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The transcription factor Glass links eye field specification with photoreceptor differentiation in *Drosophila*

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ABSTRACT

Eye development requires an evolutionarily conserved group of transcription factors, termed the retinal determination network (RDN). However, little is known about the molecular mechanism by which the RDN instructs cells to differentiate into photoreceptors. We show that photoreceptor cell identity in *Drosophila* is critically regulated by the transcription factor Glass, which is primarily expressed in photoreceptors and whose role in this process was previously unknown. Glass is both required and sufficient for the expression of phototransduction proteins. Our results demonstrate that the RDN member Sine oculis directly activates *glass* expression, and that Glass activates the expression of the transcription factors Hazy and Otd. We identified *hazy* as a direct target of Glass. Induced expression of Hazy in the retina partially rescues the *glass* mutant phenotype. Together, our results provide a transcriptional link between eye field specification and photoreceptor differentiation in *Drosophila*, placing Glass at a central position in this developmental process.

KEY WORDS: Eye formation, Photoreceptor, Cell fate, Phototransduction, *Drosophila*

INTRODUCTION

The ability to process visual information is an important feature for animal survival. Different phyla have developed various types of eyes containing specialised photoreceptor neurons (PRs). Despite the diversity of eyes, eye development across metazoans requires a group of transcription factors, called the retinal determination network (RDN), whose function is evolutionarily conserved (Silver and Rebay, 2005). In *Drosophila*, the core RDN genes are *eyeless*, *sine oculis* (*so*) and *eyes absent* (*eya*). These genes specify epithelial cells of the eye imaginal disc to form the compound eye. Flies mutant for any of the RDN genes typically lack eyes, or have eyes that are markedly reduced in size (Hoge, 1915; Bonini et al., 1993; Cheyette et al., 1994). Conversely, genetic manipulations leading to the ectopic expression of RDN genes in imaginal discs other than the eye disc induce the formation of ectopic eyes (Halder et al., 1995; Pignoni et al., 1997). Despite the importance of the RDN, little is known about the downstream mechanism by which it regulates eye formation, particularly how PR cell identity is established.

Eye disc precursors originate in the optic primordium during embryogenesis and subsequently proliferate during the first and second larval instars to form the eye-antennal imaginal disc. By the end of the third instar, cells contract apically, forming a transient groove termed the morphogenetic furrow, which sweeps across the eye disc. After the passage of the morphogenetic furrow, the proneural transcription factor Atonal (Ato) triggers specification of the R8 PR precursors, which sequentially recruit other PR precursors into the developing ommatidia by EGFR signalling: first R2/R5, then R3/R4, R1/R6 and finally, R7 (Ready et al., 1976; Treisman, 2013). A number of genes that are differentially expressed in the distinct PR subtypes control their subtype identity, and regulate how these cells develop during metamorphosis into adult PRs (Mollereau and Domingos, 2005; Tsachaki and Sprecher, 2012; Treisman, 2013).

During pupation, PR precursors undergo terminal differentiation. Proteins involved in the phototransduction cascade start to be expressed and localise to the rhabdomere, which forms on the elongating cells (Montell, 2012). In spite of broad knowledge of how the eye field is specified and how different PR subtypes are recruited, we have limited knowledge about the factors involved in the transition from neuronal specification to PR differentiation. Because the morphological changes and phototransduction proteins are common to all PR subtypes of the retina, it is plausible that these processes are regulated by a common set of transcription factors. It has been shown that rhabdomere formation, together with the expression of some of the proteins involved in phototransduction, is transcriptionally controlled by the redundant function of two homeodomain proteins: Orthodenticle (Otd) and Hazy. Both genes are expressed in all PRs and seem to act through separate pathways (Vandendries et al., 1996; Tahayato et al., 2003; Zelhof et al., 2003; Mishra et al., 2010). How the expression of Otd and Hazy is induced in PRs, and which transcription factors mediate between initial PR specification by the RDN and their final differentiation into functional PRs has not yet been resolved.

The transcription factor Glass is a good candidate to fulfil this role in specification of PR identity. Glass is primarily expressed in the visual system. Its expression starts early during eye development in all cells posterior to the morphogenetic furrow and is maintained in adult PRs (Moses and Rubin, 1991; Ellis et al., 1993). It has been suggested that *glass* mutant PR precursors die during metamorphosis. Therefore, its role in PR differentiation has not been assessed (Stark et al., 1984; Ready et al., 1986; Moses et al., 1989).

We have found that Glass is a central piece in a genetic pathway leading to PR cell formation. We show that *glass* acts downstream of the RDN member So, and that it is crucially required for the acquisition of PR cell identity by regulating the expression of Otd and Hazy. We demonstrate that contrary to previous publications, *glass* mutant PR precursors survive metamorphosis and become neurons, but fail to acquire the phototransduction machinery and do not differentiate morphologically into PRs. Ectopic expression of Glass is sufficient to induce Hazy and proteins involved in phototransduction. Taken together, our results reveal a sequence of transcriptional events in which Glass links transcription factors

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that are involved in eye field specification with genes of terminally differentiated PRs.

RESULTS

In *glass* mutants, PR precursors survive metamorphosis and are present in the adult retina

Previous publications suggest that *glass* mutant PR precursors die during metamorphosis (Stark et al., 1984; Ready et al., 1986; Moses et al., 1989). In order to assess the role of Glass during PR development, we decided to determine at which point PR precursors are lost in *glass* mutants.

We used a *spalt major* (*salm*) reporter to trace the fate of *glass* mutant PR precursors. In the eye disc of control third instar larvae, *salm* drives the expression of H2B::YFP in half of the PR precursors (R3, R4, R7, R8), as well as in cone cells (Fig. 1A). PR precursors can be distinguished from cone cells by their position and the expression of the pan-neuronal protein Elav (Tomlinson and Ready, 1987; Cagan and Ready, 1989; Robinow and White, 1991). This pattern of H2B::YFP expression was maintained during pupation and in the adult retina (Fig. 1B-D,I-I″). We analysed expression of the *salm>H2B::YFP* reporter in gl^{60j} mutant background, an amorphic mutation of *glass* (Moses et al., 1989): in the third instar eye disc, PR precursors are still specified, as indicated by the expression of Elav. However, the number of PRs was reduced and their arrangement was disorganised (Fig. 1E, Fig. S1). H2B::YFP was expressed in some, but not all PR precursors and in presumptive cone cells, comparable to expression in wild-type discs. We followed the expression of H2B::YFP during metamorphosis and found that although the regular organisation of the retina is severely compromised, double-positive cells for H2B::YFP and Elav are maintained into the adult stage of gl^{60j} mutant flies (Fig. 1F-H). We obtained similar results using the *glass* mutant allele gl^{3} (Fig. S2).

To follow the fate of the *glass* mutant cells during metamorphosis, we also induced clones by mosaic analysis with a repressible cell marker (MARCM) in the developing eye. Whereas small *glass* mutant clones often incorporated into the ommatidia and retained their positions with respect to wild-type PRs, cells in large *glass* mutant clones did not acquire their typical regular organisation and re-localised to the basal side of the retina (Fig. S3, Movie 1). Thus, we conclude that, in contrast to previous reports, Glass is not

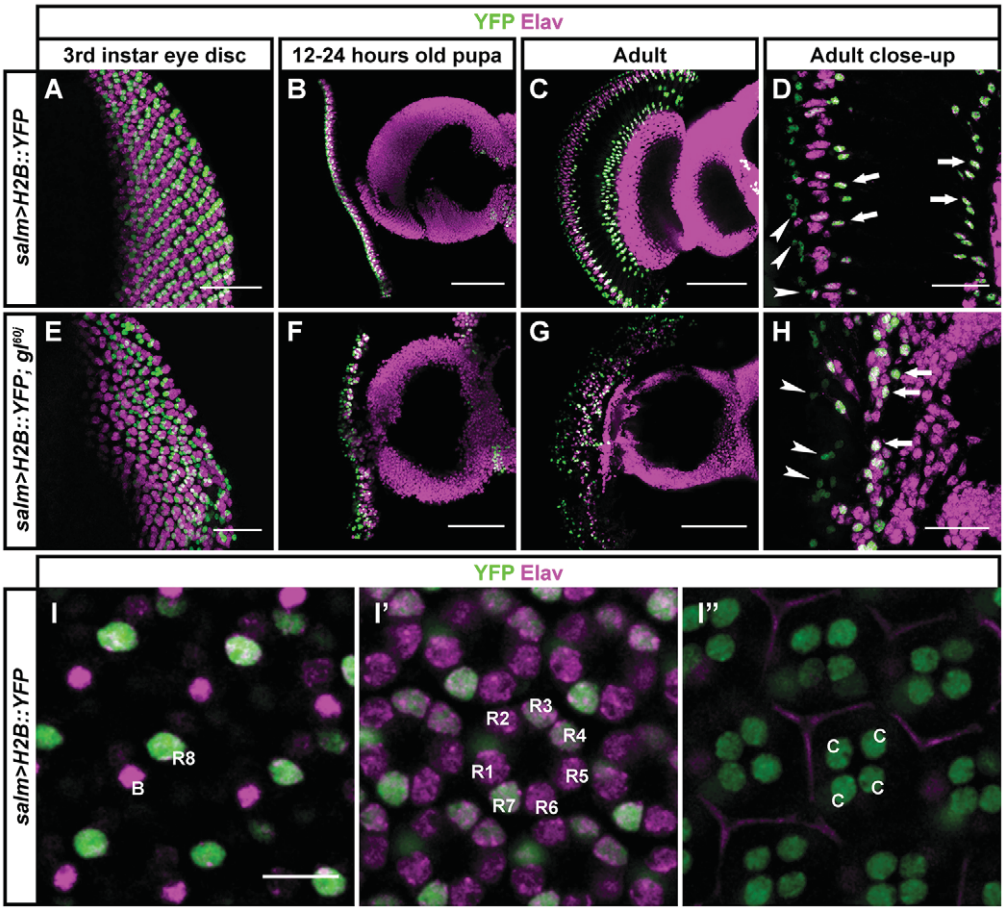

Fig. 1. *glass* mutant PR precursors survive metamorphosis and are still present in the adult retina. (A-H) Expression of YFP (green) and Elav (magenta) in a *salm>H2B::YFP* reporter line at different developmental time points in control and glass mutant background. *The salm>H2B::YFP* reporter is expressed in a fraction of PR precursors and in cone cells in the third instar eye disc (A), pupal retina (B) and adult retina (C,D). A subset of PRs can be identified by the co-expression of YFP and Elav (arrows in D), whereas cone cells do not express Elav (arrowheads in D). In *glass* mutant background, *salm>H2B::YFP* is also expressed in a fraction of PR precursors and in cone cells in the third instar eye disc (E), pupal retina (F) and adult retina (G,H). Note that PR precursors are still present and can be identified by the co-expression of YFP and Elav (arrows in H), whereas cone cells do not express Elav (arrowheads in H). (I-I″) Expression of YFP (green) and Elav (magenta) in whole mounted retinas of *salm>H2B::YFP* at 50-60 h after pupation. Images belong to the same confocal stack: YFP is detectable in proximally located R8 PR precursor nuclei (I), but not in the precursors of the mechanosensory bristle neurons, labelled 'B'. Distal to these cells, YFP is expressed in R3, R4 and R7 PR precursors, but not in R1, R2, R5 nor R6 (I′). More distal in the retina, YFP is expressed in cone cells, labelled 'C'. Scale bars: 10 µm in I (also for I′ and I″); 20 µm in D and H; 30 µm in A and E; and 80 µm in B,C,F,G.

required for the survival of PR precursors, and that presumptive PRs are still present in the adult *glass* mutant retina.

glass mutant PR precursors differentiate as neurons

PR precursors in the eye discs of *glass* mutant larvae express neuronal markers and project axons (Moses et al., 1989; Selleck and Steller, 1991; Kunes et al., 1993; Treisman and Rubin, 1996). We confirmed these results using antibodies against Elav (Fig. 1E), Futsch, Fasciclin 2 (Fas2) and horseradish peroxidase (HRP; Fig. 2A-F). This corroborates that *glass* mutant PR precursors are committed to becoming neurons. To determine whether the surviving *glass* mutant PRs fully differentiate as neurons, we tested if they maintained their axons, formed synapses or synthesised a neurotransmitter.

To track the processes of developing axons in *glass* mutant PR precursors, we labelled their membranes with *salm>mCD8::*

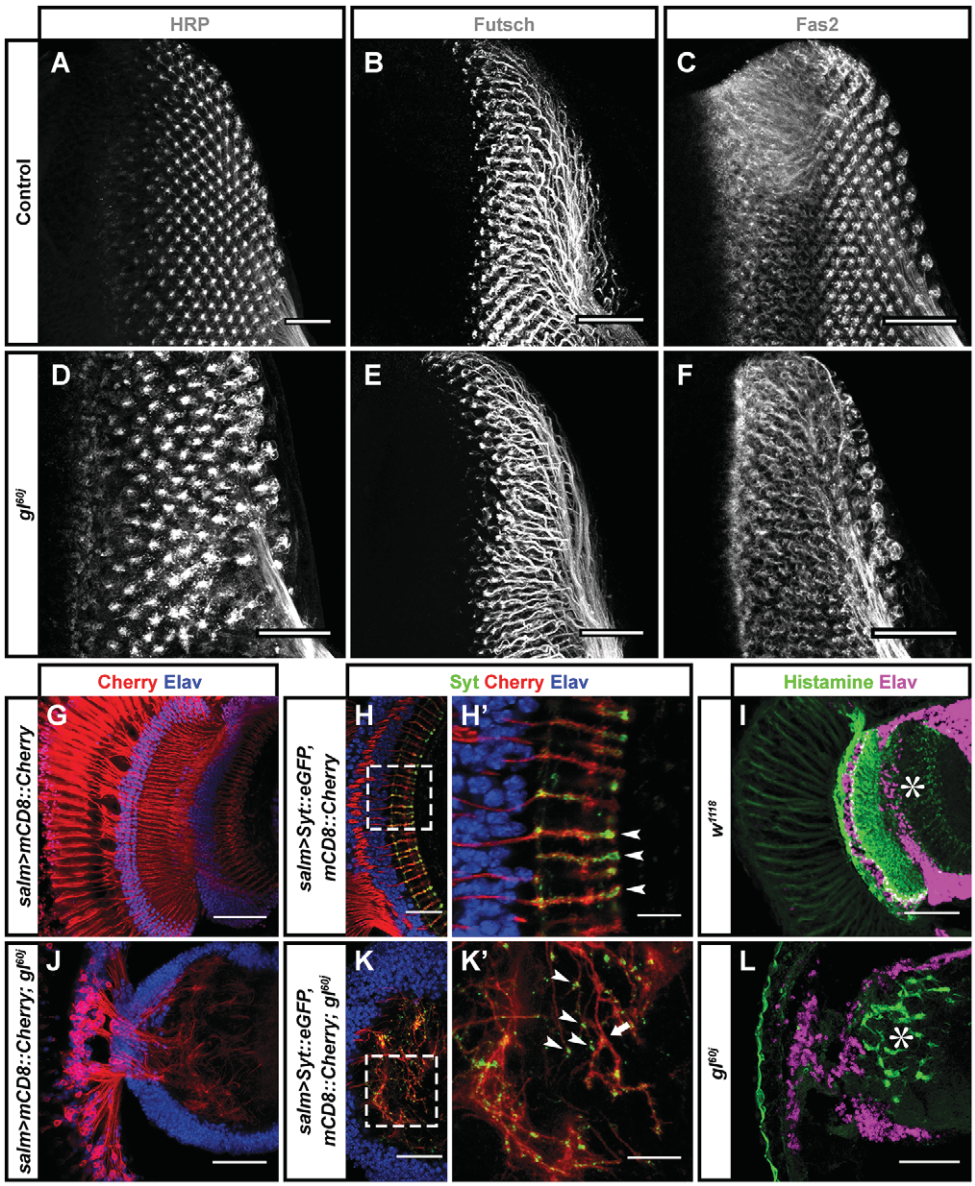

Fig. 2. *glass* mutant PR precursors differentiate as neurons. (A-F) Expression of neuronal markers in the third instar eye disc. PR precursors in the third instar eye disc of control larvae express neuronal markers as revealed by antibody staining against HRP (A), Futsch (B) and Fas2 (C). *glass* mutant PR precursors in the third instar eye disc also express these neural markers, as shown by antibody staining against HRP (D), Futsch (E) and Fas2 (F). (G,J) PR precursors at 50-60 h after pupation were labelled by the expression of *salm>mCD8::Cherry* (red) and the brain was counterstained with the neuronal marker Elav (blue). PR precursors project their axons into the optic lobe, both in control (G) and *glass* mutant pupae (J). (H,H′,K,K′) To further study the axons of the PR precursors at 50-60 h after pupation we used *salm-Gal4* to label presynaptic specialisations by expressing Syt::eGFP (green) on the axons of the PR precursors, which are labelled with mCD8::Cherry expression (red) and brains were counterstained with Elav (blue). In control pupae, PR precursors project unbranched axons into the optic lobe and Syt::eGFP accumulates at the tips of the axons (arrowheads, H,H′). In *glass* mutant pupae, PR precursors project branched axons into the optic lobe (K,K′; arrow indicates an axon branching) and Syt::eGFP accumulates both at the tips and along the length of the axons (arrowheads, K,K′). (I,L) Expression of the neurotransmitter histamine in the optic lobe of adult flies. Brains were stained for histamine (green) and counterstained with Elav (magenta). In the optic lobe of control flies (w^{1118}), histaminergic projections from the PRs innervate the lamina and the medulla (asterisk, I), whereas in *glass* mutant flies, histaminergic projections from the presumptive PRs innervate mainly the medulla (asterisk, L) and the lamina is reduced or missing. Scale bars: 10 µm in H′,K′; 30 µm in A-F,H,K; and 50 µm in G,J,I,L.

Cherry. At 50-60 h after pupation, PR precursors of control pupae projected unbranched axons in a regular pattern into the optic lobe and established synapses in the lamina and the medulla (Fischbach and Hiesinger, 2008; Fig. 2G). *glass* mutant PR precursors still projected their axons into the optic lobe; however, we found that axonal projections were highly disorganised and branched profusely (Fig. 2J). The lamina was reduced in size and labelled axons innervated primarily the medulla. To study whether the axons of *glass* mutant PR precursors differentiate presynaptic specialisations, we drove the expression of eGFP-labelled synaptotagmin (Syt), which is commonly used as a marker for synaptic vesicles (Zhang et al., 2002; Sánchez-Soriano et al., 2005; Chen et al., 2014). In control pupae, Syt::eGFP accumulated at the distal tips of PR axons (Chen et al., 2014; Fig. 2H,H′). In gl^{60j} mutants, Syt::eGFP also accumulated at distinct foci, but these were spread along the length of the axons (Fig. 2K,K′). This suggests that PR precursors in *glass* mutant flies develop axonal projections and differentiate to establish synapses.

Adult PRs express the neurotransmitter histamine and they are the only histaminergic neurons projecting into the lamina and the medulla (Pollack and Hofbauer, 1991; Fig. 2I). Histaminergic projections are still present in the optic lobe of *glass* mutant flies (Fig. 2L). These projections were disorganised compared with those in the wild type and localised primarily in the medulla, which is consistent with the irregular morphology of the *glass* mutant projections described above (Fig. 2J). Taken together, our results show that Glass is not required for PR precursors to acquire neuronal features. However, Glass is necessary for the correct organisation of axonal projections in the optic lobe.

glass mutant PR precursors fail to differentiate into mature PRs

Previous publications have analysed the *glass* mutant phenotype in the third instar eye disc, both by staining with antibodies against cell type-specific markers and RNA sequencing (Jarman et al., 1995; Treisman and Rubin, 1996; Lim and Choi, 2004; Hayashi et al., 2008; Naval-Sanchez et al., 2013). Although these data show differences in the early development of *glass* mutant PR precursors, it remains unknown what role Glass plays later in PR development. Because PR precursors survive metamorphosis and express neuronal markers in *glass* mutants, we next analysed their ability to differentiate into mature PRs. Mature PRs display a characteristic morphology due to the elongation of their cell bodies and the formation of rhabdomeres. Each rhabdomere consists of a densely packed stack of microvilli containing the components of the phototransduction pathway (Montell, 2012). *glass* mutant PR precursors did not elongate during metamorphosis (Fig. 2J) and no rhabdomeres are present in the adult *glass* mutant retina (Stark et al., 1984).

We tested whether proteins involved in phototransduction are still expressed in the adult retina of gl^{60j} and gl^{2} mutant flies. We used primary antibodies directed against different rhodopsins, which are expressed in different subsets of PRs: Rhodopsin 1 (Rh1), Rhodopsin 4 (Rh4), Rhodopsin 5 (Rh5) and Rhodopsin 6 (Rh6) (de Couet and Tanimura, 1987; Chou et al., 1999; Fig. 3A-D); and against proteins that are downstream in the phototransduction cascade and expressed in all PRs: Arrestin 1 (Arr1), G protein α q subunit (Gαq), No receptor potential A (NorpA), Transient receptor potential (Trp), Transient receptor potential-like (Trpl) and Inactivation no afterpotential D (InaD; Wong et al., 1989; Dolph et al., 1993; Zhu et al., 1993; Lee et al., 1994; Shieh and Niemeyer, 1995; Niemeyer et al., 1996; Montell, 2012; Fig. 3E,K-O). In all cases, these proteins were expressed in the retinas of control flies, but were absent in the retinas of *glass* mutant flies (Fig. 3F-J,P-T; Fig. S4). The ocelli-specific Rhodopsin 2 (Rh2) was also lost in *glass* mutants (Fig. S5). These results demonstrate that Glass is critically required during PR differentiation for the formation of rhabdomeres and the expression of phototransduction proteins.

Glass activates expression of transcription factors Hazy and Orthodenticle

Hazy and Otd are two transcription factors that are required for the differentiation of PRs. However, their mutant phenotypes are milder than that of *glass* (Vandendries et al., 1996; Tahayato et al., 2003; Zelhof et al., 2003; Mishra et al., 2010). Therefore, we tested whether *hazy* and *otd* act downstream of Glass. Indeed, although Hazy was expressed in the nuclei of PRs in control retinas, it was absent in those of *glass* mutant flies (Fig. S6A,B). By clonal analysis, we found that Glass is required in a cell-autonomous manner for the expression of Hazy (Fig. 4A). We also tested whether Glass is required for the expression of *otd*. In the *glass* mutant retina most neurons failed to express Otd (Otd was expressed in all PRs in the retina of control flies, which constitute 89% of retinal neurons, whereas in the *glass* mutant retina, Otd was only expressed in 22% of the neurons, n=280 neurons; Fig. S6C,D). By clonal analysis we found that those PRs that required Glass for the expression of Otd, did so in a cell-autonomous manner (Fig. 4B).

Whereas Otd is widely expressed in the developing nervous system, Hazy expression is restricted to PRs (Finkelstein et al., 1990; Zelhof et al., 2003). To determine whether Glass is sufficient to induce expression of *hazy*, we expressed Glass ectopically during embryonic development in clones labelled by co-expression of nuclear β-galactosidase (βGal). We found broad expression of Hazy across the larval central nervous system (CNS) in cells that ectopically expressed Glass, which shows that Glass is sufficient to induce Hazy (Fig. 4C,C′).

To address whether Glass directly activates the expression of *hazy,* we analysed a 1.1 kb genomic region upstream of the Hazy Start codon spanning the *hazy* promoter and 5′UTR (Fig. 4D). We first generated flies containing a *hazy(wt)-GFP* reporter construct, which expressed GFP specifically in PRs, reflecting the expression pattern of Hazy (Fig. 4E). When this reporter was introduced into *glass* mutant background, GFP expression was completely lost (Fig. 4F). There are two potential Glass binding sites (*gl1* and *gl2*) within the genomic fragment that we used for making the reporter (Enuameh et al., 2013), which are evolutionarily conserved across different *Drosophila* species (Fig. 4D). Expression of the GFP reporter was reduced when either *gl1* or *gl2* were mutated alone (Fig. 4G,H), and lost when both *gl1* and *gl2* were mutated (Fig. 4I). Taken together, these results suggest that Glass directly activates the expression of *hazy* through the *gl1* and *gl2* sites.

We also examined whether Glass directly activates the expression of *otd*. An eye-specific enhancer is present within the third intron of the *otd* gene. A reporter containing this enhancer is sufficient to drive βGal expression in PRs, both in control and hypomorphic gl^{3} retinas (Vandendries et al., 1996). We generated an *otd(wt)-GFP* reporter containing the same enhancer of *otd*, and placed it in the amorphic gl^{60j} background (Moses et al., 1989). *otd(wt)-GFP* was expressed primarily in PRs, and its expression pattern did not change in the gl^{60j} mutant background (Fig. S7). Although this *otd* enhancer contains a potential Glass binding site, mutating it in the reporter did not lead to changes in the GFP signal (Fig. S7), suggesting that other transcription factors can activate *otd* expression through this enhancer in the absence of Glass. Thus,

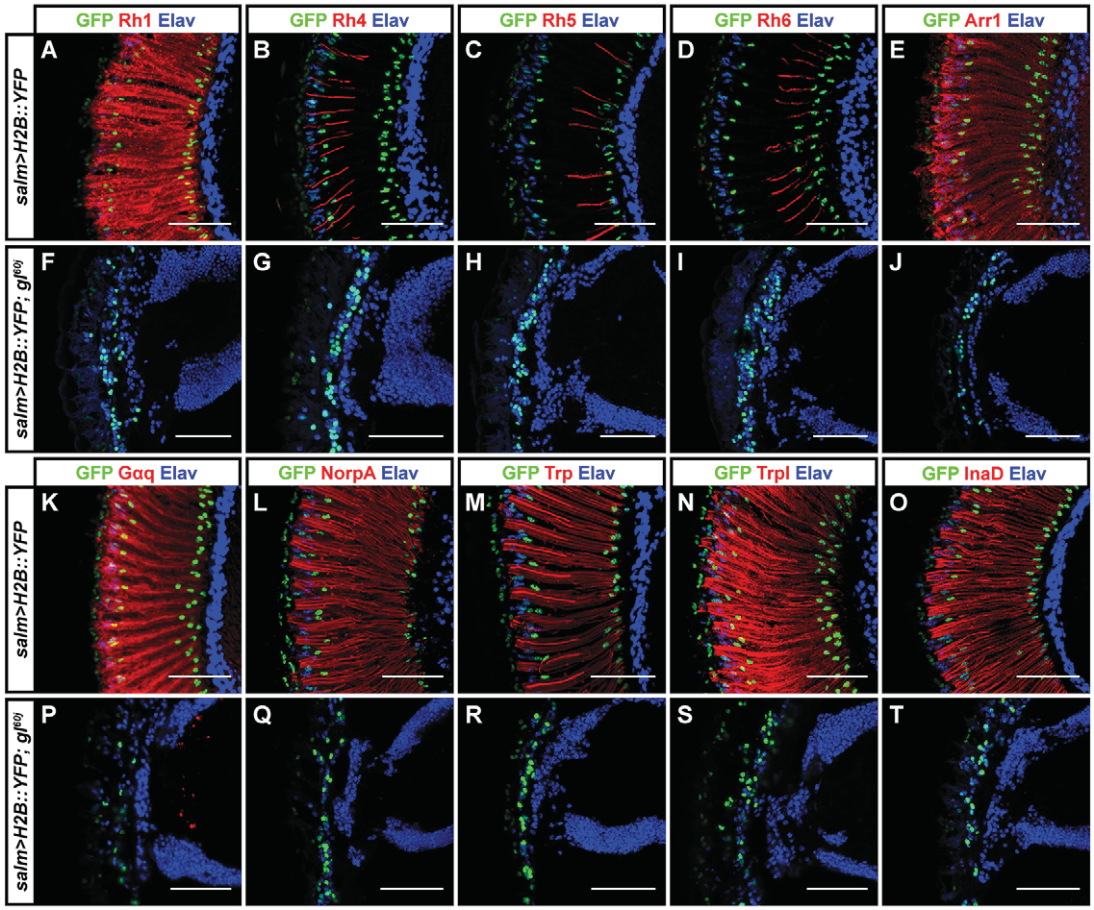

Fig. 3. Glass is required for acquisition of the phototransduction machinery. (A-T) Expression of proteins involved in the phototransduction cascade in the adult retina of *salm>H2B::YFP* (used as control) and *salm>H2B::YFP; gl60j* flies, which were stained against YFP (green), different phototransduction proteins (red) and counterstained with the neuronal marker Elav (blue). Rhodopsins Rh1 (A), Rh4 (B), Rh5 (C) and Rh6 (D) are expressed in different subsets of PRs in control retinas. In the retinas of *glass* mutant flies, 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), Gαq (K), NorpA (L), Trp (M), Trpl (N) and InaD (O). There is no expression of these proteins in the retina of *glass* mutant flies (J,P-T). Scale bars: 40 µm.

Glass is required for the correct expression of both Hazy and Otd in the retina, and sufficient to ectopically induce Hazy expression. Expression of *hazy* depends on two Glass binding motifs in its enhancer, suggesting that *hazy* is a direct target of Glass.

Hazy can partially rescue the *glass* mutant phenotype

To study the role of Hazy and Otd during PR differentiation, we attempted to rescue the *glass* mutant phenotype through Hazy and Otd expression in the retina. Hazy was expressed in clones during pupal development, labelled by co-expression of nuclear βGal. We tested the rescue of Rh1, Rh2, Rh4, Rh5, Rh6, Arr1, Gαq, NorpA, Trp, Trpl and InaD. Some Hazy-expressing cells in the adult *glass* mutant retina also stained positively for Rh6 (Fig. 5A,A′), Arr1 (Fig. 5B,B′), NorpA (Fig. 5C,C′), Trpl (Fig. 5D,D′) and InaD (Fig. 5E,E′). These results demonstrate that Hazy can partially rescue the *glass* mutant phenotype. It should be noted that, although Hazy-expressing clones were not restricted to the retina, those proteins that were rescued by Hazy were primarily expressed in the retina, thus suggesting that the ability of Hazy to activate them is context dependent.

We also attempted to rescue the *glass* mutant phenotype by expressing Otd in the developing eye of late third instar larvae. For this, we induced Otd expression in βGal-labelled clones. Otd was not able to rescue any of the phototransduction proteins that we tested: Rh1, Rh2, Rh4, Rh5, Rh6, Arr1, Gαq, NorpA, Trp, Trpl or InaD. Similarly, we tried to rescue the *glass* mutant phenotype by co-expression of Otd and Hazy. Our results for these experiments were comparable to those in which we expressed Hazy alone (Fig. S8).

Thus, activation of *hazy* by Glass is an important step for PR cell differentiation. Expression of Hazy in the *glass* mutant retina can partially rescue the *glass* mutant phenotype, whereas expression of Otd is not sufficient.

Ectopic expression of Glass and Hazy drives expression of PR proteins

Glass plays an essential role in PR terminal differentiation by activating the genes that allow PRs to transduce light into neuronal signals. Since phototransduction genes are primarily expressed in PRs, and not in most other neurons (Fig. S9), we next tested whether Glass can induce their expression ectopically. We ectopically expressed Glass in the embryonic CNS by generating *UAS-glass*-expressing clones, which were labelled by the co-expression of nuclear βGal. Subsequently, we tested whether Glass could ectopically induce the expression of PR markers in the CNS of third instar larvae. We stained against the following proteins: Chaoptin (Chp), Rh1, Rh2, Rh4, Rh5, Rh6, Arr1, Gαq, NorpA, Trp, Trpl and InaD. Of these, we found ectopic expression of Chp, Rh2 and Trpl, but none of the other PR markers in Glass-expressing cells (Fig. 6A-G′).

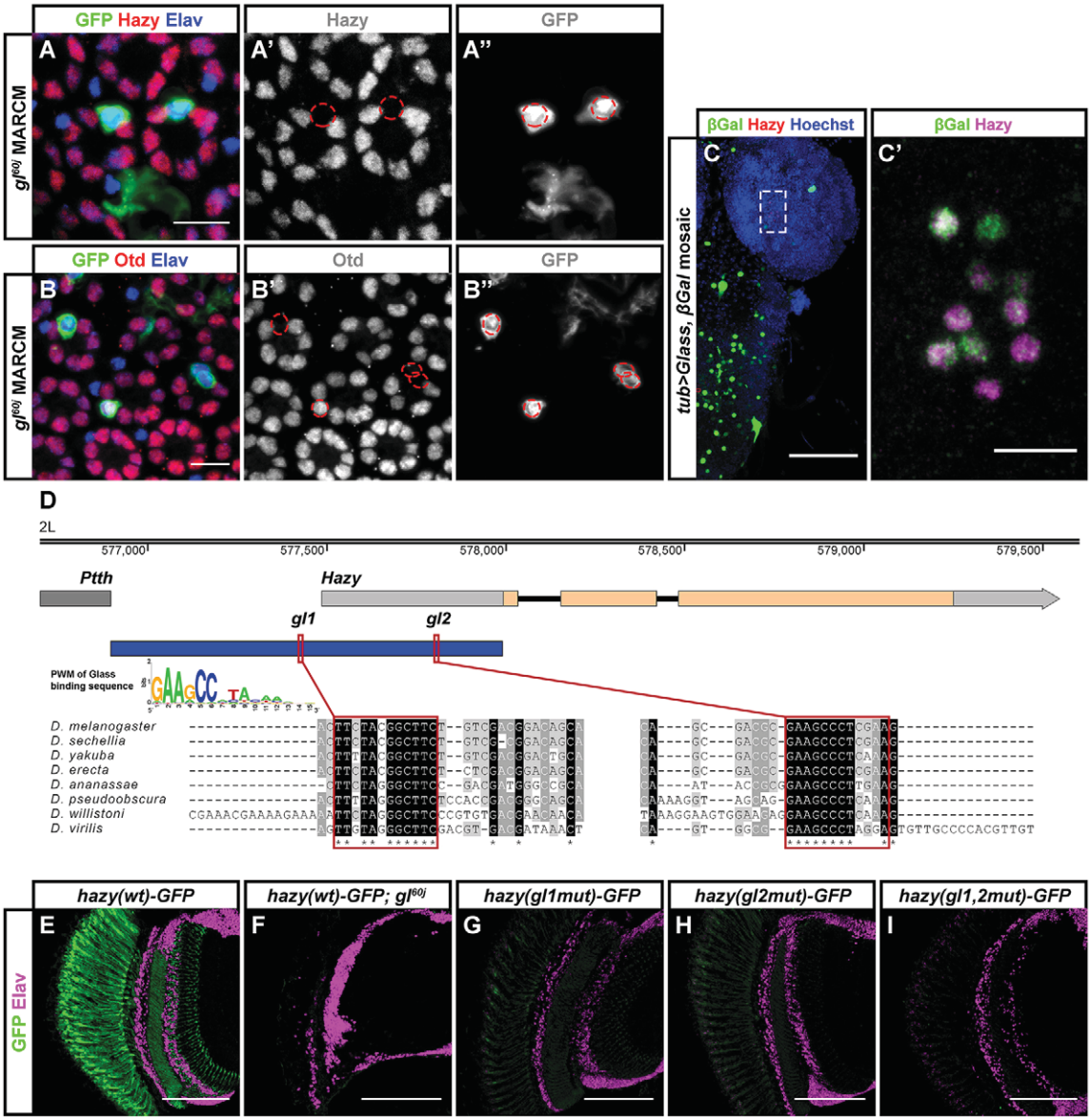

Fig. 4. Glass regulates the expression of Hazy and Otd. (A-B″) MARCM analysis of *glass* mutant cells was performed, in which homozygous gl^{60j} clones were labelled with *UAS-mCD8::GFP* expression. We dissected retinas at 50-60 h after pupation and stained them with antibodies against GFP (green), Hazy or Otd (red) and against the neuronal marker Elav (blue). (A) Expression of Hazy was lost in *glass* mutant cells. (B) Expression of Otd was also lost in most, but not all, *glass* mutant cells. The red and green channels are shown in greyscale to the right, where *glass* mutant cells are outlined in red (A′,A″,B′,B″). (C) Ectopic expression of Glass during embryonic development in clones labelled with nuclear βGal suffices to ectopically induce Hazy expression across the CNS of the larvae in Glass-expressing cells. Samples were stained with antibodies against βGal (used to mark Glass-expressing cells, green), against Hazy (red) and with Hoechst 33258 (used to label cell nuclei, blue). To the right, a close-up of the brain shows ectopic expression of Hazy (magenta) in Glass-expressing cells (green, C′). (D) Representation of the sequences of *hazy* and its enhancer region, following the conventions of FlyBase. The *hazy* promoter contains two Glass binding sites: *gl1* and *gl2*, both of which are evolutionarily conserved in different *Drosophila* species as shown by multiple sequence analysis, which was performed with MUSCLE (Edgar, 2004). Those nucleotides that are better conserved are shown on a darker background. A GFP reporter, *hazy(wt)-GFP*, was made by using the sequence upstream of *hazy* that is annotated in blue. (E-I) Analysis of expression of the *hazy(wt)-GFP* reporter in the adult eye, in which samples were stained with antibodies against GFP (green) and Elav (magenta). Similar to the Hazy protein, *hazy(wt)-GFP* is expressed in PR in control (E) but not *glass* mutant background (F). Double mutation of both Glass binding sites resulted in a complete loss of GFP expression (G,H). After mutating both the *gl1* and *gl2* sequences, GFP signal was not detected (I). Scale bars: 10 µm in A,B,C′; 80 µm in C,E-I.

Chp is an early PR marker known to require expression of Glass (Zipursky et al., 1984; Moses et al., 1989; Naval-Sanchez et al., 2013). Our finding that Glass can broadly drive Chp expression across the CNS of larvae (Fig. 6A,A′) further supports that Chp is a target of Glass. Both Rh2 and Trpl are phototransduction genes whose expression normally starts late during metamorphosis. Of these, we saw ectopic expression of Trpl confined to a dorsal region of the brain, but no expression in the ventral nerve cord (VNC; Fig. 6B,B′), whereas Rh2 is primarily expressed in the VNC (Fig. 6C,C′). Thus, Glass alone is sufficient to induce the expression of a subset of PR markers, albeit in a context-dependent manner.

We reasoned that co-expressing Glass with other downstream transcription factors might reduce the degree of context dependency in which phototransduction proteins are ectopically expressed, and thus induce more of its downstream targets. To test this, we generated clones either co-expressing *UAS-glass* and *UAS-hazy* or

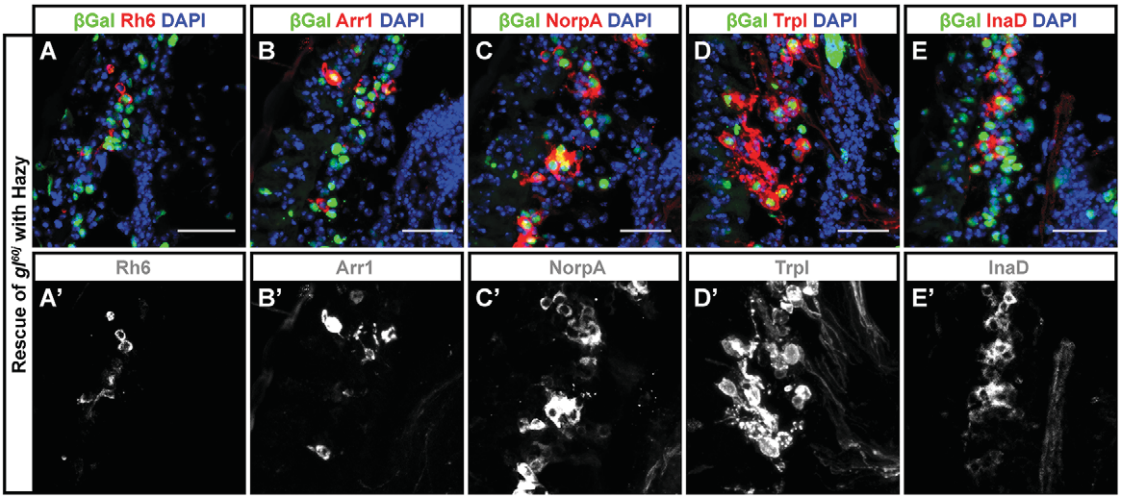

Fig. 5. Hazy expression can partially rescue the *glass* mutant phenotype. (A-E′) Hazy was expressed in the adult *glass* mutant retina in clones labelled with nuclear βGal. Samples were stained for βGal (green), different proteins involved in the phototransduction cascade (red) and with DAPI (used to label cell nuclei, blue). For each image, the red channel is shown below in greyscale. A number of Hazy-expressing cells also co-expressed Rh6 (A,A′), Arr1 (B,B′), NorpA (C,C′), Trpl (D,D′) and InaD (E,E′). Scale bars: 20 µm.

expressing *UAS-hazy* alone as a control. We found that Hazy alone is sufficient to ectopically induce the expression of Chp, NorpA and Trpl, but not other PR markers (Fig. 6H-N′). Trpl was broadly expressed in the CNS (Fig. 6I,I′) and not restricted to the dorsal brain region, in contrast to our results for the ectopic expression of Glass alone. By co-misexpressing Glass and Hazy, we confirmed the ectopic expression of Chp, Rh2, NorpA and Trpl, and found ectopic expression of additional PR markers that were not induced by either Glass or Hazy alone: Rh1, Arr1 and InaD (Fig. 6O-U′, Fig. S10).

We also induced the ectopic co-expression of *UAS-glass* and *UAS-otd*. However, our results were similar to those experiments in which we ectopically expressed *UAS-glass* alone (Fig. S10). Thus, Glass is sufficient to ectopically induce of a subset of phototransduction proteins in defined regions of the developing CNS. Interestingly, the ability of Glass to activate its targets is context dependent and can be improved by co-expressing its downstream target Hazy, suggesting that Glass and Hazy act synergistically to activate a set of common targets.

The RDN member Sine oculis is required for direct activation of *glass*

An elaborate gene regulatory network operates during development of the third instar eye disc. At this stage, the RDN member So directly activates the proneural gene *ato* at the morphogenetic furrow, and Ato induces the formation of PR precursors. Although extensive information is available on the activation of *ato*, and on how Ato specifies PR precursors to become neurons (Zhang et al., 2006; Tanaka-Matakatsu and Du, 2008; Aerts et al., 2010; Treisman, 2013; Jusiak et al., 2014), little is known about how these neurons become mature PRs. We propose that activation of *glass* by either the RDN or Ato should be a key step in this process. It has been shown that the So-Eya complex induces the formation of ectopic eyes, and is sufficient to drive expression of a *glass* reporter (Pignoni et al., 1997). To test whether So is required for the expression of *glass*, we induced *so* mutant clones using the amorphic so^3 allele (Cheyette et al., 1994; Choi et al., 2009). These clones failed to express Glass in the eye discs of third instar larvae (Fig. 7A-B″).

We next addressed whether So directly activates *glass*. It has been shown by ChIP-seq that So binds to the *glass* promoter (Jusiak et al., 2014) and we have counted 20 putative So binding sites within a 5.2 kb upstream sequence that regulates *glass* expression (Fig. 7C) (Liu et al., 1996; Jemc and Rebay, 2007). To assess the impact of mutating these So binding sites, we selected a 287 bp long fragment containing three putative So binding sites to make a GFP reporter. The resulting *glass(wt)-GFP* animals express moderate levels of GFP in the third instar eye disc behind the morphogenetic furrow, and high levels of GFP at the posterior margin of the disc (Fig. 7D,D′). After mutating the three So binding sites, GFP was no longer expressed (Fig. 7E,E′), suggesting that they are required for expression of the reporter.

Extrapolating our results to the entire 5.2 kb *glass* enhancer, we propose that So can directly activate *glass* expression in the developing eye disc by binding to about 20 sites within the upstream genomic region of glass. However, other transcription factors or more So binding sites might be required for fully activating *glass* expression in all cells posterior to the morphogenetic furrow. In this sense, we were curious to see whether Ato regulates *glass*, both because of its expression pattern right before the onset of Glass expression and because of the importance of Ato to induce neural cell fate in PR precursors (Fig. S11; Jarman et al., 1994; Aerts et al., 2010; Treisman, 2013). However, we found that Glass is still expressed in *ato* mutant clones in the third instar eye disc, suggesting that both Glass and Ato work in parallel, but independently (Fig. S11).

DISCUSSION

RDN genes have a key function in eye field specification both in vertebrates and invertebrates (Hoge, 1915; Quiring et al., 1994; Halder et al., 1995, 1998; Pignoni et al., 1997; Chow et al., 1999; Loosli et al., 1999; Silver and Rebay, 2005). Some of the cells committed to become an eye differentiate into PRs. However, the genetic mechanism by which these cells are instructed to become PRs remains unknown. Our results reveal a mechanism that molecularly links eye field specification and PR differentiation in *Drosophila* (Fig. 7F). This mechanism comprises three events:

(1) The RDN member So dimerises with Eya and activates the expression of *glass*. The So-Eya complex is required and sufficient for eye formation and drives ectopic expression of a Glass reporter (Pignoni et al., 1997). Lack of So results in the absence of *glass*

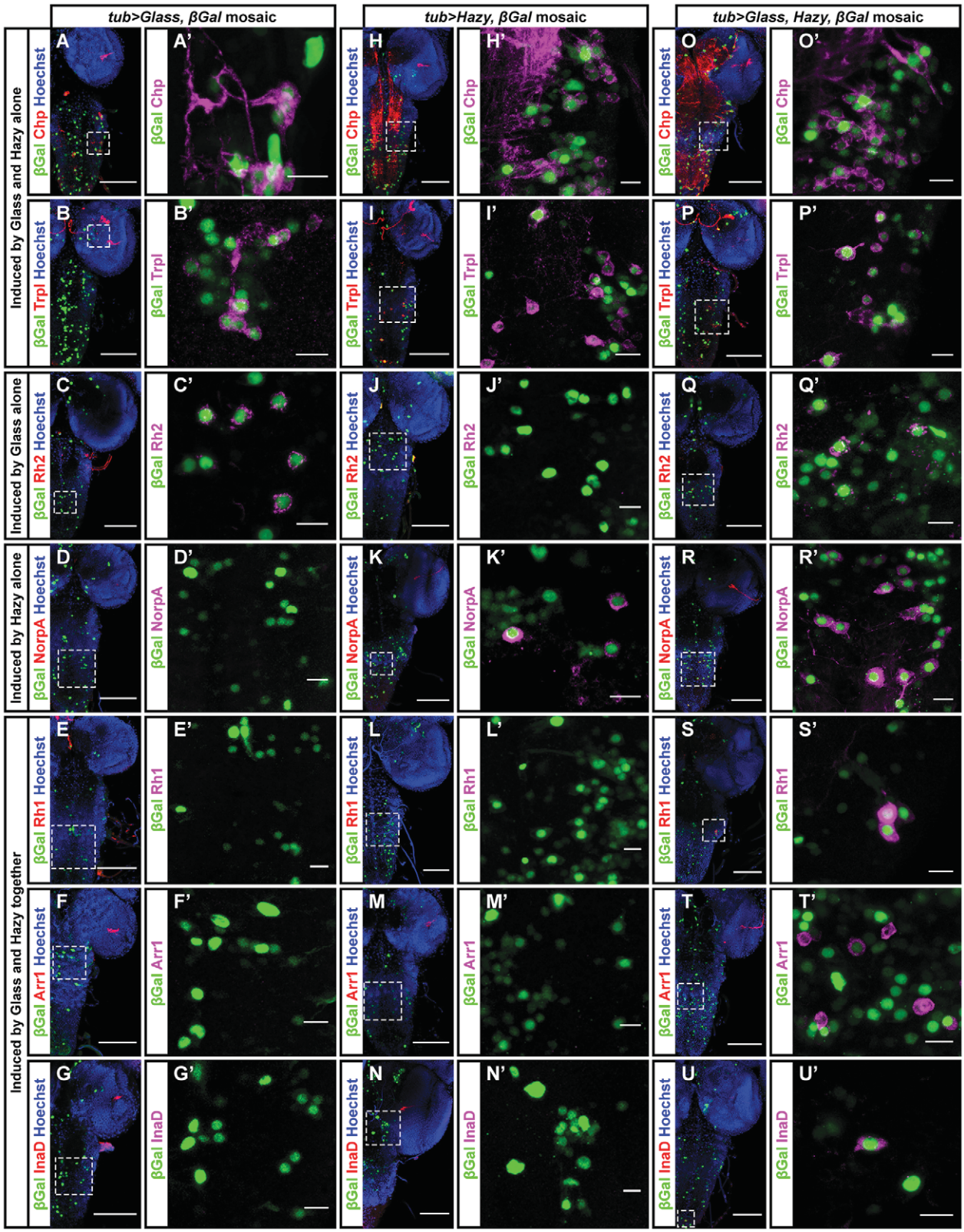

Fig. 6. Glass and Hazy can ectopically induce expression of phototransduction proteins. (A-U′) The CNS of third instar larvae, which ectopically express combinations of Glass and Hazy in clones labelled with nuclear βGal, were stained with antibodies against βGal (green), different PR proteins (red/magenta) and with Hoechst 33258 (used to label cell nuclei, blue). Close-ups of boxed regions are shown on the right of each sample. Misexpression of Glass was sufficient to ectopically induce Chp (A,A′), Trpl (B,B′) and Rh2 (C,C′); but not NorpA (D,D′), Rh1 (E,E′), Arr1 (F,F′) or InaD (G-G′). Misexpression of Hazy was sufficient to ectopically induce Chp (H,H′), Trpl (I,I′) and NorpA (K,K′); but not Rh2 (J,J′), Rh1 (L,L′), Arr1 (M,M′) or InaD (N,N′). Co-misexpression of Glass and Hazy was sufficient to ectopically induce more phototransduction proteins than either Glass or Hazy alone: Chp (O,O′), Trpl (P,P′), Rh2 (Q,Q′), NorpA (R,R′), Rh1 (S,S′), Arr1 (T,T′) and InaD (U,U′). Scale bars: 10 μm in A′-U′; 80 μm in A-U.

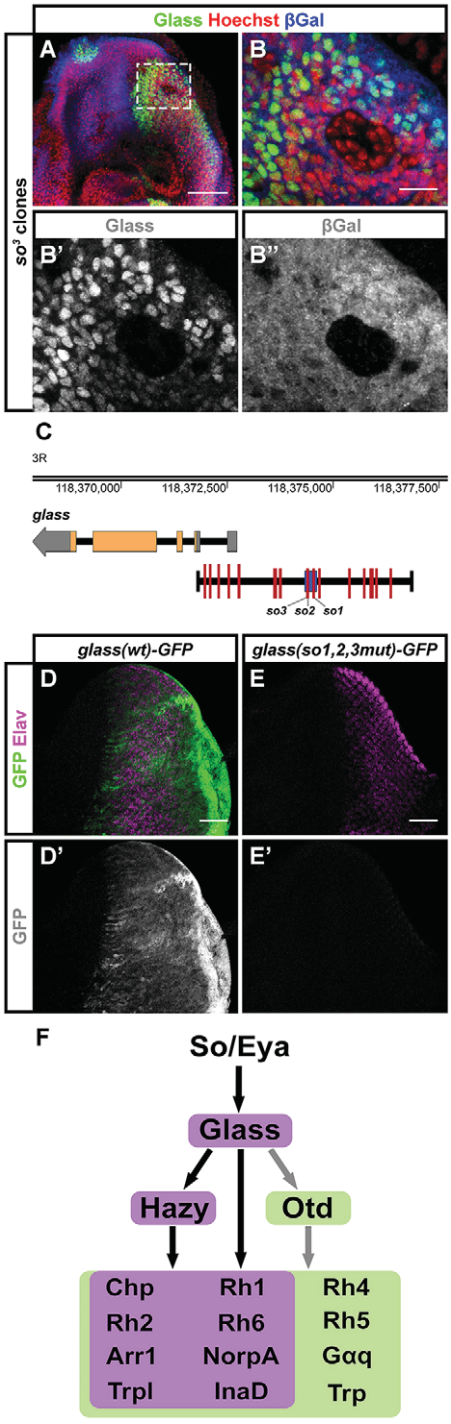

Fig. 7. Glass transcriptionally links the RDN with the expression of proteins involved in PR terminal differentiation. (A-B″) The RDN member So is required for expression of Glass. Third instar eye discs carrying *so*^{3} mutant clones were stained for Glass (green), βGal (blue) and with Hoechst 33258 (used to label cell nuclei, red). A disc containing *so*^{3} mutant clones is shown (A), together with a close-up of one of the clones (B). Glass expression is lost in *so*^{3} mutant clones, which are labeled by the absence of βGal staining (B-B″). (C) Representation of the *glass* genomic region, following the conventions of Flybase. Below the *glass* gene, a line segment indicates its 5.2 Kb regulatory region, containing 20 So binding sites (red lines; Liu et al., 1996). The blue box on the line segment indicates the enhancer that was used for *glass(wt)GFP* flies. (D-E′) Expression analysis of the *glass-GFP* reporters, for which third instar eye discs were stained against GFP (green) and Elav (magenta). For each image, the green channel is shown below in greyscale. This reporter contains three So binding sites and drives GFP expression after the morphogenetic furrow (D,D′). Mutating the three So binding sites abolishes GFP expression (E,E′). (F) Model for PR development. Black arrows indicate the sufficiency of an upstream transcription factor to activate its targets (either in misexpression or rescue experiments), which are shown within the magenta box. Grey arrows indicate that, although the upstream transcription factor regulates some of the indicated targets (green box), we did not find it sufficient to ectopically induce nor rescue any of them. (1) The Eya-So complex instructs eye field specification and is sufficient to directly activate the expression of *glass*. (2) Glass instructs neuronal precursors in the developing eye to become PRs and is sufficient to directly activate the expression of *hazy*. (3) Hazy synergises with Glass and directly activates the expression of some PR proteins. Scale bars: 10 µm in B; 40 µm in A,D,E.

expression. The So-Eya complex activates *glass* directly, because So binds to the promoter of *glass in vivo*, as shown by ChIP-seq (Jusiak et al., 2014). Also, we show that the expression of a *glass*-reporter in the eye disc depends on the presence of So binding sites.

(2) Subsequently, Glass is required for the expression of the transcription factors *hazy* and *otd*. Hazy expression is restricted to PRs, and we show that expression of Glass is sufficient to induce Hazy ectopically across the CNS of the larva. Activation of the *hazy* promoter crucially depends on two Glass binding sites, suggesting that *hazy* is a direct target of Glass.

(3) Hazy and Otd regulate PR differentiation downstream of Glass. Both genes are required for rhabdomere formation and for the expression of several phototransduction genes (Vandendries et al., 1996; Tahayato et al., 2003; Zelhof et al., 2003; Mishra et al., 2010). Expression of *Rh3* and *Rh5* is directly regulated by Otd binding to their enhancers (Tahayato et al., 2003). Also, Hazy binding sites are found in the regulatory regions of many phototransduction genes. For instance *Rh2*, *Rh6*, *G protein β-subunit 76C* (*Gβ76C*), *trp* and *trpl* appear to be direct targets of Hazy (Zelhof et al., 2003; Rister et al., 2015; Mishra et al., 2016). In addition, we show that Hazy is sufficient to partly rescue the *glass* mutant phenotype and, together with Glass, ectopically induces the expression of phototransduction proteins.

Our model might be taken as a blueprint for the transcriptional network underlying PR formation. In this sense, we extend previous computational predictions on the early development of the eye by adding genes that are expressed later in PRs, and functionally demonstrate the roles of So, Glass and Hazy for activating their targets (Aerts et al., 2010; Naval-Sanchez et al., 2013; Potier et al., 2014).

A comparison among transcriptional networks reveals analogous features between the development of various neuronal types. This is normally a multi-step process in which earlier regulators confer broad cell identities, and activate the expression of subsequent transcription factors that cooperate with each other to provide cell-type information in a more specific manner. In several instances, early regulators also play a role in later steps by co-activating gene expression through feedforward mechanisms (Alon, 2007; Baumgardt et al., 2007, 2009; Etchberger et al., 2007, 2009). In the case of PRs, our model resembles these other networks in that, because So is more broadly expressed than Glass, and Glass is more broadly expressed than Hazy (Moses and Rubin, 1991; Cheyette et al., 1994; Zelhof et al., 2003), the information to make PRs seems to be also sequentially refined. Related to this, there are two questions that should be addressed in the future. First, it remains unclear what role *glass* plays during the development of other cell types that are not PRs. Second, given that co-misexpression of Glass and Hazy together is sufficient to ectopically induce more targets than either Glass or Hazy alone, it could be that Glass and Hazy co-activate a set of common direct targets among the phototransduction

proteins (through a feed-forward loop) or that Glass activates the expression of other transcription factors that, together with Hazy, directly regulate the expression of phototransduction proteins. This might be studied by identifying the full repertoire of direct targets of Glass and Hazy using DamID or ChIP-seq. However, it strikes us that, although Glass is required for expression of all the proteins involved in the phototransduction cascade that we have tested, including Hazy, it is only sufficient to ectopically induce a few of them. It could be that the timing and relative levels of Glass and Hazy expression are relevant to produce ectopic, fully differentiated PRs, or that additional signals are needed, such as cell-cell interactions, chromatin regulators or additional transcription factors.

The function of the RDN genes during eye development is evolutionarily conserved (Quiring et al., 1994; Chow et al., 1999; Loosli et al., 1999; Silver and Rebay, 2005). However, it remains unknown whether the genetic network downstream of the RDN is also conserved. In the case of Glass, clear homologues exist in a wide range of animal phyla, based on the amino acid sequence of their zinc finger domain (Liu and Friedrich, 2004; Etchberger et al., 2007). We were able to identify Glass homologues up to the basal chordate *Branchiostoma floridae*. However, it remains challenging to identify a clear homologue of Glass in vertebrates, despite the existence of zinc finger proteins with some degree of similarity. Intriguingly, in *Caenorhabditis elegans*, which does not have canonical photoreceptors (Diaz and Sprecher, 2011), the Glass homologue CHE-1 is crucially required for the development of the ASE cell type of chemosensory neurons, and is also sufficient to ectopically induce the expression of ASE cell markers in a small number of neurons (Uchida et al., 2003; Etchberger et al., 2007; Tursun et al., 2011). Thus, given that CHE-1 acts as key regulator for a specific sensory neuronal identity, it is possible that the role of Glass proteins in determining specific neural identities is evolutionarily conserved. However, it remains unexplored whether Glass homologues in other phyla are involved in specification of PR identity.

MATERIALS AND METHODS

Fly stocks and genetics

All crosses and staging were made at 25°C. For further information and a list of the stocks used, see supplementary Materials and Methods.

Generation and analysis of clones

glass mutant clones were induced in 2-day-old larvae with a 20 min heat shock at 37°C and were analysed as described in supplementary Materials and Methods by MARCM.

Generation of transgenic flies

hazy WT and mutant reporter constructs were amplified by PCR and site-directed mutagenesis from wild-type flies as described in supplementary Materials and Methods using primers listed in Table S1. To generate 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, as described in the supplementary Materials and Methods.

Immunohistochemistry and imaging

Samples were dissected and fixed at room temperature for 20 min with 3.7% formaldehyde in 0.01 M phosphate buffer (PB; pH 7.4), taking care in the case of adult heads for cryosections to remove the proboscis and air sacs in order to improve the penetration of the reagents. Because the primary antibody against Glass is sensitive to methanol, we ensured that the formaldehyde solution did not contain methanol as a stabiliser. An exception was made for histamine staining: in this case, fixation was carried out for 30-60 min with 4% ethyldimethylaminopropyl carbodiimide (EDAC) in PB. After fixation, we followed previously described methods (Wolff, 2000a,b). For further details on the protocol and antibodies used, see supplementary Materials and Methods. Imaging was carried out with a Leica SP5 confocal microscope. Final processing of the images and composition of the figures was done with Adobe Photoshop CS6.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.J.B.-G. and S.G.S. conceived the study. F.J.B.-G. and C.F. performed the experiments. All authors contributed to writing the manuscript.

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Supplementary information

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