SUPPLEMENTARY MATERIAL

Supplementary methods

List of *Drosophila* stocks

The following strains were used in this work: *w*^{1118} (courtesy of R. Stocker); *Canton-S* (courtesy of R. Stocker); *salm-Gal4, UAS-H2B::YFP* (courtesy of C. Desplan; Mollereau et al., 2000); *UAS-syt::eGFP, UAS-mCD8::Cherry* (courtesy of O. Urwyler; Zhang et al., 2002); *FRT42D, so^{3}* (courtesy of F. Pignoni; Pignoni et al., 1997), *UAS-hazy* (courtesy of J. Bischof; Bischof et al., 2013), *UAS-otd* (courtesy of H. Reichert; Blanco et al., 2011), *UAS-mCD8::GFP* and *FRT82B, ato^{w}* (courtesy of B. Hassan; Choi et al., 2009). Stocks containing *glass* mutant alleles were previously characterised in G. Rubin’s lab (Moses et al., 1989), and obtained from the Bloomington stock center: *gf^{60}* (No. 509); *FRT82B, gf^{60}* (No. 6333); *gf^{2}, e^{4}* (No. 507) and *gf^{3}* (No. 508). We used the following stocks as drivers for analysing mutant clones: *eyFLP;; FRT82B, ubiGFP* (courtesy of B. Hassan), *eyFLP; FRT42D, arm-lacZ* (courtesy of J. Curtiss) and *hsFLP; tub-Gal4, UAS-mCD8::GFP; FRT82B, tub-Gal80* (courtesy of B. Bello). For flip-out misexpression experiments we used *hsFLP; tub(FRT cassette)Gal4, UAS-lacZ.nls* (courtesy of E. Piddini).

We took advantage of commonly used balancers and phenotypic markers for performing crosses and selecting flies of the desired genotype, in particular *noc^{ Sco}, Sp, CyO, TM2, MKRS, TM6b* (Lindsley and Zimm, 1992) and *CyO dfd-eYFP* (Le et al., 2006).

Antibodies and fluorescent dyes

We used the following primary antibodies: rabbit anti-βGal (1:1000, Cappel, No. 55976), chicken anti-βGal (1:1000, Abcam, ab9361), rabbit anti-GFP (1:1000, Molecular probes, A-
6455), chicken anti-GFP (1:1000, Abcam, ab13970), rabbit anti-DsRed (1:1000, Clontech, No. 632496), rabbit anti-HRP (1:200, Sigma, P7899), guinea pig anti-Otd (1:750, courtesy of T. Cook; Ranade et al., 2008), rabbit anti-Hazy (1:500, courtesy of A. Zelhof; Zelhof et al., 2003), rabbit anti-Rh2 (1:40, Mishra et al., 2016), rabbit anti-Rh6 (1:10000, courtesy of C. Desplan; Tahayato et al., 2003), rabbit anti-histamine (1:1000, ImmunoStar, No. 22939) and rabbit anti-Ato (1:5000, courtesy of B. Hassan). Mouse monoclonal antibodies anti-Rh4, Rh5, and Rh6 were obtained from S. Britt and used 1:40 (Chou et al., 1999). A number of rabbit polyclonal antibodies against proteins of the phototransduction cascade were produced in C. Zuker’s lab and kindly provided by N. Colley: anti-Arr1 (1:100), anti-Gq (1:100), anti-NorpA (1:100), anti-Trpl (1:100) and anti-InaD (1:100). We obtained the following antibodies from Developmental Studies Hybridoma Bank (DSHB) at The University of Iowa: mouse anti-βGal (1:20, 40-1a), rat anti-Elav (1:30, No. 7E8A10), mouse anti-Chp (1:20, No. 24B10), mouse anti-Fas2 (1:20, ID4), mouse anti-Fas3 (1:20, 70G10), mouse anti-Futsch (1:20, 22C10), mouse anti-Glass (1:10, 9B2.1), mouse anti-Rh1 (1:20, 4C5) and mouse anti-Trp (1:20, MAb83F6).


**Generation and analysis of clones**

MARCM analysis of glass mutant clones was performed in hsFLP; tub-Gal4, UAS-mCD8::GFP; FRT82B, tub-Gal80/FRT82B, gfpO animals. Clones were induced in larvae two
days after the flies had laid the eggs with a 20 minute long heat shock at 37°C. We could identify \( g^{60j} \) clones in the pupal retina positively labelled with mCD8::GFP.

Rescue of the glass mutant retina was tested by inducing Hazy and Otd-expressing clones. For this, we crossed \( hsFLP; \text{tub}(FRT\ \text{cassette})Gal4, \text{UAS-lacZ.nls}; \ g^{60j} \) flies with others carrying combinations of the UAS-hazy and UAS-Otd constructs with the \( g^{60j} \) mutation. Hazy expression was induced alone and together with Otd in 6 day old animals (mainly pupae, at about 24 hours after pupation) with a 5 minute long heat shock at 37°C. We induced the expression of Otd alone in 5 day old animals (mainly late third instar larvae) with a 5 minute long heat shock at 37°C. For both Hazy and Otd we aimed to express them at the time point in which they should be expressed during development in wild-type PRs (Vandendries et al., 1996; Zelhof et al., 2003). We were able to identify Hazy and Otd-expressing cells in the adult glass mutant retina because of the co-expression of nuclear βGal.

To test the potency of Glass to ectopically induce PR markers we generated clones in which combinations of Glass with Hazy and Otd were ectopically expressed. For this we crossed \( hsFLP; \text{tub}(FRT\ \text{cassette})Gal4, \text{UAS-lacZ.nls} \) flies with others carrying the UAS-glass, UAS-hazy and UAS-otd constructs. Clones were induced in 4-6 hour old embryos by a 6 minute heat shock at 37°C. Expression of the UAS promoters was driven by \( \text{tub}(FRT\ \text{cassette})Gal4 \), which we activated by removing the FRT cassette through \( hsFLP \) mediated recombination (Blair, 2003; Struhl and Basler, 1993). Gal4-expressing cells in the CNS of 4 day old larvae were labelled with nuclear βGal.

\( so^{3} \) mutant clones were obtained in the eye discs of \( \text{eyFLP}; \text{FRT42D}, \text{arm-lacZ/FRT42D}, \ so^{3} \) larvae, and could be identified as groups of cells negatively labelled for βGal.

\( ato^{n} \) mutant clones were generated in the eye discs of \( \text{eyFLP};\text{FRT82B}, \text{ubiGFP/FRT82B}, \ ato^{n} \) larvae, and we could recognise them as groups of cells negatively labelled for GFP.
Generation of transgenic flies

In order to generate the *hazy*(wt)-GFP reporter construct, a 1085 bp fragment upstream of the *hazy* start codon was amplified by PCR from wild-type flies and cloned into pBluescript using KpnI and NotI sites attached to the primers. The two Glass binding motifs were then mutated individually and in combination using site directed mutagenesis (Stratagene), to produce the *hazy*(gl1mut)-GFP, *hazy*(gl2mut)-GFP and *hazy*(gl1,2mut)-GFP reporter constructs. The wild-type and mutant sequences were then transferred into an attB-GFP-hsp70 3'UTR reporter vector (modified from a plasmid provided by J. Rister). All constructs were injected into nos-PhiC31; attP40 flies. For primer sequences see table S1.

To generate the *otd*(wt)-GFP reporter construct, the 1.5 kb PR enhancer element (Vandendries et al., 1996) was amplified by PCR from wild-type flies and cloned into pBluescript using the endogenous KpnI and BamHI sites flanking this element. For making *otd*(glmut)-GFP, the Glass binding motif was mutated by PCR amplification from wild-type flies of two fragments of the enhancer with the 5' and 3' flanking primers combined with primers extending to and from the Glass binding motif with a XhoI restriction site replacing the Glass binding motif. The wild-type and mutant constructs were then transferred into the attB-GFP-hsp70 3'UTR reporter vector. Both constructs were injected into nos-PhiC31; attP40 flies. For primer sequences see table S1.

For generating the UAS-*glass* flies we used the Glass PA isoform (REFSEQ:NP_476854, FBpp0083005), containing all five zinc fingers, which has been reported to be functional (O'Neill et al., 1995). To obtain this isoform we had to remove the last intron from the only fully sequenced BDGP DGC *glass* cDNA clone (GH20219) available encoding the Glass PB isoform. This isoform lacks the last 47 amino acids including half of the last zinc-finger due to the presence of a stop codon within the last intron. We removed this intron by PCR amplification of the sequences encoding the Glass PA isoform using primers with overhangs...
that match the coding sequence at the other side of the intron, ligating the two fragments together and PCR amplifying the entire Glass PA coding region and 5'UTR. We cloned this PCR product into the BamHI and XhoI sites of pBluescript using restriction sites added to the flanking primers. We next PCR amplified the Glass PA coding region with primers for gateway cloning and inserted it into a pUASg.attB plasmid (courtesy of J. Bischof; Bischof et al., 2013). The construct was injected into nos-PhiC31; attP40 flies. We tested the ability of the UAS-glass flies to ectopically express the protein by antibody staining against Glass, and found that this construct rescues the glass mutant phenotype when expressed in the eye during development (data not shown). For primer sequences see table S1.

For the glass-GFP reporter constructs a 293 bp BamHI-EcoRI fragment from the middle of the 5.2 kb upstream genomic region of glass was cloned in front of a minimal hsp70 promoter + GFP reporter gene using the endogenous BamHI and EcoRI sites. The BamHI site present in our genomic sequence is missing in the Flybase sequence due to a single nucleotide polymorphism. The putative So binding sites were mutated by PCR amplifications using primers with overhangs replacing the So sites with restriction sites for SpeI (so1) and Ncol (so2 and so3). Since so2 and so3 are very close to each other (within 25 bp) they were mutated together. The PCR fragments were ligated and cloned in front of the minimal hsp70 promoter + GFP reporter gene. Both transgenes were injected into nos-PhiC31;; attP2 flies. For primer sequences see table S1.
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**Immunohistochemistry**

In the case of adult heads, we incubated them in cryoprotectant solution (sucrose 25% in PB) at 4°C overnight. Next we embedded them in OCT and cut 14 µm cryosections in the transverse plane, after which we proceeded to stain them.

For staining both cryosections and whole mounted samples, we first washed them at room temperature with PBT (Triton X-100 0.3% in PB) at least three times for a minimum of 20 minutes each: this procedure was repeated in all washing steps that follow. Incubation in primary antibody solution was done overnight at 4°C and was followed by PBT washes. Next, we incubated our samples in secondary antibody solution overnight at 4°C, after which we washed them. We mounted our samples either in 50% glycerol or Vectashield.
Fig. S1. *glass* mutant PR precursors are not correctly recruited into the developing ommatidia in the third instar eye disc. It has been reported that, at this stage, *glass* mutant PR precursors fail to acquire a correct subtype identity, based on the expression of subtype specific PR markers (Hayashi et al., 2008; Jarman et al., 1995; Lim and Choi, 2004; Treisman and Rubin, 1996). To analyse in detail the order in which PR precursors are recruited in *glass* mutant, we counted the number of Elav-positive cells in the third instar eye disc of wild-type and *gl60j* larvae. Each ommatidium was pseudo-coloured according to the number of PR precursors that it contains. This image illustrates how PR precursors in the wild-type eye disc are orderly recruited into the developing ommatidia (A). By comparison, in the *glass* mutant eye disc PR precursors are recruited slower and disorderly (B). Scale bars represent 30 μm.
Fig. S2. *glass* mutant PR precursors survive metamorphosis and are still present in the adult retina. Expression of YFP (green) and Elav (magenta) in the *salm>H2B::YFP* reporter line in the adult retina of control (A) and *gl* mutant flies (B). In both cases, a subset of presumptive PRs can be identified by the co-expression of YFP and Elav, while cone cells express YFP but not Elav. Scale bars represent 40 μm.
Fig. S3. *glass* mutant PR precursors are irregularly distributed in the retina. (A-C'') We used MARCM analysis to induce the formation of *glo* mutant clones, labeled with the expression of UAS-*mCD8::GFP*. Retinas were dissected ~50 hours after pupation, and stained against
GFP (green), Elav (used to label the nuclei of neurons, red) and Fas3 (used to label the membranes of interommatidial cells, blue). For each image, these three channels are shown below in greyscale. All images belong to the same confocal stack (Movie S1), in which those cells that are wild-type for glass are located in the upper half of the area that is shown, while a big homozygous gl<sup>60</sup> clone crosses the lower half. Distally in the retina, cones strongly express Fas3 and can be seen as groups of 4 cells in the wild-type (GFP-negative) region of the image (arrowheads; A, A”). This kind of organization is not present in the GFP-labeled glass mutant clone (A, A”). More proximally, PR precursors are abundant in the wild-type area, where they distribute in rosettes of 8 Elav-positive cells (arrows; B, B”). Rosettes are separated from each other by pigment and bristle cells, which form the hexagonal lattice of the ommatidia, and are strongly stained for Fas3 (arrowheads; B, B”). By contrast, in the glass mutant region there are fewer Elav-positive cells, and cells do not group in any structure resembling an ommatidium (B, B”, B”). This is different from earlier developmental stages, since ommatidial clusters can still be seen in the glass mutant eye disc (Figs. 1E, S1; Moses et al., 1989; Treisman and Rubin, 1996). The most proximal region of the wild-type retina contains the nuclei of bristle neurons, which are orderly arranged between the ommatidia (arrowheads; C, C”). At this level, the glass mutant clone contains densely packed groups of neurons (C, C”), including the PR precursors missing in the medial section. Scale bar represents 30 μm.
Fig. S4. Glass is required for the acquisition of the phototransduction machinery. (A-T) Expression of different proteins involved in the phototransduction cascade in the adult retina of control (salm>H2B::YFP) and gl2 mutant flies. Samples were stained against phototransduction proteins (green) and counterstained with DAPI (magenta). Rhodopsins Rh1 (A), Rh4 (B), Rh5 (C), and Rh6 (D) are expressed in different subsets of PRs in control retinas. In the gl2 mutant retina there is no expression of Rh1 (F), Rh4 (G), Rh5 (H) or Rh6 (I). Proteins downstream in the phototransduction cascade are expressed in all PRs in the retina of control flies: Arr1 (E), Gaq (K), NorpA (L), Trp (M), Trpl (N) and InaD (O). These proteins are not expressed in the retina of gl2 mutant flies: Arr1 (J), Gaq (P), NorpA (Q), Trp (R), Trpl (S), or InaD (T). Scale bars represent 40 μm.
Fig. S5. Glass is required for Rh2 expression. Both control and glass mutant flies were stained against Rh2 (green) and counterstained with Elav (magenta). Rh2 is expressed in ocellar PRs in control flies (A). In glass mutant, there is no expression of Rh2 in the presumptive ocelli PRs (arrowheads, B). Scale bars represent 40 μm.
Fig. S6. Glass is required for the correct expression of Hazy and Otd. (A-D') We used the *salm>H2B::YFP* reporter to label the retina of adult flies, and stained against GFP (green), either Hazy or Otd (red), and the neuronal marker Elav (Blue). For each image, the red channel is shown below in greyscale. There is expression of Hazy in the nuclei of PRs in control flies (A, A') but not in the presumptive PRs of *glass* mutant flies (B, B'). Otd is expressed in the PRs of control flies (C, C') but only a fraction of presumptive *glass* mutant PRs express Otd (D, D'). Scale bars represent 50 μm.
Fig. S7. Expression of the otd(wt)-GFP reporter is independent of Glass. Samples were stained against GFP (green) and against the neuronal marker Elav (magenta). (A-C) In adult flies, otd(wt)-GFP is expressed both in control (A) and glass mutant background (B). This reporter is also expressed when the Glass binding motif is mutated (C). (D, E) At 40-50 hours after pupation, all PR precursors express the otd(wt)-GFP reporter in control animals (D). After mutation of the Glass binding motif the reporter is still expressed in all PRs (E). Scale bars represent 10 μm in panels D-E, and 50 μm in panels A-C.
Fig. S8. Induced co-expression of Hazy and Otd does not rescue the glass mutant phenotype better than Hazy alone. (A-F’) Hazy and Otd were expressed in the glass mutant retina in clones labelled with nuclear βGal. Samples were stained against βGal (green),
different proteins involved in the phototransduction cascade (red) and with DAPI (used to label cell nuclei, blue). For each image, the red channel is shown below in greyscale. We did not observe the rescue of any of those proteins that were not rescued by Hazy alone (the rescue of *glass* mutant with Hazy is shown in Fig. 5), namely the expression of Rh1 (A, A’), Rh2 (B, B’), Rh4 (C, C’), Rh5 (D, D’), Goq (E, E’) and Trp (F, F’) was not induced in Hazy-Otd-expressing clones. Scale bars represent 40 μm.
Fig. S9. Expression of PR markers in the CNS of third instar larvae. (A-H') The CNS of control (Canton-S) animals was stained with antibodies against different PR proteins (green) and counterstained with Hoechst 33258 (used to label cell nuclei, magenta). For each image, the green channel is shown below in greyscale. Hazy is expressed in the nuclei of PRs in the Bolwig organ (Zelhof et al., 2003), and cannot be seen in the CNS (A, A'). Chp is primarily expressed in the axons of PRs in the Bolwig organ, which project into the optic lobe (arrowheads; B, B'). In addition, a small number of cells in the brain are stained (arrow; B, B').
Neither Rh1 nor Rh2 are expressed in the CNS of the larvae (C, D). Both Arr1 and NorpA are expressed in the axon projections of the Bolwig organ PRs (arrowheads; E, E', F, F'). Trp1 is expressed in the axons of PRs in the Bolwig organ (arrowheads; G, G') and in 3-4 cells located rostrally in each of the brain lobes (arrows; G, G'). InaD is expressed in the axon projections of the Bolwig organ PRs (arrowheads; H, H'). Scale bars represent 80 μm.
Fig. S10. Co-misexpression of Glass and Hazy is not sufficient to ectopically induce all the phototransduction proteins that we have tested, and co-misexpression of Glass and Otd does not ectopically induce more phototransduction proteins than Glass alone (for a comparison, see Fig. 6). Misexpression of these transcription factors was induced during embryonic development in clones, which were labelled by the presence of nuclear βGal. We dissected and stained the CNS of third instar larvae with antibodies against βGal (green), different phototransduction proteins (red/magenta) and with Hoechst 33258 (used to label cell nuclei, blue). Close-ups are shown below each sample. (A-E') Co-misexpressing Glass and Hazy is not sufficient to ectopically induce Rh4 (A, A'), Rh5 (B, B'), Rh6 (C, C'), Gaq (D, D') nor Trp (E, E'). (F-N') Co-misexpressing Glass and Otd is not sufficient to ectopically induce Rh4 (F, F'), Rh5 (G, G'), Rh6 (H, H'), Gaq (I, I'), Trp (J, J'), Rh1 (K, K'), Arr1 (L, L'), NorpA (M, M') nor InaD (N, N'). Scale bars represent 10 μm in A'-N', and 80 μm in A-N.
Fig. S11. The proneural transcription factor Ato is not required for glass expression. (A) During the development of the third instar eye disc, Ato expression (red) precedes that of Glass (green). Counterstaining with phalloidin (blue) serves to locate the position of the morphogenetic furrow (arrow), where both transcription factors overlap in a narrow band of cells. The three channels are shown in greyscale on the right (A'-A''). (B) To test whether Ato is required for the expression of glass we induced the formation of ato\textsuperscript{w} mutant clones in the third instar eye disc, which were labelled by the absence of GFP staining. Samples were stained against Glass (red), GFP (green) and with Hoechst 33258 (used to label cell nuclei, blue). A close-up on the right shows that Glass (magenta) is expressed in atonal mutant clones, which lack GFP (green) (B''). Scale bars represent 10 \mu m in B', and 40 \mu m in A and B.
Movie S1. Confocal stack showing the structure of a retina that contains a GFP-labeled \( gl^{60j} \) clone, induced by MARCM. Images from this stack were used for Fig. S1. Z-stack frames are ordered from distal to proximal, and show staining against GFP (green, A'), Elav (used to label the nuclei of neurons, red, A'') and Fas3 (used to label the membranes of interommatidial cells, blue, A'''). The typical structure of the ommatidia can be recognized in the upper half of the stack, where cells possess the wild-type version of \( glass \), but not in the lower half of the stack, where cells are homozygous for \( gl^{60j} \) and are disarrayed. Taking the expression of Elav as a guide, it seems that most PR precursors relocate basally in the \( gl^{60j} \) clone. Scale bar represents 30 \( \mu \)m.
Supplementary references

Development 140, 2434-2442.

Development 130, 5065-5072.


