LETTERS

Coordinate control of synaptic-layer specificity and rhodopsins in photoreceptor neurons

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How neurons make specific synaptic connections is a central question in neurobiology. The targeting of the *Drosophila* R7 and R8 photoreceptor axons to different synaptic layers in the brain provides a model with which to explore the genetic programs regulating target specificity. In principle this can be accomplished by cell-type-specific molecules mediating the recognition between synaptic partners[1]. Alternatively, specificity could also be achieved through cell-type-specific repression of particular targeting molecules. Here we show that a key step in the targeting of the R7 neuron is the active repression of the R8 targeting program. Repression is dependent on NF-YC, a subunit of the NF-Y (nuclear factor Y) transcription factor[2]. In the absence of NF-YC, R7 axons terminate in the same layer as R8 axons. Genetic experiments indicate that this is due solely to the derepression of the R8-specific transcription factor Senseless[3] (Sens) late in R7 differentiation. Sens is sufficient to control R8 targeting specificity and we demonstrate that Sens directly binds to an evolutionarily conserved DNA sequence upstream of the start of transcription of an R8-specific cell-surface protein, Capricious (Caps) that regulates R8 target specificity. We show that R7 targeting requires the R7-specific transcription factor Prospero[4,5] (Pros) in parallel to repression of the R8 targeting pathway by NF-YC. Previous studies demonstrated that Sens[6,7] and Pros[8] directly regulate the expression of specific rhodopsins in R8 and R7. We propose that the use of the same transcription factors to promote the cell-type-specific expression of sensory receptors and cell-surface proteins regulating synaptic target specificity provides a simple and general mechanism for ensuring that transmission of sensory information is processed by the appropriate specialized neural circuits.

The compound eye comprises about 750 simple eyes (ommatidia), each containing a cluster of eight photoreceptor neurons (R1–R8). These neurons form synaptic connections in two regions of the optic lobe, the lamina and the medulla (Fig. 1a). The R1–R6 neurons innervate the lamina; the R7 and R8 neurons form connections in the M6 and M3 medulla layers, respectively. Genetic studies have led to the identification of cell-surface proteins regulating R7 and R8 target specificity[9–18]. Notably, mis-targeting mutant R7 neurons terminate selectively in M3 (refs 9–14), the layer in which wild-type R8 axons terminate, suggesting a close relationship between the genetic programs controlling R7 and R8 target specificity. Here we describe transcriptional regulatory pathways that control the differential targeting specificity of these neurons.

In a screen for R7 targeting mutants[10], we identified a strong loss of function mutation in the *NF-YC* gene (Supplementary Fig. 1), which encodes a subunit of NF-Y, an evolutionarily conserved heterotrimeric transcription factor. Although NF-Y function has not been studied extensively in the fly[19,20], it has been shown to act as both an activator[21] and a repressor[22] in other organisms. The targeting of visual-system neurons was assessed in mosaic animals[9] to generate large patches of mutant retinal tissue early in development (Fig. 1b, b′). About 75% of *NF-YC* mutant R7 axons ($n = 892$ of 1,148)

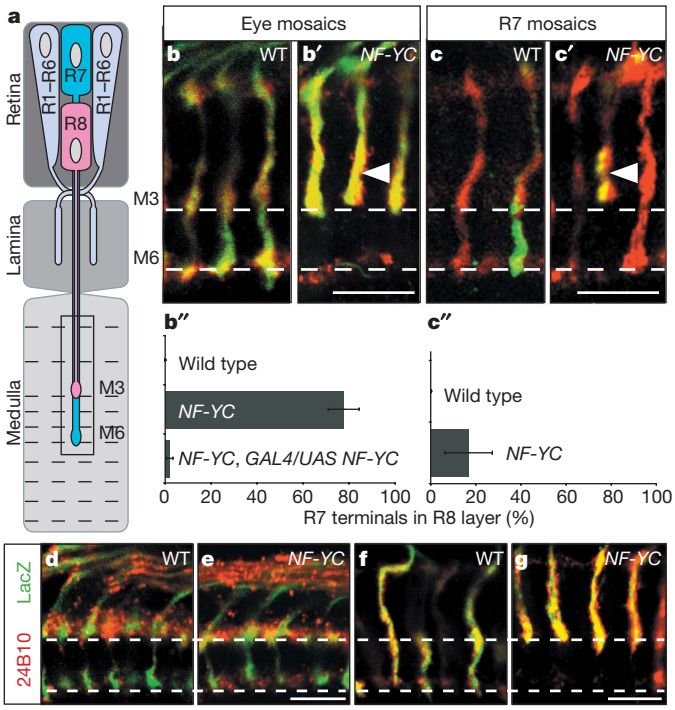

Figure 1 | *NF-YC* mutant R7 axons mis-target to the M3 layer. **a**, Schematic diagram of photoreceptor neurons (R cells) in the *Drosophila* visual system. The box indicates the area shown in **b**, **b′**, **c**, **c′** and **d**–**g**. **b**–**c″**, Targeting of *NF-YC* mutant R7s (green) in mosaic animals (**b**–**b″**, *ey3.5FLP*; **c**–**c″**, *GMRFLP*). Monoclonal antibody 24B10 (red) labelled all R7 and R8 axons. Arrowheads, mutant R7s terminating in M3. Scale bar, 10 μm. Error bars indicate s.d. **b**–**b″**, Large patches of control (wild type (WT), **b**) and mutant (*NF-YC*, **b′**) R cells. WT R7s target exclusively to M6 (11 brains, 895 R7 neurons; **b**, **b″**), whereas *NF-YC* mutant axons target to M3 (77.7 ± 6.7%, 8 brains, 1,148 R7s; **b′**, **b″**). The mis-targeting phenotype was rescued by the expression of *NF-YC* cDNA (2.0 ± 1.4%, 6 brains, 1,076 R7s; **b″**). **c**–**c″**, All WT R7s targeted to M6 (9 brains, 812 R7s; **c**, **c″**), whereas isolated mutant R7s (16.8 ± 10.5%, 10 brains, 807 R7s; **c′**, **c″**) targeted to M3. **d**–**g**, Targeting of WT and *NF-YC* mutant R7s at different developmental stages (green). At 30% APF, both WT (**d**) and mutant (**e**) R7s target correctly to their temporary layer. At 70% APF, WT R7s (**f**) target to the M6 layer. Mutant R7s (**g**) terminate in M3. Scale bar, 10 μm.

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terminated in M3, the same layer as wild-type R8 axons (Fig. 1b′, b″). This phenotype was fully rescued by an *NF-YC* complementary DNA (Fig. 1b″). In contrast with the marked effect of *NF-YC* mutations on R7, targeting of R8 to the M3 layer and targeting of R1–R6 to the lamina were unaffected (Supplementary Fig. 2).

To assess whether *NF-YC* is required in a cell-autonomous fashion in R7 neurons, we generated mosaic flies in which a fraction of R7 neurons was rendered mutant and labelled with green fluorescent protein (GFP), whereas the remaining R7 neurons and all the R8 neurons were wild-type and unlabelled (Fig. 1c, c′). We observed that about 17% of the mutant R7 neurons ($n = 144$ of 807) mis-targeted to M3 (Fig. 1c′, c″). The decrease in penetrance of the phenotype, in comparison with mutant R7 neurons generated by mitotic recombination induced earlier in the eye primordium (compare Fig. 1b″ with Fig. 1c″), probably reflects perdurance of NF-YC protein present in precursor cells. *NF-YC* is therefore required autonomously for R7 targeting but not for the targeting of other classes of photoreceptor neurons. As NF-YC is expressed in all R cells (Supplementary Fig. 3), NF-YC must function in combination with other factors or signals selectively activating NF-YC function in R7.

Given that NF-YC is part of a transcription factor complex[20] and is expressed in the nucleus of R7 neurons, it is likely that the change in targeting specificity reflects a change in gene expression. Wild-type R7 neurons initially target to the temporary R7 layer in the medulla and then, during mid-pupal development, extend to their final target layer[11]. Targeting of *NF-YC* mutant R7 neurons to the temporary layer is indistinguishable from the wild type (Fig. 1d, e). Extension to the final target layer at 70% after puparium formation (APF) is frequently disrupted, with many R7 neurons terminating in the layer within which R8 terminates (Fig. 1f, g). Consistent with this finding was our observation that *NF-YC* mutant R7 neurons expressed all five early R7 markers tested in wild-type patterns (Table 1). We reasoned, then, that *NF-YC* might repress a subset of R8-specific genes in the R7 neuron that later in development control final target layer selection. Indeed, the R8-specific transcription factor Sens was expressed ectopically in *NF-YC* mutant R7 neurons (Table 1 and Fig. 2).

sens is a key regulator of R8 development[23]. In wild-type larval eye discs, Sens is expressed in two or three cells that have the potential to become R8 before becoming restricted to a single differentiating R8

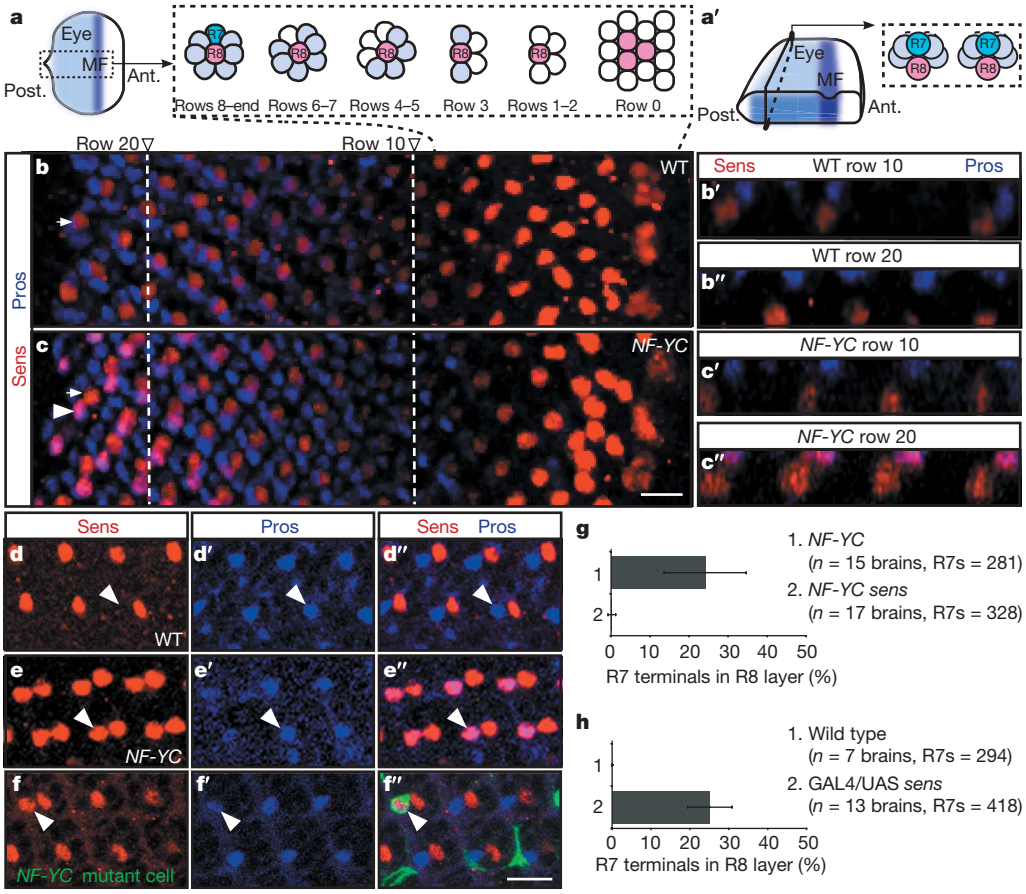

Figure 2 | Mis-expression of Sens in *NF-YC* mutant R7s is necessary and sufficient for the R7 mis-targeting phenotype. **a**, **a′**, Diagrams of R-cell recruitment to the ommatidium in the eye disc. **a**, Eye disc viewed from above the epithelium. R-cell clusters form as a wave of differentiation moves from posterior (post.) to anterior (ant.) with specific R cells joining the cluster at different times (that is, correlating with the distance from row 0, morphogenetic furrow (MF)). Red, R8 nuclei; blue, R7 nuclei; white, undifferentiated cells; light blue, R1–R6. During development, clusters rotate by 90°. **a′**, Cross-section along the apicobasal axis of the disc. **b**–**c″**, WT (**b**–**b″**) and *NF-YC* mutant (**c**–**c″**) eye discs stained for Sens (red) and Pros (blue). **b**–**b″**, In WT, Sens is expressed in two or three precursor cells before becoming restricted to a single R8 (arrow, **b**). Pros expression in R7 begins as R7 differentiation commences (about row 8). Pros is expressed at later stages in non-neuronal cells. Cross-sections are shown in **b′** and **b″**. **c**–**c″**, *NF-YC* mutant R7s express Pros like WT R7s. Sens is detected in mutant R7s 10–12 rows after R7 differentiation (arrowhead, **c**). R8s express Sens in a WT pattern (arrow). Co-expression of Sens and Pros in mutant R7s is seen at row 20 (**c″**), but not at row 10 (**c′**). Scale bar, 10 μm. **d**–**f″**, WT (**d**–**d″**), mutant (**e**–**e″**), and mosaic (**f**–**f″**) retinas at 40% APF stained for Sens (red) and Pros (blue). Arrowheads, R7s. **d**–**d″**, WT R8s express Sens, whereas WT R7s express Pros. **e**–**e″**, *NF-YC* mutant R7s also express Sens (**e″**). **f**–**f″**, In mosaics, single *NF-YC* mutant R7s (arrowhead, **f″**) mis-express Sens. Scale bar, 10 μm. **g**, **h**, Sens is necessary (**g**) and sufficient (**h**) for R7 targeting to M3 in *NF-YC* mutants. Error bars indicate s.d. **g**, Mis-targeting of *NF-YC sens* double-mutant R7s (0.2 ± 1.0%) and *NF-YC* control (24.1 ± 10.5%). **h**, Mis-targeting of WT R7s mis-expressing Sens (25.1 ± 5.7%).

Table 1 | Marker expression in *NF-YC* mutant eye discs

Markers	Name	Wild-type R8	*NF-YC* R8	Wild-type R7	*NF-YC* R7
R7 markers	H214 (Klingon)*	–	–	+	+
	PM181-GAL4 (Sevenless)†	–	–	+	+
	Prospero‡	–	–	+	+
	Deadpan-LacZ†	–	–	+	+
R7 & R8 markers	Runt‡	+	+	+	+
R8 markers	Atonal‡	+	+	–	–
	Boss‡	+	+	–	–
	BBO2*	+	+	–	–
	RO-156*	+	+	–	–
	109-68 (Scabrous)*	+	+	–	–
	Senseless‡	+	+	–	+

Markers were analysed by using enhancer trap lines (*), promoter fusion (†) or antibodies (‡).

neuron[23] (Fig. 2a–b″). Sens remains expressed in R8 into the adult[6,7] (see Supplementary Fig. 6b). It is required at a very early stage of eye development to regulate R8 specification[23] and, much later during pupal development and in the adult, to regulate the transcription of R8-specific rhodopsins directly[7]. In *NF-YC* mutant larval eye discs, Sens expression in R8 begins before overt R8 differentiation as in the wild type. By contrast, Sens mis-expression in mutant R7 neurons (Fig. 2c) was first observed 15–20 h after the onset of differentiation as assessed by the expression of the R7-specific marker *pros* (Table 1, and compare Fig. 2c′ with Fig. 2c″). Expression of Sens in mutant R7 neurons persists throughout pupal development (Fig. 2d–e″) and into the adult (see Supplementary Fig. 6d) and is cell-autonomous (Fig. 2f–f″). As Sens mis-expression occurs after the onset of R7 differentiation and *NF-YC* mutant R7 neurons mis-target to the M3 layer during the late phase of R7 targeting, *sens* may promote an R8 targeting program that is distinct from the role of *sens* in cell fate earlier in development.

If upregulation of Sens in *NF-YC* mutant R7 neurons is responsible for targeting to the M3 layer, removal of Sens from *NF-YC* mutant cells should suppress the targeting defect. To test this, we induced mitotic recombination on two different chromosomes (namely chromosomes X and 3) to generate R7 neurons that were simultaneously mutant for both *NF-YC* and *sens*, and we assessed their targeting in an otherwise wild-type background (Supplementary Fig. 4a). Removing *sens* from *NF-YC* mutant R7 neurons completely suppressed the mis-targeting phenotype (Fig. 2g). Thus, during wild-type development the *NF-YC* mediated repression of *sens* in R7 is necessary to prevent inappropriate targeting to M3.

To test whether Sens is sufficient to implement an R8 targeting program, we mis-expressed *sens* in R7 neurons (Supplementary Fig. 4b; see Methods). Under these conditions about 25% ($n = 104$ of 418) of the R7 neurons were redirected to the M3 layer, thus phenocopying *NF-YC* loss-of-function mutations (Fig. 2h). Additional experiments using the method[7] in which Sens was provided conditionally early in development to promote R8 cell fate, but removed later, support the view that Sens functions at later stages of R8 development to promote targeting (Supplementary Fig. 5). Taken together, these data raise the possibility that Sens could directly control the expression of cell-surface proteins regulating R8 target specificity.

Caps is the only cell-surface molecule that has been shown to be both specifically expressed in the R8 neuron and required for R8 targeting[18] and it is therefore an excellent candidate for direct regulation by Sens. Indeed, like Sens, Caps is expressed ectopically in R7 in *NF-YC* mutants (Fig. 3a–f). Expression of Caps, as detected with an enhancer trap, is specifically activated in *NF-YC* mutant R7 neurons about 9 h after the onset of Sens expression. Furthermore, a previous study[18] showed that ectopic expression of Caps in R7 respecified their connections to the R8 layer. Both *NF-YC* mutant R7 neurons and R7 neurons mis-expressing Caps initially target correctly but then select the inappropriate M3 layer during mid-pupal development. Taken together, these observations indicate that *caps* could be a downstream target of Sens.

Examination of the DNA sequences 1 kilobase upstream of *caps* and within the first large intron led to the identification of four and three putative Sens-binding sites, respectively. We identified an

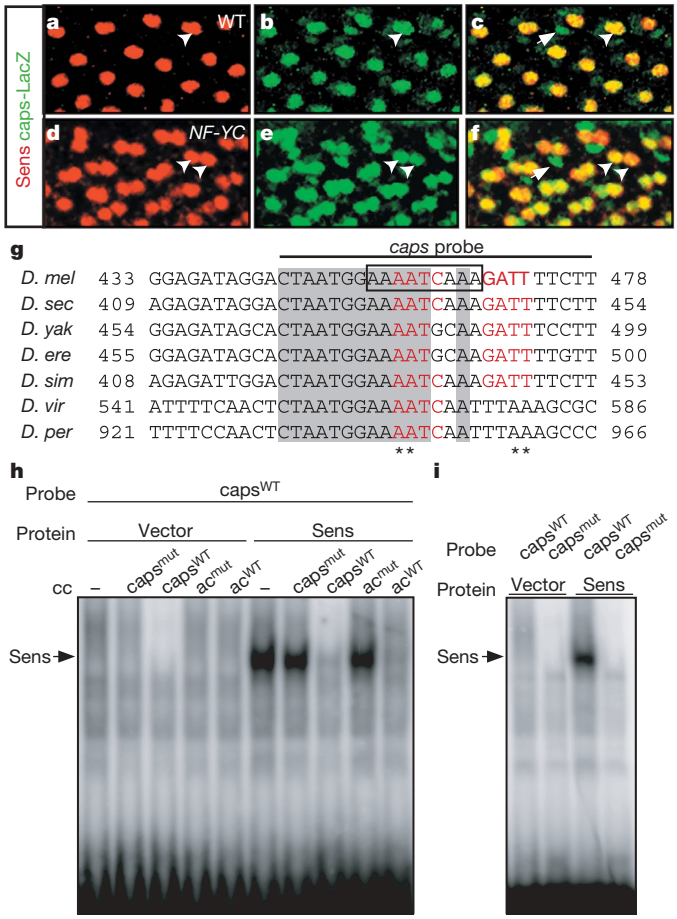

Figure 3 | Sens regulates *caps* expression. **a–f**, WT (**a–c**) and *NF-YC* mutant (**d–f**) eye discs carrying *caps-lacZ*, stained for Sens (red) and LacZ (green). **a–c**, In WT, R8s express LacZ (**c**, arrowhead). **d–f**, In *NF-YC* mutant, LacZ is also detected in R7s (**f**, arrowheads). Arrows indicate LacZ expression in non-neuronal cone cells both in WT (**c**) and in *NF-YC* mutant tissue (**f**). Original magnification, ×40 (**a–f**). **g**, Alignment showing conservation (grey) of Sens-binding site (box) in *caps*. In closely related species there is another predicted Sens-binding site (core sequence, red). Line, *caps* probe in **h** and **i**. Asterisk, AA→CC nucleotide change in the mutant probe. *D.mel*, *Drosophila melanogaster*; *D.sec*, *Drosophila sechellia*; *D.yak*, *Drosophila yakuba*; *D.ere*, *Drosophila erecta*; *D.sim*, *Drosophila simulans*; *D.vir*, *Drosophila virilis*; *D.per*, *Drosophila persimilis*. **h**, **i**, Sens binds to predicted Sens sites on *caps* probe. **h**, Gel-shift assay with WT *caps* probe and either 'vector' control or Sens protein. cc, 50× unlabelled competitor probes added as indicated. caps^{WT}, *caps* probe; ac^{WT}, *achaete* probe[29] (positive control); caps^{mut} and ac^{mut}, *caps* and *ac* probes with Sens-binding sites mutated. **i**, Gel-shift assay with WT or mutant *caps* probes.

evolutionarily conserved Sens-binding site 500 base pairs upstream of the *caps* transcriptional start site (Fig. 3g; see Supplementary Methods). Sens protein binds specifically to this site in gel-shift assays, making it likely that *caps* is a direct target of Sens (Fig. 3h, i). However, Sens must regulate R8 target specificity by controlling the expression of other genes in addition to *caps*, because loss of *caps* does not suppress the *NF-YC* mutant phenotype (*NF-YC caps* 20.3 ± 11.0%, 23 brains, 441 R7 neurons; *NF-YC* 24.1 ± 10.5%, 15 brains, 281 R7 neurons; means ± s.d.). This is consistent with the finding that loss of *caps*, in an otherwise wild-type background, results in targeting defects in about 50% of the R8 neurons[18]. Together, these data suggest that Sens directly regulates the expression of Caps, a cell-surface protein controlling R8 target specificity, and must also regulate the expression of other genes involved in this process.

Specific repression of *sens* in R7 neurons could arise through interactions between NF-YC and the R7-specific transcription factor Pros. Like NF-YC, Pros is also required for R7 target specificity[24,25]. It is expressed in R7 from an early stage of its development through to the adult[8,24] in a similar fashion to Sens expression in R8. About 20% of the *pros*-null mutant R7 neurons terminate in M3 (Fig. 4a). Two lines of evidence support the view that Pros works in parallel with NF-YC: first, the loss of *pros* in R7 neurons does not lead to ectopic expression of Sens (Fig. 4b, c), and second, the frequency of mis-targeting R7 axons in single *pros*-null mutant cells is markedly increased by removing *NF-YC* (Fig. 4a; see Methods). Thus, Pros could either promote R7 targeting directly or, like NF-YC, act to repress an R8 targeting program, or both.

Thus, R7 targeting requires NF-YC and, in parallel, Pros, whereas R8 targeting relies on Sens-dependent regulation of *caps* and other genes. Mutations in many other genes required for R7 targeting cause R7 neurons to mis-target to the M3 layer specifically rather than terminating promiscuously in the medulla. This underscores a tight inter-relationship between the mechanisms regulating targeting to these two layers. On the basis of the strong M3 mis-targeting phenotype of *NF-YC* mutant R7 neurons and complete suppression of the phenotype by the removal of *sens*, a key mechanism regulating R7 layer specificity is repression of an R8 targeting program. More generally, repression of inappropriate pathways may promote differential targeting in closely related neurons[26].

The roles of Pros and Sens in target layer selection are analogous to their function in controlling the expression of R7-specific and R8-specific rhodopsins. R7 and R8 neurons express different rhodopsins and hence detect different wavelengths of light. In R8, Sens directly represses the transcription of R7 rhodopsins and directly activates the transcription of an R8 rhodopsin[7]. In the R7 neuron, Pros binds to an upstream regulatory sequence in the R8 rhodopsin genes and represses their expression[7,8]. *NF-YC* mutant R7 neurons no longer express R7 rhodopsins, and all express R8 rhodopsins (Supplementary Fig. 6). This is consistent with the finding that *NF-YC* mutant R7 neurons in adults express Sens but no longer express Pros. Thus, transcription of both R8-specific rhodopsins and, as we have shown here, an R8-specific targeting protein Caps is directly regulated by Sens (Fig. 4d).

These observations suggest a simple solution to the mechanisms by which sensory neurons connect to the neural circuits specialized for the reception of different sensory stimuli (for example, different wavelengths of light or different odours). Although the molecular basis of this coupling is understood in considerable detail for vertebrate olfactory neurons, in which odorant receptors have a direct function in controlling target specificity[27], little is known about the coupling in other sensory systems. Coupling is likely to be regulated in a different fashion in other neurons, because even in the fly olfactory system, for example, targeting is independent of sensory receptor expression[28]. On the basis of our studies on Sens we propose that the same transcription factors directly control both rhodopsin expression and the cell-surface proteins that control target layer specificity. More generally, we speculate that in many sensory neurons a common set of transcription factors may directly control, and thereby coordinate, the expression of cell-surface proteins regulating target specificity and the receptors detecting specific sensory stimuli.

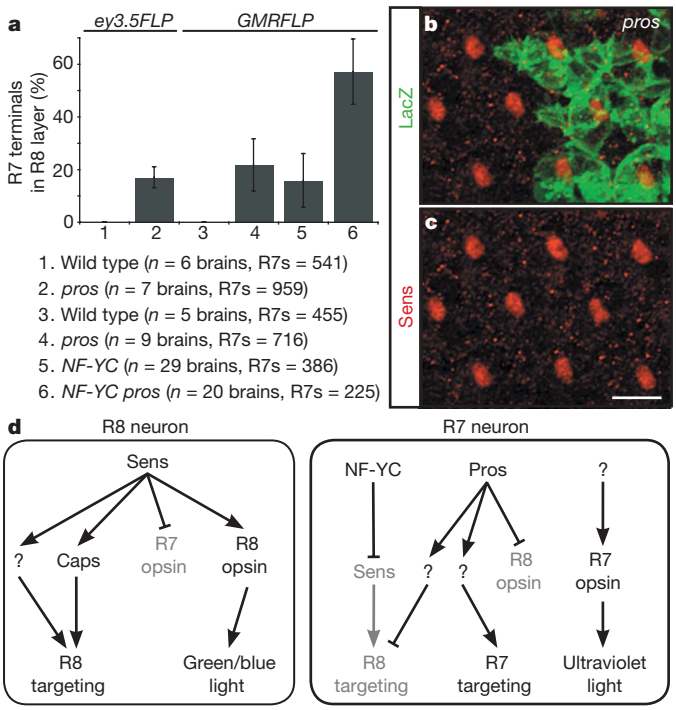

Figure 4 | Pros and NF-YC regulate R7 targeting in parallel pathways. **a**, *pros* and *NF-YC pros* R7 phenotypes in large (*ey3.5FLP*) and isolated (*GMRFLP*) patches of mutant tissue. WT R7s target to M6 (1, 3), whereas *pros* R7s mis-target to M3 (2, 17.1 ± 4%; 4, 21.8 ± 9.9%). *NF-YC pros* R7s have a more severe mis-targeting phenotype (6, 57.2 ± 12.4%; *NFYC* control, 5, 15.9 ± 10.2%). Error bars indicate s.d. **b**, **c**, A 40% APF retina with large patches of *pros* mutant cells (green) stained for Sens (red). R8s express Sens; *pros* mutant R7s do not mis-express Sens. Scale bar, 10 μm. **d**, Summary of the genetic control of layer specificity and rhodopsin expression in R8 and R7 neurons.

METHODS SUMMARY

To generate *NF-YC* or *pros* mutant R7s, we used the MARCM (mosaic analysis with a repressible cell marker) technique as described previously[10]. To generate R7 mosaics (that is, mutant R7 neurons surrounded by wild-type cells) *GMR-FLP* was used as a source of recombinase. For larger patches of mutant tissue in the eye, including R7 neurons (eye mosaics), the *ey3.5-FLP* construct was used. A similar version of the MARCM technique with *GMR-FLP* was used to generate double-mutant R7 neurons. Mutant R7 neurons were marked only when flipping occurred on both chromosomes X and 3. *GMR-FLP* was used with MARCM to generate R7 neurons expressing Sens (*UAS-sens*), in addition to the inducible marker. For assessing expression of R7 and R8 markers, hemizygous *NF-YC*[40] mutant third-instar eye discs and late pupae were analysed.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

The stocks used to assess the targeting of *NF-YC* mutant R7 neurons in large clones were *ey3.5-FLP, tubP-GAL80, FRT19; act-GAL4, UAS-lacZ/CyO* and *NF-YC^{40}, FRT19/FM7, Kr-GFP*. To assess whether *NF-YC* was autonomously required in R7 neurons we used *GMR-FLP*, which induces mitotic recombination in R7, but not R8 cell precursors. The stocks used were *GMR-FLP, tubP-GAL80, FRT19; act-GAL4, UAS-syt-GFP/CyO* and *NF-YC^{40}, FRT19/FM7, Kr-GFP*. *FRT19/FRT19* was used as a control. Because *NF-YC^{40}* mutant animals die before eclosion, analysis of R7 and R8 markers was performed on hemizygous mutant *NF-YC^{40} FRT19/Y* and wild-type *FRT19* eye discs and pupal retinas. To assess the possibility that mis-expression of *sens* in R7 clones was autonomous we used the same stocks used to assess the *NF-YC* requirement in R7. The stocks used to generate flies with R7 neurons double mutant for *NF-YC* and *sens* or *NF-YC* and *caps* were *NF-YC^{40}, FRT19/FM7, Kr-GFP; +; sens^{E1}, FRT79/TM6B* or *NF-YC^{40}, FRT19/FM7, Kr-GFP; +; caps^{C18fs}, FRT79/TM6B* crossed to *GMRFLP, tubP-GAL80, FRT19; act-GAL4, UAS-syt-GFP/CyO; tubP-GAL80, FRT79/TM6B*. A similar scheme was used to generate single R7 neurons mutant for both *NF-YC* and *pros* (*FRT82B, pros$^{17.17}$*). As a control, to generate animals with R7 neurons mutant for *NF-YC* only, *NF-YC^{40}, FRT19/FM7, Kr-GFP; +; FRT79* or *FRT82B* flies were crossed to *GMRFLP, tubP-GAL80, FRT19; act-GAL4, UAS-syt-GFP/CyO; tubP-GAL80, FRT79* or *tubP-GAL80 FRT82B/TM6B*. The stocks used to mis-express *sens* in clones of wild-type R7 neurons were *FRT40/FRT40; UAS-sens^{C5}/TM6B* and *GMR-FLP, tubP-GAL80, FRT40/CyO; tub-GAL4, UAS-Nsyb-GFP/TM6B*. Similar results were obtained with independent insertions on chromosomes X (*UAS-sens^{C1}*) and 3 (*UAS-sens^{C6}*). We confirmed that wild-type R7 neurons mis-expressing *sens* also expressed the R7-specific marker Pros at 40% APF. As a control we generated clones of wild-type R7 neurons by crossing *FRT40/FRT40* to *GMR-FLP, tubP-GAL80, FRT40/CyO; tub-GAL4, UAS-Nsyb-GFP/TM6B*.