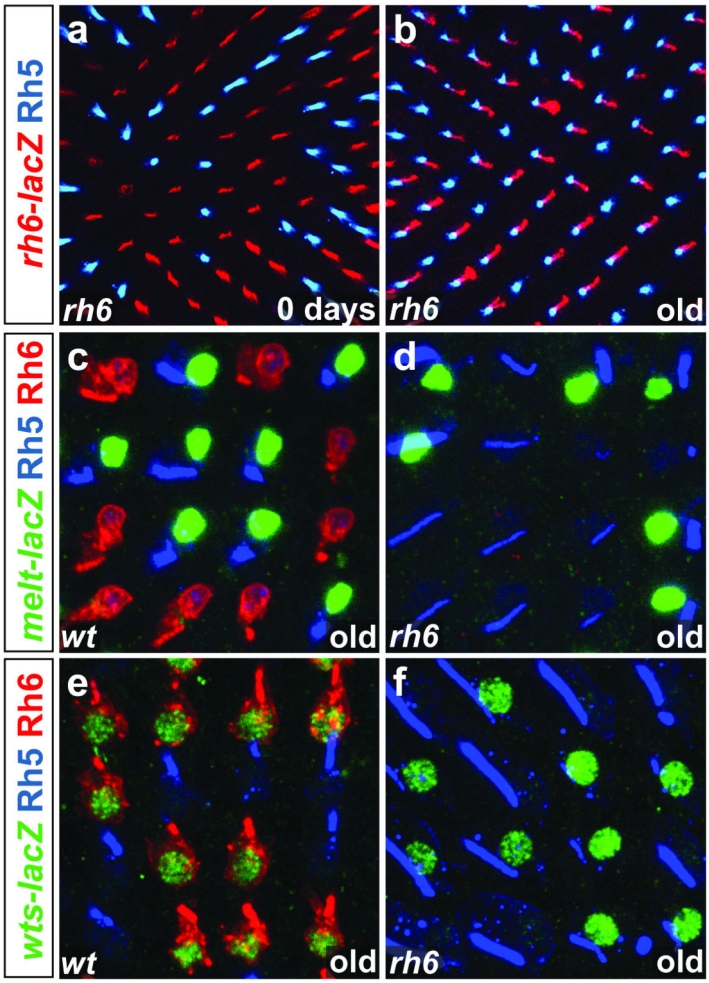

Figure 3. Mutation of *rh6* does not lead to change in yR8 cell identity

a, b: A *rh6-lacZ* reporter (red) is expressed normally in *rh6* mutants. It is induced in a pattern complementary to the expression of Rh5 (blue) in young flies (**a**). In 2 week old *rh6* mutants, Rh5 expression expands into the *lacZ* positive, yR8 cells (**b**).

c-f: Z-projections of confocal stacks encompassing nuclei and Rh-containing rhabdomeres of R8 PRs.

c, d: Expression of the nuclear pR8 marker *melt-nlacZ* (green) does not change in *rh6* mutants. It is normally expressed together with Rh5 (blue) in pR8 and never in Rh6-expressing yR8 cells (red) (**c**). In 5 week old *rh6* mutants, *melt-nlacZ* is not de-repressed along with Rh5 and remains restricted to pR8 (**d**).

e, f: Expression of nuclear yR8 marker *wts-nlacZ* does not change in *rh6* mutants. It is normally expressed together with Rh6 (red) in yR8 and never in Rh5-expressing pR8 cells (blue) (**e**). In *rh6* mutants, *wts-nlacZ* remains in yR8 of 4 week old flies as Rh5 is de-repressed (**f**).

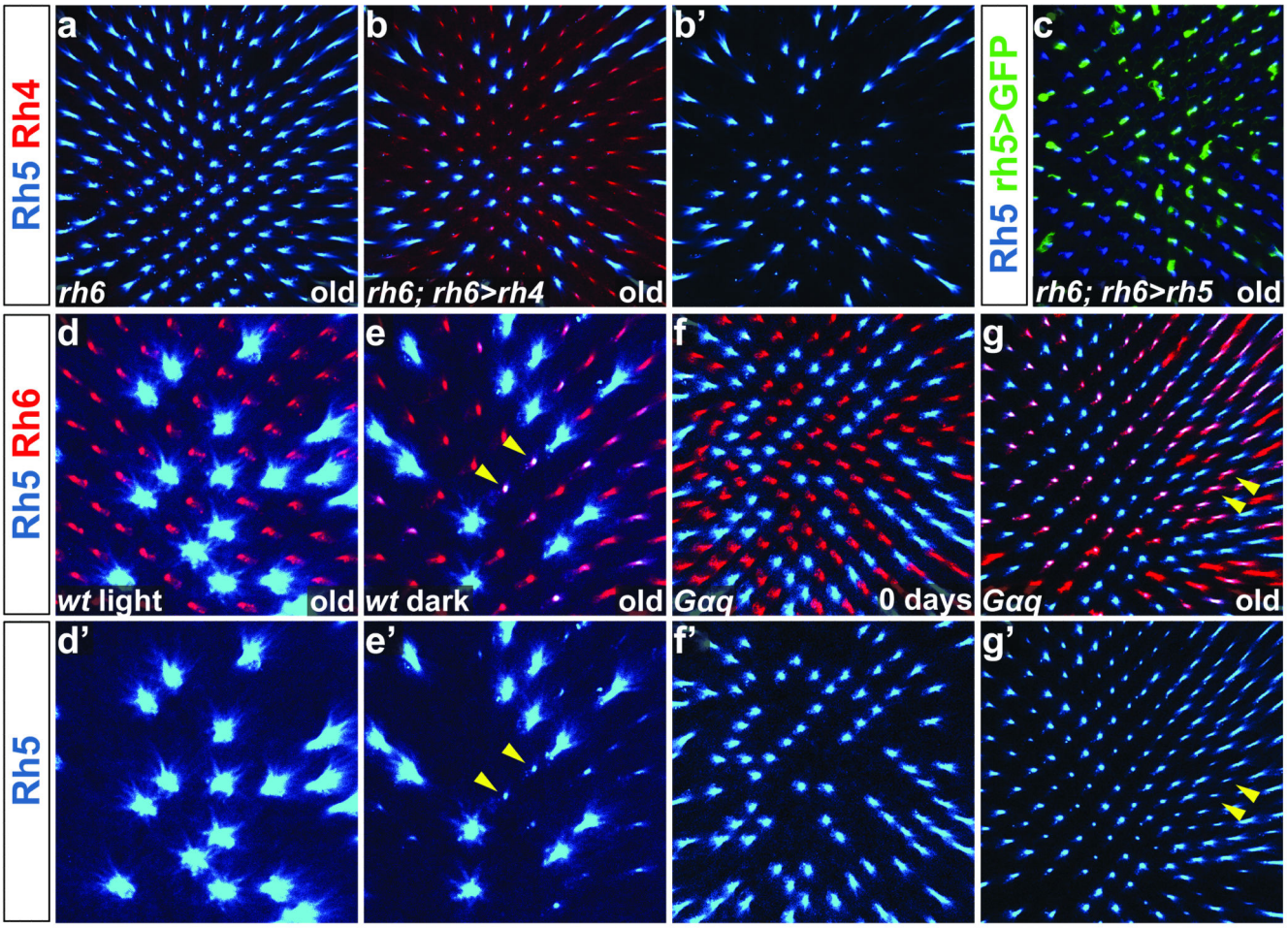

Figure 4. Part of the phototransduction pathway is required to maintain repression of Rh5

a, b: Forced expression of Rh4 (red) in yR8 with *rh6*-Gal4 in $rh6^1$ mutants prevents Rh5 (blue) de-repression (**b**) observed in $rh6^1$ mutant flies (**a**).

c: Forced expression of Rh5 (blue) in yR8 with *rh6*-Gal4 in $rh6^1$ mutants prevents rh5>GFP (green) de-repression observed in $rh6^1$ mutant flies (compare to Fig. 2f and Supplementary Fig. 7f).

d-e: Dark-reared flies partially de-repress Rh5 in yR8 PRs. In the light, *wt* flies do not de-repress Rh5 (blue) in Rh6-expressing yR8s (red) (**d**). After 2-3 weeks in complete darkness, a significant number of yR8s of *wt* flies express low levels of Rh5 in addition to Rh6 (arrowheads, **e**). **d, e** show close ups of dorsal retinas, just dorsal to the equator.

f, g: $G_{\alpha q}$ is required to maintain repression of Rh5 in yR8. In 2-3 week old (**g**), but not in just eclosed (**f**) $G_{\alpha q}{}^1$ mutants, Rh5 (blue) is expressed in yR8 and thus is co-expressed (arrowheads) with Rh6 (red).

b', d'-g': Rh5 expression alone as in **b**, **d-g**.