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

Daniel Vasiliauskas[1], Esteban O. Mazzoni[1]†, Simon G. Sprecher[1]†, Konstantin Brodetskiy[1], Robert J. Johnston Jr[1], Preetmoninder Lidder[1], Nina Vogt[1], Arzu Celik[1]† & Claude Desplan[1]

Sensory systems with high discriminatory power use 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 ageing adult retina. The Rh6 feedback signal results in repression of the *rh5* promoter and can be mimicked by other *Drosophila* rhodopsins; it is partly 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]. Each ommatidium, the unit eye of the adult retina, contains eight photoreceptors. Six outer photoreceptors, R1–R6, express Rh1 and are involved in motion detection and dim light vision (reviewed in ref. 4). Inner photoreceptors R7 and R8 mediate colour vision and define two main ommatidial subtypes based on the rhodopsins they express: in pale (p) ommatidia, pR7 expresses ultraviolet-sensitive Rh3 whereas pR8 expresses Rh5; in yellow (y) ommatidia, yR7 expresses a distinct ultraviolet-sensitive Rh4 whereas yR8 expresses Rh6[4]. Subtypes p and y are distributed stochastically throughout the main part of the retina with an approximate 30:70 ratio (Fig. 1c)[6]. An exception to the exclusive rhodopsin 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 rhodopsin expression pattern is established by a well-understood developmental program executed during pupal stages[4,8,9] (Supplementary Fig. 1a, b).

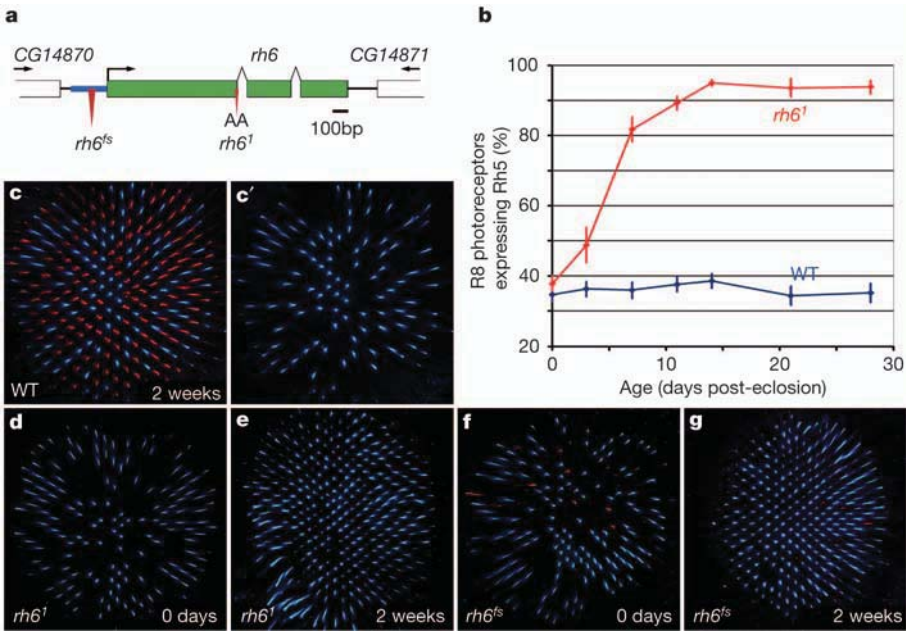

Figure 1 | Rh6 acts to repress Rh5 expression in yR8 photoreceptors. **a**, Genomic *rh6* locus. The promoter region sufficient to drive *rh6* expression in yR8 is in blue, exons are in green and mutations in red. In $rh6^{fs}$ mutants, 58 bp of the promoter are deleted. In $rh6^{1}$ mutants, 21 bp at the first exon–intron junction are replaced with AA, leading to an immediate truncation of the open reading frame. **b**, Percentage of R8 photoreceptors expressing Rh5 as a function of time (days post-eclosion) in wild-type (WT, blue) and $rh6^{1}$ mutants (red). Error bars, 84% confidence intervals. **c–g**, Wholemount retinas stained with specific antibodies for Rh5 (blue) and Rh6 (red). **c**, **c′**, Normal expression of Rh5 and Rh6 in 2-week-old flies. **c′**, 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**).

[1]Center for Developmental Genetics, Department of Biology, New York University, New York, New York 10003, USA. †Present addresses: Departments of Pathology, Neurology, and Neuroscience, Columbia University Medical Center, 630 West 168 Street, New York, New York 10032, USA (E.O.M.); Department of Biology, University of Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland (S.G.S.); Department of Molecular Biology and Genetics, Bogazici University, 34342 Bebek, Istanbul, Turkey (A.C.).

It is unknown, however, how p and y photoreceptor 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 rhodopsins themselves participate in maintaining their mutual exclusion by analysing rhodopsin 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 and Supplementary Table 1). In 1-day-old *rh6*^{1} mutant flies, Rh5 expression appears normal, with approximately 38% of R8s expressing uniformly high levels of Rh5 protein. In 3-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 and Supplementary Table 1).

We next asked if other rhodopsins are controlled by rhodopsin-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 rhodopsins in R7s in either young or old (over 3 weeks) flies (Supplementary Figs 3 and 4, and data not shown). Thus, a rhodopsin-dependent mechanism for controlling rhodopsin expression occurs only in yR8s. Moreover, Rh5 is the only rhodopsin that is actively repressed by Rh6.

In the *rh6*^{1} allele, commonly found in laboratory stocks, a short deletion that spans the first exon–intron junction leads to a truncation of the protein after its fifth transmembrane domain[15] (Fig. 1a). The levels of *rh6* messenger RNA (mRNA) measured by quantitative PCR with reverse transcription (qRT–PCR) are more than tenfold lower in *rh6*^{1} mutants than in wild-type flies (Supplementary Fig. 9a), probably because of 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. Also, *rh6*^{1} can be rescued by a 2,575 base pair (bp) genomic fragment encompassing the *rh6* locus (Supplementary Figs 7a and 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 2 weeks post-eclosion or older.

We identified a second *rh6* allele, also in a laboratory stock, which we named *frank sinatra* (*rh6*^{fs}) after the singer known as "Ol' blue eyes" (Fig. 1a). This mutation removes 58 bp 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 rhodopsin expression. However, Rh5 becomes broadly de-repressed in R8s of old flies (Fig. 1g and 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 RNA interference (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 visualize this phenotype more clearly, we repeated the experiment in a *sevenless* (*sev*) mutant background in which R7 photoreceptors 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 photoreceptors[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 analysed the expression of a *rh5* reporter (*rh5*>GFP (green fluorescent protein)) 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 and 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 and Supplementary Fig. 9c), suggesting correct specification of the yR8 subtype. As the fly ages, these cells de-repress Rh5 but remain positive for β-galactosidase (βGal) (Fig. 3b and Supplementary Fig. 9c). We also tested for a possible yR8-to-pR8 fate transition using the marker genes that specify these cells. The p versus y subtype specification of R8 cells depends on an R8-intrinsic bistable

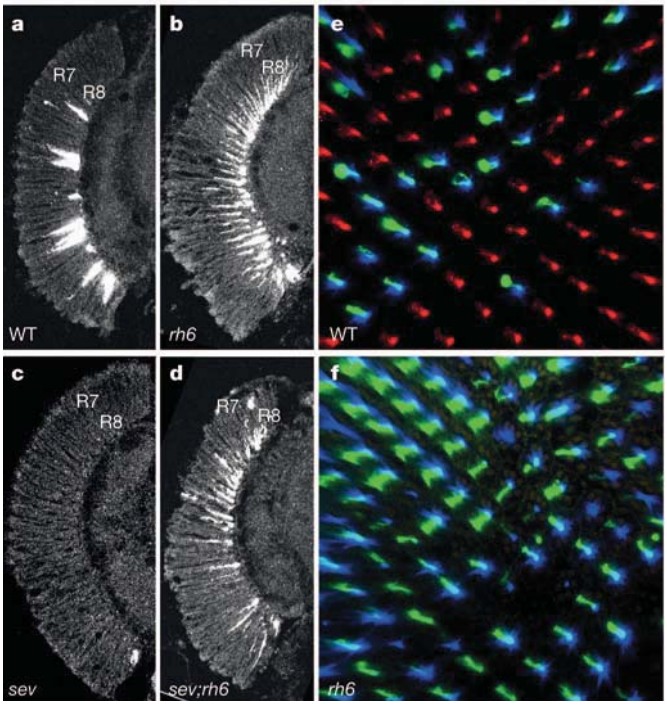

Figure 2 | Rh6 represses transcription of the *rh5* gene. a–d, *rh5* mRNA, detected by *in situ* hybridization in transverse cryo-sections of 3-week-old fly eyes. Many more cells are expressing *rh5* mRNA in the R8 layer of *rh6* mutants (**b**) compared with wild-type flies (**a**). In *sev* mutants, very few cells express *rh5* (**c**). However, in *sev;rh6* double mutants, *rh5* is extensively de-repressed in R8 photoreceptors (**d**). **e**, **f**, In 3-week-old control flies, a *rh5* reporter (*rh5>GFP*) (green) is expressed in pR8 cells 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**).

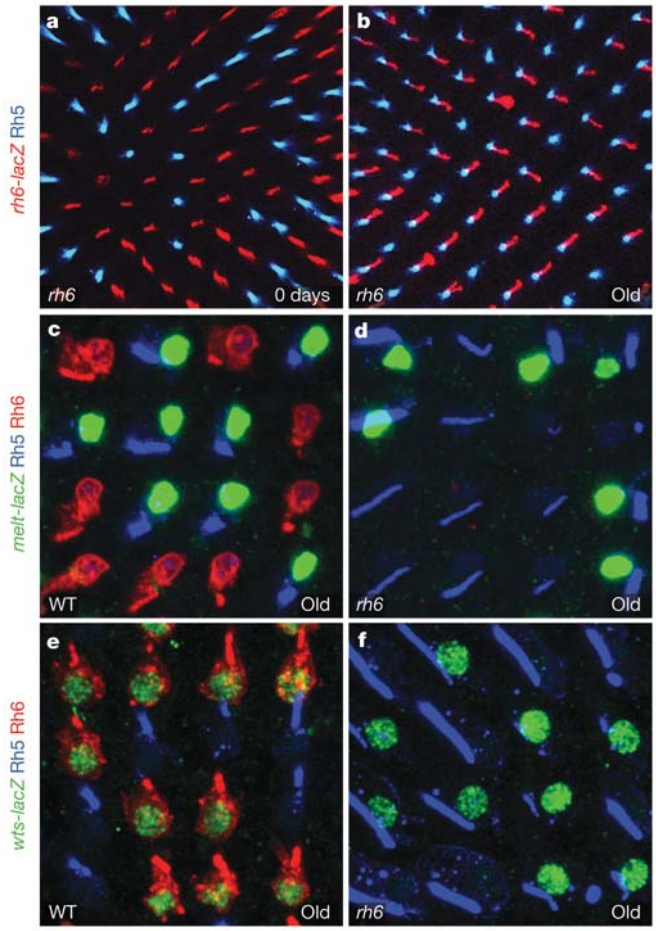

Figure 3 | Mutation of *rh6* does not lead to change in yR8 cell identity. **a**, **b**, A *rh6-lacZ* reporter (red) is expressed normally in *rh6* mutants. It is induced in a pattern complementary to the expression of Rh5 (blue) in young flies (**a**). In 2-week-old *rh6* mutants, Rh5 expression expands into the *lacZ*-positive, yR8 cells (**b**). **c–f**, Z-projections of confocal stacks encompassing nuclei and Rh-containing rhabdomeres of R8 photoreceptors. **c**, **d**, Expression of the nuclear pR8 marker *melt-nlacZ* (green) does not change in *rh6* mutants. In wild-type flies it is expressed 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. In wild-type flies it is expressed 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**).

switch involving mutual transcriptional repression between *warts* (*wts*) and *melted* (*melt*) (Supplementary Fig. 1b). During pupal development, Wts represses *melt* to specify yR8 photoreceptors. In response to an extrinsic signal originating in pR7, *melt* is upregulated in pR8, leading to repression of *wts* transcription and expression of Rh5 (ref. 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, whereas Rh5 expression expands broadly to cells that do not express *melt-nlacZ* (Fig. 3d and Supplementary Fig. 9d). In addition, we do not observe downregulation of a *wts* reporter (*wts-nlacZ*) in yR8s of old *rh6* mutants, leading to co-expression of *wts* with ectopic Rh5 (Fig. 3f and Supplementary Fig. 9d). Although 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 photoreceptor fate is established, these inputs do not depend on extrinsic signals from R7 cells because, as described earlier, the absence of R7s in *sev* mutants does not suppress the *rh6* mutant phenotype.

yR8 cells are not the only photoreceptors expressing Rh6. The larval eye, Bolwig's organ, is composed of about 12 photoreceptors[21,22]. Four primary photoreceptors express Rh5 whereas the eight secondary photoreceptors express Rh6 (Supplementary Fig. 6a). During metamorphosis, secondary photoreceptors die while the primary photoreceptors downregulate Rh5 and upregulate Rh6 (ref. 23). The newly Rh6-expressing cells form the eyelet, an adult extra-retinal visual organ[24,25] (Supplementary Fig. 6c). In *rh6* mutants, neither the secondary Bolwig photoreceptors nor the eyelet photoreceptors ever express Rh5 and are thus devoid of any rhodopsin (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 rhodopsin signal, de-repression of Rh5 can only occur in yR8 photoreceptors.

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 rhodopsins 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 photoreceptors (Fig. 4 a, b and Supplementary Figs 7b–e and 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 and Supplementary Fig. 7f-i, 9f), suggesting that a generic *Drosophila* rhodopsin signal is sufficient to maintain exclusion of Rh5 in yR8 cells. Because 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 rhodopsin-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 and 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 and 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 photoreceptor neurons remain plastic with respect to rhodopsin exclusion and that simply preventing activation of Rh6 by light can evoke Rh5 expression in yR8s. This de-repression 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 partly 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 rhodopsins 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 rhodopsin converts the $G_{\alpha q}$ subunit of a

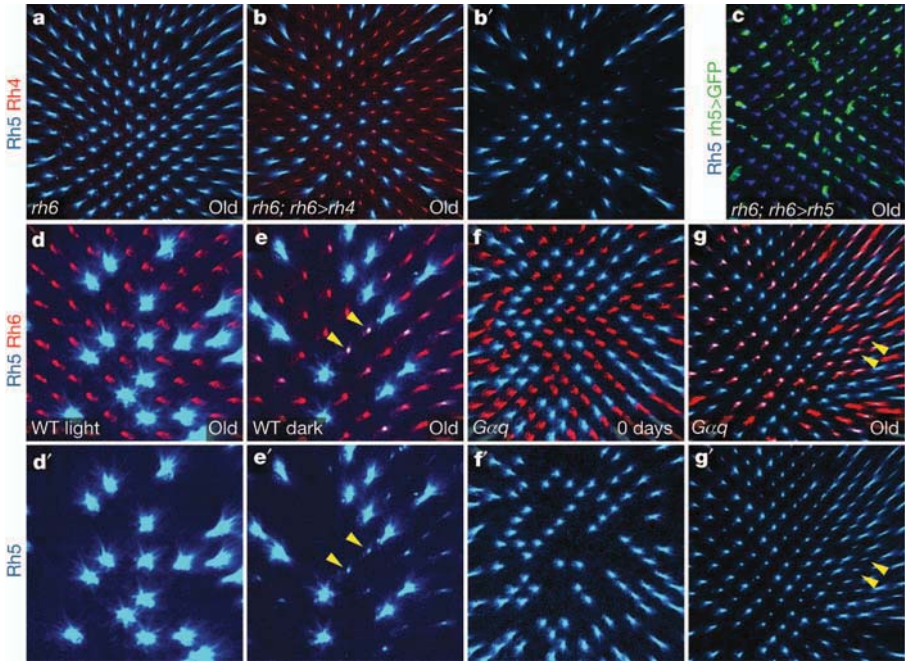

Figure 4 | Part of the phototransduction pathway is required to maintain repression of Rh5. a, **b**, Forced expression of Rh4 (red) in yR8 with *rh6*-Gal4 in $rh6^1$ mutants prevents Rh5 (blue) de-repression (**b**) observed in $rh6^1$ mutant flies (**a**). **c**, Forced expression of Rh5 (blue) in yR8 with *rh6*-Gal4 in $rh6^1$ mutants prevents rh5>GFP (green) de-repression observed in $rh6^1$ mutant flies (compare with Fig. 2f and Supplementary Fig. 7f). **d**, **e**, Dark-reared flies partly de-repress Rh5 in yR8 photoreceptors. In the light, wild-type flies do not de-repress Rh5 (blue) in Rh6-expressing yR8s (red) (**d**). After 2–3 weeks in complete darkness, a significant number of yR8 cells of wild-type flies express low levels of Rh5 in addition to Rh6 (arrowheads, **e**). **d**, **e**, 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- to 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**.

heterotrimeric G-protein to a GTP-bound form which dissociates from the $G_{\beta\gamma}$ dimer and activates phospholipase C encoded by the *norpA* gene. Phospholipase C then catalyses hydrolysis of PIP_2, which leads to the activation of TRPC channels[26], inflow of Ca^{2+} and depolarization of the photoreceptors. 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 and 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, removal of neither phospholipase C (in $norpA^{36}$ mutants) nor 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.

Rhodopsins 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 photoreceptors[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 yR8 photoreceptors[30], limiting the ability of the visual system to discriminate colours. 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 photoreceptor neurons, 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 photoreceptors 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 genes in vertebrate olfactory neurons[14], which encode G-protein-coupled receptors similar to Rhodopsins. With rare exception, each olfactory neuron expresses only one allele of one olfactory receptor gene. This exclusion mechanism is not well understood, but requires an active olfactory receptor to generate a feedback signal for repression of other olfactory receptor genes[10–14]. There, however, the feedback control of exclusion appears to be a common mechanism in all olfactory neurons, in contrast to the fly retina where only *rh5* is regulated by another rhodopsin, and only in the yR8 photoreceptor 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 G-protein-coupled receptor-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 G-protein-coupled receptor can exclude expression of other seven-transmembrane 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 RNAi experiments (at 29 °C) and dark isolation experiments (in complete darkness). Dissected adult retinas were stained wholemount 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-labelled antisense probe transcribed from *rh5* 3′ untranslated region (UTR) 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 per sample and complementary DNA (cDNA) amplified using SYBR-Green PCR Mix (Stratagene).

Full Methods and any associated references are available in the online version of the paper

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Supplementary Information is linked to the online version of the paper

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METHODS

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

***Drosophila* strains.** For wild-type controls we used $y^1\ w^{67};\ Sp/CyO;\ wt^{isoB}$ flies, where '*isoB*' represents an isogenized wild-type third chromosome.

We used the following *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 (lower case in TGACCATCATCTTCTcctac tggcacatcatgaaggTATGACATTCGTTA) at the end of the first exon with two As, removing a splice donor site and introducing a stop codon immediately afterwards. This results in the truncation of the open reading frame within the fifth transmembrane 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 *Drosophila* Stock Center (Stock 1385, named genotype $z^{v77h}\ w^{67c23}$) which mapped to the third chromosome. Sequencing of *rh6* locus revealed a 58-bp deletion upstream of the *rh6* transcription start site, which removes sequence AGCGGCAATCGAAAGCC CAATTCGAACGGTTAGCTTTGGATTGGCCAAGTGCCGGCTA within the *rh6* promoter. We named this mutation after the singer Frank Sinatra, for his nickname "Ol' blue eyes", because 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 and spans 3R:11154443–11154444 ... 11363188 (ref. 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 (ACGAATTCA CCTCGGCCTGAACACCTAC) primers to produce a 2,575-bp 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 the second 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 wholemount retinas: normal Rh6 expression was detected in *rh6* mutant background.

UAS-rh6RNAi (transformant 102152) was obtained from the Vienna *Drosophila* RNAi Center[34].

We generated the following additional mutants.

$rh3^1$ mutant (a nucleotide change C278T resulting in Q46* truncation) was obtained by TILLING (Seattle TILLING Project)[35]. The mutation was backcrossed into wild-type background four times (confirmed by genomic PCR and by stain of wholemount retinas with anti-Rh3 antibody).

$rh4^1$ mutant (a nucleotide change T727A resulting in Y203* truncation between fourth and fifth transmembrane domains) was obtained by TILLING (Seattle TILLING Project)[35]. The mutation was back crossed into wild-type background four times (confirmed by genomic PCR and by stain of wholemount retinas with anti-Rh4 antibody).

We generated the following transgenic lines.

rh5>GFP flies carry two transgenes recombined on the second chromosome: *rh5-lexA* and *lexAop-GFP*.

rh5-lexA. *lexA* (from pBS-lexA SV40 3′ UTR[36]) was cloned into pBS-LoxP-white-Lox2272 (ref. 33) and named LexA-Lox, a 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 (TCGGAAAATGT CGTGCAAGTG) 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 second chromosome[33].

lexAop-GFP. GFP with SV40 3′ UTR was PCR amplified from the pIRES2-eGFP vector (Clontech) with the primers oBJ78 (TAATACTAGTATGGTGA GCAAGGGCGAGGAG) and oBJ79 (GTCAGGATCCACCACAACTAGAATG CAGTG) 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 … AAGGACAAAAAAAAACTCA AC + 15A) from rh1-pFLC-1 plasmid (*Drosophila* Genomics Resource Center clone RH01460 (ref. 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 second chromosome landing sites *attP-51D*, *attP-58A* and *attP40* (refs 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 … AATATAGTAAAATTACAGC AAGCT + 19A) from rh3-pOT2 (*Drosophila* Genomics Resource Center clone GH02505 (ref. 37)) was ligated into EcoRI–XhoI sites of pUASTattB vector[38] to produce pDV133 plasmid. φC31-mediated integration was used to insert this construct into second chromosome landing sites *attP-51D*, *attP-58A* and attP40 (refs 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 clone RH33063 (ref. 37)) was ligated 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 CAGAGCGAAAC GGGTAGCGGT … AACTTATTGCAAACGAAGTAG + 16A. φC31-mediated integration was used to insert this construct into second chromosome landing sites *attP-51D* and *attP40* (refs 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 … TACAAACCAAAAAAAGTTGGCA TT + 78A) from rh5-pOT2 (*Drosophila* Genomics Resource Center clone GH28578 (ref. 37)) was ligated into EcoRI–XhoI sites of pUASTattB vector[38] to produce pDV135 plasmid. φC31-mediated integration was used to insert this construct into second chromosome landing site *attP40* (ref. 39).

UAS-Rh6. It has proved 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 … ACAG CAATTGATACAAAATC) into EcoRI–KpnI sites of pUASTattB vector[38] to produce pDV160 plasmid. φC31-mediated integration was used to insert this construct into second chromosome landing site *attP40* (ref. 39).

We used the following other strains.

$G_{\alpha q}{}^1$ (ref. 40), $norpA^{36}$ (ref. 41), $rh5^2$ (ref. 42), sev^{14} (ref. 43), $trpl^{302}$;trp^{301} (ref. 44), *melt-nlacZ*[8], *rh6-Gal4* (ref. 20), *rh6-lacZ*[45] and *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); rabbit anti-Rh4 (1:100) (gift from C. Zuker); rabbit anti-Rh6 (1:2,000) (ref. 20); goat anti-βGal (1:5,000) (Biogenesis); mouse anti-βGal (1:500) (Promega); rat anti-Elav (1:40) (DSHB, clone Rat-Elav-7E8A10); 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 1:1,000, 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 min with 4% formaldehyde at room temperature, 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-h 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-h 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-labelled 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 10 μm distal to R8 nuclei in the centre 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) and treated with DNase1 (Qiagen). Three micrograms 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.

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