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

F. Javier Bernardo-Garcia, Cornelia Fritsch and Simon G. Sprecher*

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 (Hogep, 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; Tsakani 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 (Vandendriess et al., 1996; Tahaya and 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...
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 gl60j 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 gl60j mutant flies (Fig. 1F-H). We obtained similar results using the glass mutant allele gl3 (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::Cherry.

---

**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 (H,H’; arrow indicates an axon branching) 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,H’; 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 gl460 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 gl460 and gl2 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 (Gqα), No receptor potential α (NorPα), 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-I,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 (Emameh 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 gl1 retinas (Vandendries et al., 1996). We generated an otd(wt)-GFP reporter containing the same enhancer of otd, and placed it in the amorphic gl460 background (Moses et al., 1989). otd(wt)-GFP was expressed primarily in PRs, and its expression pattern did not change in the gl460 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,
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, Gqα, 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, Gqα, 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, Gqα, 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′).
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...
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') 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 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.
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 (Gb76C), 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 Reyb, 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 Brachionus floridus. 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.

Acknowledgements

We thank the Bloomberg Stock Center, R. Stocker, C. Desplan, F. Pignoni, O. Unwyler, J. Curtis, J. Bischof, H. Reichert, B. Bello, E. Piddini and B. Hassan for fly stocks, the DSHB, T. Cook, A. Zeilhof, C. Desplan, S. Brit, B. Hassan and N. Colley for antibodies, the BDGP DGC, J. Rister and J. Bischof for plasmids. We are also grateful to colleagues for valuable discussions, and to C. Desplan, B. Egger and M. Brauchle for comments on the manuscript.

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.

Funding

This work was funded by the Swiss National Science Foundation [31003A_149499 to S.G.S.] and the European Research Council [ERC-2012-StG 309832; PhotoNavNet to S.G.S.].

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.128801/-/DC1

References


