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Genetic control of photoreceptor terminal differentiation in *Drosophila melanogaster*

THESIS

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by

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CONTENTS

LIST OF ABBREVIATIONS

ac-Tub	acetylated Tubulin
Arr1	Arrestin 1
Ath5	atonal-homologue 5
Ato	Atonal
BF	brightfield
Chp	Chaoptin
CNG	cyclic nucleotide-gated
CNS	central nervous system
CRX	cone-rod homeobox protein
DAG	1,2-diacylglycerol
EDAC	ethyldimethylaminopropyl carbodiimide
EGFR	Epidermal growth factor receptor
Elav	Embryonic lethal abnormal vision
EM	electron microscopy
Ey	Eyeless
Eya	Eyes absent
Fas2	Fasciclin 2
GCAP	guanylate cyclase-activating protein
Gl	Glass
Gαq	G protein α q subunit
Gβ76C	G protein β-subunit 76C
HRP	horseradish peroxidase
Htr	halobacterial transducer
Hyb-Mix	Hybridisation mix
InaD	Inactivation no afterpotential D
IP3	inositol 1,4,5-triphosphate
ipRGC	intrinsically photosensitive retinal ganglion cell
MARCM	mosaic analysis with a repressible cell marker
MATH5	mouse atonal homologue 5
MF	morphogenetic furrow
NorpA	No receptor potential A
Otd	Orthodenticle
PB	phosphate buffer
PBT	phosphate buffer with Triton X-100
PDE	phosphodiesterase

PIP2	4,5-bisphosphate
PLC	phospholipase C
PR	photoreceptor neuron
RDN	retinal determination network
Rh1	Rhodopsin 1
Rh2	Rhodopsin 2
Rh4	Rhodopsin 4
Rh5	Rhodopsin 5
Rh6	Rhodopsin 6
r-opsin1	rhabdomeric opsin 1
Salm	Spalt major
Shh	sonic hedgehog
So	Sine oculis
SSC	saline-sodium citrate buffer
SSCT	saline-sodium citrate buffer with Tween 20
Syt	Synaptotagmin
TBR2	T-box transcription factor 2
TNT	Tris NaCl Tween 20 buffer
Trp	Transient receptor potential
Trpl	Transient receptor potential-like
VNC	ventral nerve cord
W	White
βGal	β-galactosidase

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Larisa, Tim, Siran, Shanaz, Lucía, David, Yves, Magali, Jules, Clarisse, Silvia and Jenifer. Particularly, thanks to Jenifer Kaldun for encouraging me to register electroretinograms, and to Magali Jungo for helping me to improve my German. Also, thanks to Yves Widmer for organising theater evenings, helping me to take the right tram in Basel, and for sharing his knowledge of how to tame a stray dog in Crete! I am equally grateful to G. Larisa Maier and Martín Baccino Calace (with whom I used to work in the lab until crazy hours in the night) for countless discussions (now I know a lot about Uruguayan music and about evil Artigas!), and to (now Dr) Tim-Henning Humberg for his contribution in one of our papers, as well as for several cultural exchanges. Importantly, Tim is a very efficient fly flipper and virgin collector, the right person to trust your crosses on holidays! Special thanks to Dr Boris Egger, who first (and best) reviewed our favourite paper, and to Lucía Ruiz Roca and Rubén Ramírez Rodríguez for their continued friendship during my Ph.D.

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Finally, I sincerely thank you, whoever you are, for reading at least this part of my Ph.D. thesis. I hope it helps you to find any information that you need, and I will be very happy if you like some of my work. If not, 'Time flies like an arrow; fruit flies like a banana.'

SUMMARY

Why do photoreceptors differentiate in the eye? Though simple, biologically this is an important question, and it may prove complex to answer. To present a bigger picture: animals have evolved a diversity of highly specialised sensory organs, which they use to obtain information from their environment and thus survive. These organs contain different types of receptor neurons. For example, there are chemoreceptors in the labellum and in the antennae of insects, or mechanoreceptors in the inner ear of vertebrates... and each of these types of receptor neurons specifically possesses the molecular machinery to detect and transduce stimuli from one particular sensory modality. In the case of the eye, it contains photoreceptor neurons, which are specialised in light detection. Neither photoreceptors nor most of the components of the phototransduction cascade appear commonly outside the eye. Therefore, what are mechanisms that ensure that photoreceptors differentiate correctly in the eye, and not in other body parts?

To start answering this question, first it might be useful to understand the early-acting process of eye field specification. This depends on a group of transcription factors that are collectively called the 'retinal determination network' (RDN), and work in combination with each other to confer eye identity to the developing, multipotent tissue. RDN genes are both necessary and sufficient for eye formation in different animal species, from *Drosophila* to vertebrates, and they tend to act through an evolutionarily conserved sequence of transcriptional events. First in this sequence, following the *Drosophila* nomenclature, the transcription factor Eyeless activates the expression of *sine oculis* and *eyes absent*. Then, Sine oculis and Eyes absent form a heterodimer and direct eye formation. Despite the importance of the RDN, until recently, little was known about its targets, or about the molecular mechanisms by which it coordinates eye development. In particular, how does it instruct photoreceptor differentiation?

Our work suggests that a key step in this process is coordinated by the zinc finger transcription factor *glass*, which is a direct target of Sine oculis. While previous literature has shown that the Glass protein is primarily expressed in photoreceptors, its role in these cells was not known because it was believed that *glass* mutant photoreceptor precursors died during metamorphosis. Contrary to former studies, we demonstrate that *glass* mutant photoreceptor precursors survive and are present in the adult retina, but fail to mature as functional photoreceptors. Importantly, we have found that Glass is required for the expression of virtually all

the proteins that are involved in the phototransduction cascade, and thus *glass* mutant flies are blind. Consistent with this, ectopic expression of Glass is able to induce some phototransduction components in the brain. Another step in the formation of photoreceptors is regulated by the homeodomain transcription factor Hazy, which is a direct target of Glass. While we show that both Glass and Hazy act synergistically to induce the expression of phototransduction proteins, we have also found that Glass can initiate the expression of most of the components of the phototransduction machinery in a Hazy-independent manner, and that *hazy* mutant flies only fail to detect white light after they are older than five days. Glass seems to be both required and sufficient for the expression of Hazy, and inducing Hazy in the retina partly rescues the *glass* mutant phenotype.

Taken together, our results show a transcriptional link between the RDN and the expression of the proteins that adult *Drosophila* photoreceptors need to sense light, placing Glass at a key position in this developmental process. Finally, we compare the expression pattern of Glass in *Drosophila* and in the annelid *Platynereis*, and discuss the possibility that Glass plays an evolutionarily conserved role across different phyla.

ZUSAMMENFASSUNG

Warum bilden sich Fotorezeptoren gerade im Auge aus? Obwohl diese Frage einfach erscheint, ist sie aus biologischer Sicht doch sehr bedeutend und bedarf eventuell einer komplexen Antwort. Allgemein lässt sich sagen, dass Tiere eine Vielfalt von hoch spezialisierten Sinnesorganen entwickelt haben, durch die sie Informationen aus ihrer Umwelt aufnehmen und auf diese Weise ihr Überleben sichern. Diese Organe enthalten verschiedene Arten von Rezeptorneuronen. Zum Beispiel gibt es Chemorezeptoren im Labellum und in den Antennen der Insekten, oder Mechanorezeptoren im Innenohr von Wirbeltieren... und jedes dieser Rezeptorneuronen besitzt eine spezifische molekulare Maschinerie, um Reize einer bestimmten Sinnesmodalität wahrzunehmen und umzuwandeln. Beim Auge sind es Fotorezeptorneuronen, die auf die Wahrnehmung von Lichtreizen spezialisiert sind. Weder die Fotorezeptoren noch die meisten der Komponenten der Fototransduktionskaskade kommen außerhalb des Auges vor. Welche Mechanismen sind demzufolge ausschlaggebend, damit sich Fotorezeptoren im Auge und nicht in anderen Körperteilen entwickeln?

Um diese Frage zu beantworten, ist es zunächst wichtig die frühen Mechanismen der Augenspezifizierung zu verstehen. Diese erfolgt unter Einfluss einer Gruppe von Transkriptionsfaktoren, die als „Retinales Determinations Netzwerk" (RDN) bezeichnet werden. Diese Transkriptionsfaktoren interagieren, um aus dem sich entwickelnden multipotenten Gewebe ein Sehorgan zu bilden. RDN-Gene sind für die Augenentwicklung verschiedener Tierarten, von *Drosophila* bis zu Wirbeltieren, sowohl notwendig als auch ausreichend. Sie agieren durch eine evolutionär konservierte Sequenz transkriptioneller Mechanismen. An erster Stelle dieser Sequenz, nach der *Drosophila* Nomenklatur, aktiviert der Transkriptionsfaktor Eyeless die Expression von *sine oculis* und *eyes absent*. Anschließend bilden Sine Oculis und Eyes absent ein Heterodimer und induzieren die Entwicklung des Auges. Trotz der Bedeutung des RDNs war bis vor Kurzem nur sehr wenig über seinen Zweck oder die molekularen Mechanismen durch die es die Augenentwicklung koordiniert, bekannt. Vor allem stellt sich die Frage, wie es die Differenzierung der Fotorezeptoren reguliert?

Unsere Arbeit legt nahe, dass ein wesentlicher Schritt in diesem Prozess durch den Zinkfinger-Transkriptionsfaktor *glass* koordiniert wird. Dabei handelt es sich um ein direktes Zielgen von Sine oculis. Obwohl in früheren wissenschaftlichen Arbeiten belegt wurde, dass das Glass-Protein in erster Linie in Fotorezeptoren exprimiert

wird, war seine Rolle in diesen Zellen nicht bekannt, da angenommen wurde, dass Fotorezeptoren von *glass* Mutanten während der Metamorphose absterben. Im Gegensatz zu früheren Studien belegen wir das Überleben der Fotorezeptor-Vorläuferzellen von *glass* Mutanten und ihre Präsenz in der Retina adulter Fliegen, wobei sie jedoch nicht zu funktionsfähigen Fotorezeptoren heranreifen. Insbesondere konnten wir zeigen, dass Glass für die Expression fast aller Proteine, die in der Fototransduktionskaskade involviert sind, erforderlich ist. Daher sind *glass* Mutanten blind. In Übereinstimmung mit diesen Erkenntnissen bewirkt die ektopische Expression von Glass die Induktion einiger Komponenten der Fototransduktion im Gehirn. Ein weiterer Schritt in der Bildung von Fotorezeptoren wird reguliert durch den Homeodomänen-Transkriptionsfaktor Hazy, der ein direktes Ziel von Glass ist. Wir zeigen zum einen die synergetische Wirkung von Glass und Hazy bei der Expression von Fototransduktionsproteinen, zum anderen belegen wir, dass Glass die meisten Komponenten der Fototransduktionsmaschinerie unabhängig von Hazy induzieren kann, und dass *hazy* Mutanten ab dem Alter von fünf Tagen weißes Licht nicht mehr wahrnehmen können. Glass scheint notwendig und ausreichend für die Expression von Hazy zu sein und die Induktion von Hazy in der Retina rettet teilweise den Phänotyp von *glass* Mutanten.

Insgesamt beweisen unsere Ergebnisse einen transkriptionellen Zusammenhang zwischen dem RDN und der Expression von Proteinen, die in Fotorezeptoren von adulten *Drosophila* Fliegen notwendig sind um Licht wahrzunehmen. Bei diesem Entwicklungsprozess hat Glass eine Schlüsselposition. Schließlich vergleichen wir die Expressionsmuster von Glass in *Drosophila* und im Anneliden *Platynereis* und diskutieren die Möglichkeit, dass Glass eine evolutionär konservierte Rolle über verschiedene Phyla hinweg spielt.

1. GENERAL INTRODUCTION

Some sections of this chapter are currently being modified, and may be published as part of a review on photoreceptor development in the future.

1.1. Why do we study photoreceptor development in *Drosophila*?

Living beings need to obtain information from their environment in order to adapt their activity to changes in their surroundings and survive. In the case of metazoans, this information is usually obtained via sensory organs located on the surface of their body. Importantly, different sensory organs possess different types of receptor neurons (e.g. in mammals, the inner ear contains auditory mechanoreceptors, the tongue has gustatory chemoreceptors...) and each of these types of receptor neurons specifically possesses the molecular components to detect and transduce stimuli from one particular sensory modality (Julius and Nathans, 2012; Yamamoto and Koganezawa, 2013). In the case of the eye, it contains photoreceptor neurons (PRs), which are specialised in detecting light.

But, what are the mechanisms that ensure that PRs are formed correctly in the eye, and not in other parts of the body? And how is it that all the components of the phototransduction cascade are coordinately expressed in PRs, and not in other cell types? Here, we have investigated these questions using the fruit fly, *Drosophila melanogaster*, as a model organism.

Drosophila is a small, easy-to-care insect that has already been used for over a century to study the mechanisms behind a wealth of biological processes, such as heredity, mutation, body patterning or immunity (Kohler, 1994; Lemaitre et al., 1996; Morgan, 1919; Muller, 1927; Nüsslein-Volhard and Wieschaus, 1980). Its fast development, low genetic redundancy, and the amount of tools and information available make *Drosophila* ideal for studying a process such as PR development (http://flybase.org/) (Adams et al., 2000). Of note, a number of evolutionarily conserved transcription factors that play a role during early eye development were initially identified in *Drosophila* (Bonini et al., 1993; Cheyette et al., 1994; Hoge, 1915; Silver and Rebay, 2005). Understanding the function of these genes has helped us to understand eye development in vertebrates, as well as the mechanisms behind a number of congenital visual disorders in humans (Azuma et al., 2000; Gallardo et al., 1999; Quiring et al., 1994). Given that inducing the expression of these transcription factors causes the appearance of PR markers in

cultured stem cells, it may be possible to use them in future therapies to treat blindness (Ikeda et al., 2005; MacLaren and Pearson, 2007). Therefore, studying the role of additional genes that act during *Drosophila* PR development might not only teach us something about PR formation in vertebrates, but it could also prove useful for understanding and treating visual disorders in human patients.

1.2. Evolutionary origin of light sensing mechanisms

1.2.1. Origin of metazoan opsins

The function of PRs depends on opsins, which are specialised, light-sensing proteins (Terakita et al., 2012). It is believed that metazoans evolved from colonial choanoflagellates about $700 \cdot 10^6$ years ago (Mikhailov et al., 2009; Morris, 1998). Genome analyses of the choanoflagellate *Monosiga* and early metazoans (placozoans and sponges) have revealed the presence of opsin-like genes in these organisms, but their ability to respond to light was questioned. However, it has been recently shown that one choanoflagellate opsin is able to activate an enzymatic cascade in a light-dependent manner in *Salpingoeca*. Therefore, it seems that light-sensing opsins were already present in the common ancestor of all metazoans (Feuda et al., 2012; Porter et al., 2012; Yoshida et al., 2017).

Opsins are seven-transmembrane receptors that use retinal as a cofactor. Typically, opsins possess a lysine residue in their seventh transmembrane domain (except for choanoflagellate and early metazoan opsin-like proteins), which covalently binds to retinal through a Schiff's base: this molecular feature allows them to modify their spatial conformation in response to light. Interestingly, based on this definition, opsins also exist outside metazoans, i.e. in bacteria, archaeans, and other eukaryote lineages (Findlay and Pappin, 1986; Spudich et al., 2000). For this reason, it has been controversial whether opsins evolved independently in different groups or not. According to one hypothesis, called the 'Russian doll model', opsins would be a monophyletic family of proteins originated in cyanobacteria, and metazoans would have acquired opsin genes during a series of horizontal DNA transfer events (Gehring, 2005). Alternatively, a second hypothesis proposes that two types of opsins evolved independently: type I opsins would have originated very early in the history of life, and be present in bacteria, archaeans, and some eukaryotes, while type II opsins would be specific of metazoans. A number of studies supporting this latter hypothesis have already been reviewed (Spudich et al., 2000). Basically, some critical differences between type I and type II opsins

suggest that they are too different from each other to share a common ancestor among the opsins. This suggestion is based on:

- Aminoacid sequence: when sequences are compared, type I and II opsins cluster separate from each other.
- 3D structure: the seven transmembrane domains are arranged differently in type I and II opsins.
- Activation mechanism: during the activation of type I opsins all-*trans*-retinal transforms into 13-*cis*-retinal. In the case of type II opsins, 11-*cis*-retinal transforms into all-*trans*-retinal.
- Downstream mechanism: the activation of different type I opsins may cause various effects (Yizhar et al., 2011). They can work as ion pumps, ion channels, or activate a transduction cascade via halobacterial transducer (Htr). By contrast, type II opsins typically activate a transduction cascade through interaction with heterotrimeric G proteins.

Thus, it seems most likely that light-sensing opsins originated independently twice during the history of life. Remarkably, both times retinal was selected as a cofactor and covalently bound to a lysine, which was located in the seventh transmembrane domain of a seven-transmembrane receptor.

1.2.2. Division of metazoan opsins in four clades

It is believed that metazoan opsins first appeared in choanoflagellates through gene duplication of a melatonin receptor. After this event, opsins have diversified, and they are typically grouped by sequence comparison in at least four clades (Fig. 1.1) (Feuda et al., 2012; Porter et al., 2012; Shichida and Matsuyama, 2009):

- Placozoan opsins: members of this group appear only in placozoans, and their role has not been addressed yet (Feuda et al., 2012).
- Go/RGR opsins: they are widely present in metazoans, and are heterogeneous in their functions. However, more research will be necessary to clarify their exact roles. For example, in the case of annelid Go opsins, one report has shown that they work in combination with other opsins to adjust the wavelength-specific sensitivity of PRs (Gühmann et al., 2015). In the case of RGR opsins, they are expressed in the eyes of vertebrates in cells adjacent to PRs (retinal pigment epithelium and Müller cells), where they contribute to recycling retinal by photoisomerising all-*trans*-retinal to 11-*cis*-retinal, which is then transferred to and used by PRs (but this role is not essential, since it is redundant with an enzymatic pathway which also

recycles retinal) (Maeda et al., 2003). Retinochrome is another opsin of this group which plays a similar role to that of RGR opsins in the eye of molluscs (Hara and Hara, 1972). Other members of this group are peropsins and neuropsins, for which little information is available (Shichida and Matsuyama, 2009).

- Rhabdomeric opsins: this group is widely distributed across metazoans. It includes melanopsins in vertebrates (which play a role in irradiance detection) and rhodopsins in invertebrates (which classically act in image-forming vision) (Shichida and Matsuyama, 2009).
- Ciliary opsins: widely present in metazoans. This group includes the classical vertebrate visual opsins: rod and cone opsins. It also contains vertebrate extraretinal opsins, including pinopsins, parietopsins, parapinopsins, encephalopsins (these four groups may function in circadian rhythm regulation) and invertebrate brain opsins, called pteropsins (with a yet unknown function) (Shichida and Matsuyama, 2009).

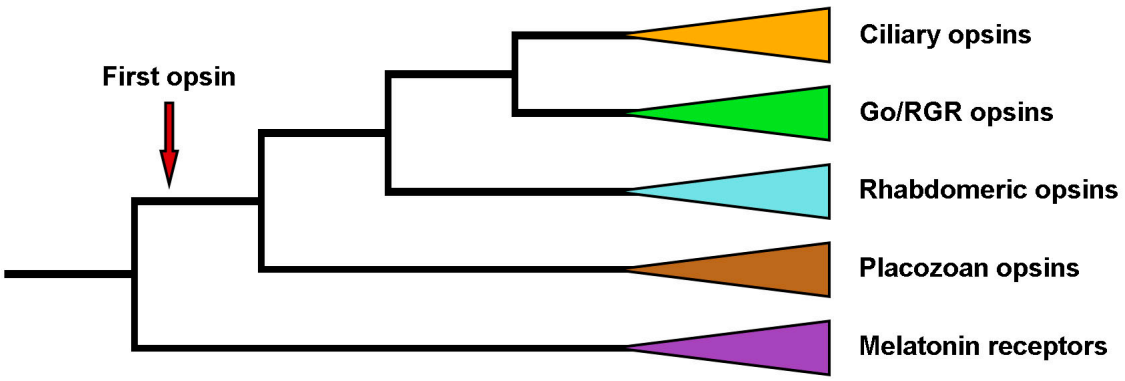

Fig. 1.1: Classification of opsins. Sequence comparison suggests that melatonin receptors are the sister group of opsins, and that opsins can be divided into four clades: placozoan, rhabdomeric, Go/RGR and ciliary opsins (Feuda et al., 2012).

1.2.3. Functional diversity of visual opsins and the morphology of photoreceptors

Despite their diversity, all opsins are believed to be able change their spatial conformation in response to light. For this, they have retained a lysine residue (called K296, using a bovine opsin as reference for its position) in their seven-transmembrane domain, to which retinal binds. Apart of this, modifications in other residues result in opsin diversity (Feuda et al., 2012; Porter et al., 2012). In the case of the classical visual opsins – rhabdomeric and ciliary opsins – there are three main properties that vary among different homologues:

- Light absorption curve: different opsins have the ability to respond preferentially to light photons with a particular wavelength or intensity. For example, in humans, rod opsin OPN2 is very sensitive under dim light conditions, while cone opsins require better illumination to work and are used for colour detection: OPN1SW (blue, 420 nm), OPN1MW (green, 534 nm) and OPN1LW (red, 564 nm) (Purves et al., 2004b).
- Heterotrimeric G protein subtype-specific interaction: upon light stimulation, both rhabdomeric and ciliary opsins directly interact with one particular type of heterotrimeric G proteins, thus initiating the phototransduction cascade. In the case of rhabdomeric opsins they interact specifically with the Gq group, while classical ciliary opsins interact with Gt proteins. Importantly, most metazoan PRs only express one type of opsin, and phototransduction works through a different signalling cascade depending on whether a Gq or a Gt protein is activated (Fain et al., 2010; Shichida and Matsuyama, 2009; Vopalensky and Kozmik, 2009). It could be possible that, initially, individual opsins were able to interact with more than one type of heterotrimeric G proteins, as melatonin receptors (the sister group of opsins) do (Dubocovich et al., 2003), and that they have progressively become more selective during their evolution.
- Reversibility of the opsin's activated conformation: rhabdomeric opsins can switch back to their inactive form by simply absorbing a second photon of light, reconverting all-*trans*-retinal back to 11-*cis*-retinal in this process. This is a fast and efficient restoration mechanism, which allows rhabdomeric opsins to undergo many activation/deactivation cycles before they have to be recycled (Hamdorf, 1979; Terakita et al., 2012). By contrast, classical ciliary opsins (i.e. rod and cone opsins) are bleached by light: they cannot easily reverse their activated conformation, nor the isomerisation of retinal. Therefore, the process to restore ciliary opsins is longer and more costly than that for rhabdomeric opsins, which at first sight appears to be a disadvantage. But then, why was this trait selected during evolution? It seems that ciliary opsins acquired the ability to experiment a larger change of their spatial conformation after light stimulation, leading to a more efficient activation of Gt proteins, which was advantageous (Terakita et al., 2012; Tsukamoto et al., 2009). However, this increased conformational change is more difficult to reverse and, as a side effect, ciliary opsins are bleached by light.

In metazoans, opsins are most abundantly expressed in the eyes, which typically need to detect and process various features of light. For this, different opsins are expressed in separate PR cells. This has resulted in different types of PRs co-evolving with the opsins that they express, and thus they have adopted distinct morphologies. A remarkable morphological feature of most PRs is the expansion of one region of their cytoplasmic membrane, where opsins and other phototransduction components accumulate, which, therefore, generates a light-sensing organelle. In the case of those PRs that express a rhabdomeric opsin (also called rhabdomeric PRs) this organelle is called the 'rhabdomere', and consists of a brush-like structure formed by densely packed microvilli. In the case of those PRs that express a ciliary opsin (also called ciliary PRs) this organelle is known as the 'outer segment', and consists of a stack of disc-shaped membranes that are connected to each other and to the soma through a cilium. Both rhabdomeric and ciliary PRs are present in most metazoans, but serve different functions that vary among phyla (Arendt, 2003; Fain et al., 2010; Lamb, 2009) (Fig. 1.2). There are also a few cases in which PRs do not display any obvious membranous specialisation, such as the 'intrinsically photosensitive retinal ganglion cells' (ipRGCs) in vertebrates (Do and Yau, 2010), or PRs in the eyes of acoel worms (Yamasu, 1991).

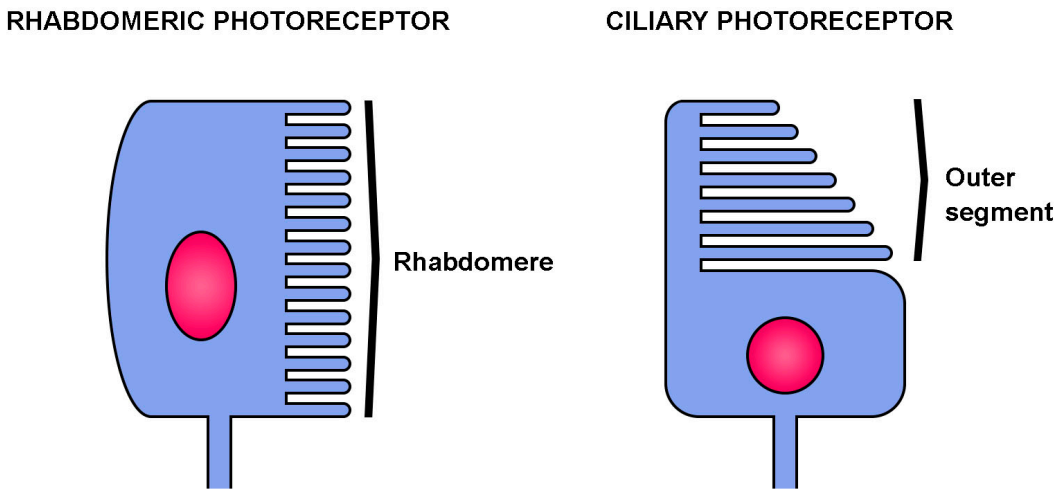

Fig. 1.2: PR morphology. Typically, the cytoplasmic membrane of PRs expands to increase its surface, forming a light-sensing organelle, where the components of the phototransduction cascade are located. This organelle may have appeared early during the evolution of PRs, and has adopted two different morphologies, which are present in two classes of functionally different PRs. It is called 'rhabdomere' in rhabdomeric PRs, where it consists of a brush-like specialisation formed by stacked microvilli. In the case of ciliary PRs, it is called 'outer segment', and is formed by a pile of disc-shaped membranes, connected to each other and to the soma through a cilium. Importantly, both rhabdomeric and ciliary PRs are broadly distributed across metazoans, and may co-exist in the same eye, but they employ two different phototransduction cascades (Arendt, 2003; Fain et al., 2010; Lamb, 2009).

1.3. Transduction of light stimuli

1.3.1. Mechanisms of opsin activation and deactivation

Metazoan opsins are mainly expressed in PRs. In dark conditions, they consist of an apoprotein (which derives from one single polypeptide sequence) and the chromophore 11-*cis*-retinal. The apoprotein has a pocket where 11-*cis*-retinal is inserted, and both are covalently bound to each other through a Schiff base that forms on an evolutionarily conserved lysine residue of the apoprotein (Fu and Yau, 2007; Montell, 2012; Nelson and Cox, 2013; Park et al., 2008; Scheerer et al., 2008; Shichida and Matsuyama, 2009).

Upon light stimulation, 11-*cis*-retinal absorbs one photon and modifies its spatial conformation, transforming into its stereoisomer all-*trans*-retinal. This conformational change is propagated to the apoprotein, and thus opsins become activated and are able to interact with heterotrimeric G proteins. Then, heterotrimeric G proteins activate other downstream components of the phototransduction cascade (Fig. 1.3) (Fu and Yau, 2007; Montell, 2012; Nelson and Cox, 2013; Oldham and Hamm, 2008; Park et al., 2008; Scheerer et al., 2008; Shichida and Matsuyama, 2009).

Typically, PRs can still respond after multiple consecutive light stimuli. Therefore, how do PRs maintain enough molecules of 11-*cis*-retinal-bound opsin? As explained above (section 1.2.3), opsins are bistable in the case of rhabdomeric PRs: they can reconvert all-*trans*-retinal back to 11-*cis*-retinal by absorbing a second photon, and thus they go through several activation/deactivation cycles before being recycled (Hamdorf, 1979; Terakita et al., 2012). By contrast, in vertebrate ciliary PRs each opsin molecule is normally bleached after being activated by light, and needs to be recycled before it can function again (Terakita et al., 2012). Opsins are recycled after all-*trans*-retinal is enzymatically modified, which causes its dissociation from the apoprotein and its release from PRs. Adjacent cells (pigment cells in *Drosophila*, and both pigment epithelium and Muller glial cells in vertebrates) absorb it and convert it to 11-*cis*-retinal, which is then released and absorbed by PRs. There, 11-*cis*-retinal binds again to the opsin apoprotein (Arshavsky, 2010; Kiser et al., 2014; Maeda et al., 2003; Wang et al., 2010; Wang et al., 2012).

After PRs have responded to light the phototransduction cascade needs to be deactivated, and part of this process involves blocking opsin signalling. For this,

arrestins play an important role. In the case of *Drosophila* PRs, under dark conditions, arrestins are sequestered in Myosin III-bound vesicles. But, upon light stimulation, Ca^{2+} (which enters the cell in a latter step of the phototransduction cascade) signals via calmodulin the release of arrestins, which translocate to the rhabdomere (Hardie et al., 2012; Lee and Montell, 2004). In the case of vertebrate rods and cones, arrestins also translocate to the outer segment upon light stimulation, but the mechanism by which this happens is not yet fully understood (Calvert et al., 2006; Mirshahi et al., 1994; Peterson et al., 2003; Slepak and Hurley, 2008). Once they are in either the rhabdomere or the outer segment, arrestins associate with the activated opsins, blocking their interaction with heterotrimeric G proteins, and thus terminate opsin signalling (Fu and Yau, 2007; Montell, 2012; Nelson and Cox, 2013). In the case of *Drosophila* rhabdomeric PRs, arrestins also promote clathrin-mediated endocytosis of opsins, which contributes to the desensitisation of these receptors (Kiselev et al., 2000; Kristaponyte et al., 2012; Orem et al., 2006).

1.3.2. Heterotrimeric G protein signalling

Heterotrimeric G proteins are formed by the assembly of three subunits: Gα, Gβ and Gγ. They are typically associated with the cytoplasmic membrane via covalent bonds to fatty acids in the Gα and the Gγ subunits (Wedegaertner et al., 1995), and possess several homologues that participate in various transduction cascades in different regions of the body, where they signal through a similar mechanism to that which they use during phototransduction (Oldham and Hamm, 2008; Wettschureck and Offermanns, 2005).

In PRs, in dark conditions, the three subunits that form heterotrimeric G proteins are assembled together, and, of these, Gα is bound to GDP. Upon light stimulation, opsin binds and activates the Gαβγ complex, which changes its spatial configuration. This allows Gα to exchange GDP for GTP, resulting in a second conformational change that causes, simultaneously, the dissociation of the Gαβγ complex from the opsin, and of the Gα subunit from the Gβγ dimer. Still associated to the cytoplasmic membrane, GTP-bound Gα is now able to diffuse and activate the following component of the phototransduction cascade: either phospholipase C (PLC) in rhabdomeric PRs or phosphodiesterase (PDE) in ciliary PRs (Fig. 1.3) (Fain et al., 2010; Fu and Yau, 2007; Montell, 1999; Nelson and Cox, 2013; Oldham and Hamm, 2008; Purves et al., 2004b).

G protein signalling is active for as long as Gα remains bound to GTP. Importantly, Gα possesses GTPase activity, which hydrolyses GTP to GDP, and thus stops G protein signalling. Hydrolase activity is increased when Gα associates to the downstream component of the cascade – either PLC or PDE – (Chidiac and Ross, 1999; Pagès et al., 1993). The activation cycle of heterotrimeric G proteins ends when GDP-bound Gα reassociates with the Gβγ dimer (Oldham and Hamm, 2008).

Similar to arrestins, heterotrimeric G proteins can be translocated to the cytoplasm upon prolonged illumination, which contributes to the light adaptation of PRs (Kosloff et al., 2003; Sokolov et al., 2002). However, the mechanism by which this happens is unclear. According to one model, the assembled Gαβγ complex, which is bound to two fatty acid molecules, has a greater affinity for the cytoplasmic membrane than either the Gα subunit or the Gβγ dimer alone, since each of them is bound to just one fatty acid molecule. This model proposes that, after opsins activate the Gαβγ assembly, both the dissociated Gα subunit and the Gβγ dimer diffuse to the cytoplasm (Slepak and Hurley, 2008). However, it has been observed that Gα diffuses slower than it would be expected from this model (Kerov and Artemyev, 2011; Kerov et al., 2007), suggesting that it is sequestered somehow. In this sense, it has been proposed that Gα may associate to cholesterol in lipid microdomains in the cytoplasmic membrane of *Xenopus* PRs, which would limit its diffusion rate (Wang et al., 2008). Also, in most heterotrimeric G protein homologues, Gα can be reversibly modified through palmitoylation (Mumby, 1997; Wedegaertner et al., 1995), and thus it could be that both palmitoylation and depalmitoylation play a role for the translocation of Gα in rhabdomeric PRs (Kosloff et al., 2003), but not in ciliary PRs, in which Gα is irreversibly modified by myristoylation (Wedegaertner et al., 1995).

1.3.3. Formation and function of the second messengers

As explained above, different subtypes of heterotrimeric G proteins – Gq and Gt – activate either PLC or PDE in rhabdomeric and ciliary PRs, respectively (Fain et al., 2010). Both PLC and PDE are enzymes, and possess various homologues and isoforms that participate in diverse physiological processes in different regions of the organism by catalysing the formation of second messengers. Second messengers are intracellular signalling molecules, which couple the detection of extracellular signals at the cytoplasmic membrane with an intracellular response (Francis et al., 2011; Keravis and Lugnier, 2012; Rebecchi and Pentyala, 2000). In the case of the phototransduction cascade, these second messengers affect the

opening or closing of cation (Ca^{2+} and Na^{+}) channels in the cytoplasmic membrane, and thus modify the membrane potential (Fig. 1.3) (Fu and Yau, 2007; Hardie and Juusola, 2015; Nelson and Cox, 2013).

In the case of rhabdomeric PRs, PLC forms from a single polypeptide sequence and it associates to lipids in the cytoplasmic membrane, mainly via its PH domain (Ferguson et al., 1995; McKay et al., 1994; Rebecchi and Pentyala, 2000; Yoon et al., 2004). During light transduction in rhabdomeric PRs, GTP–Gαq-activated PLC cleaves one of the lipids that compose the cytoplasmic membrane, phosphatidylinositol 4,5-bisphosphate (PIP2). This results in the formation of inositol 1,4,5-triphosphate (IP3), 1,2-diacylglycerol (DAG), and one proton (H^{+}) (Hardie and Juusola, 2015). Of these products, IP3 diffuses to the cytoplasm and it seems to play a role during phototransduction in some animal species. Particularly, it is believed that IP3 induces the release of Ca^{2+} from the smooth endoplasmic reticulum of PRs in the ventral eye of *Limulus*, which leads to the opening of cation channels in their cytoplasmic membrane, and thus modifies the membrane potential. Intriguingly, IP3 does not seem to play a role in *Drosophila* phototransduction (Bollepalli et al., 2017; Yau and Hardie, 2009). As a consequence, it is still controversial how PLC signalling works in *Drosophila* PRs. However, three hypothesis have been proposed, and studies in support or against them have been recently reviewed (Hardie and Juusola, 2015):

- It could be that either DAG or a yet unidentified fatty acid derived from DAG directly binds to the cation channels in the cytoplasmic membrane of *Drosophila* PRs, activating their opening.
- PLC-mediated depletion of PIP2 from the cytoplasmic membrane results in the contraction of the rhabdomeres. This may be sufficient to mechanically activate the opening of cation channels.
- A PLC-dependent increase in the H^{+} concentration acidifies the cytoplasm inside the rhabdomeres during phototransduction. This could contribute to the opening of cation channels in the cytoplasmic membrane.

Indeed, it would also be possible that these three mechanisms act in combination in *Drosophila* PRs (Hardie and Juusola, 2015). Interestingly, the mechanism by which PLC mediates the opening of cation channels is also unclear in vertebrate ipRGCs. These cells signal through the rhabdomeric phototransduction cascade, and require PLC for this. However, neither IP3 nor DAG seem to play a role as second messengers during phototransduction in ipRGCs (Do and Yau, 2010).

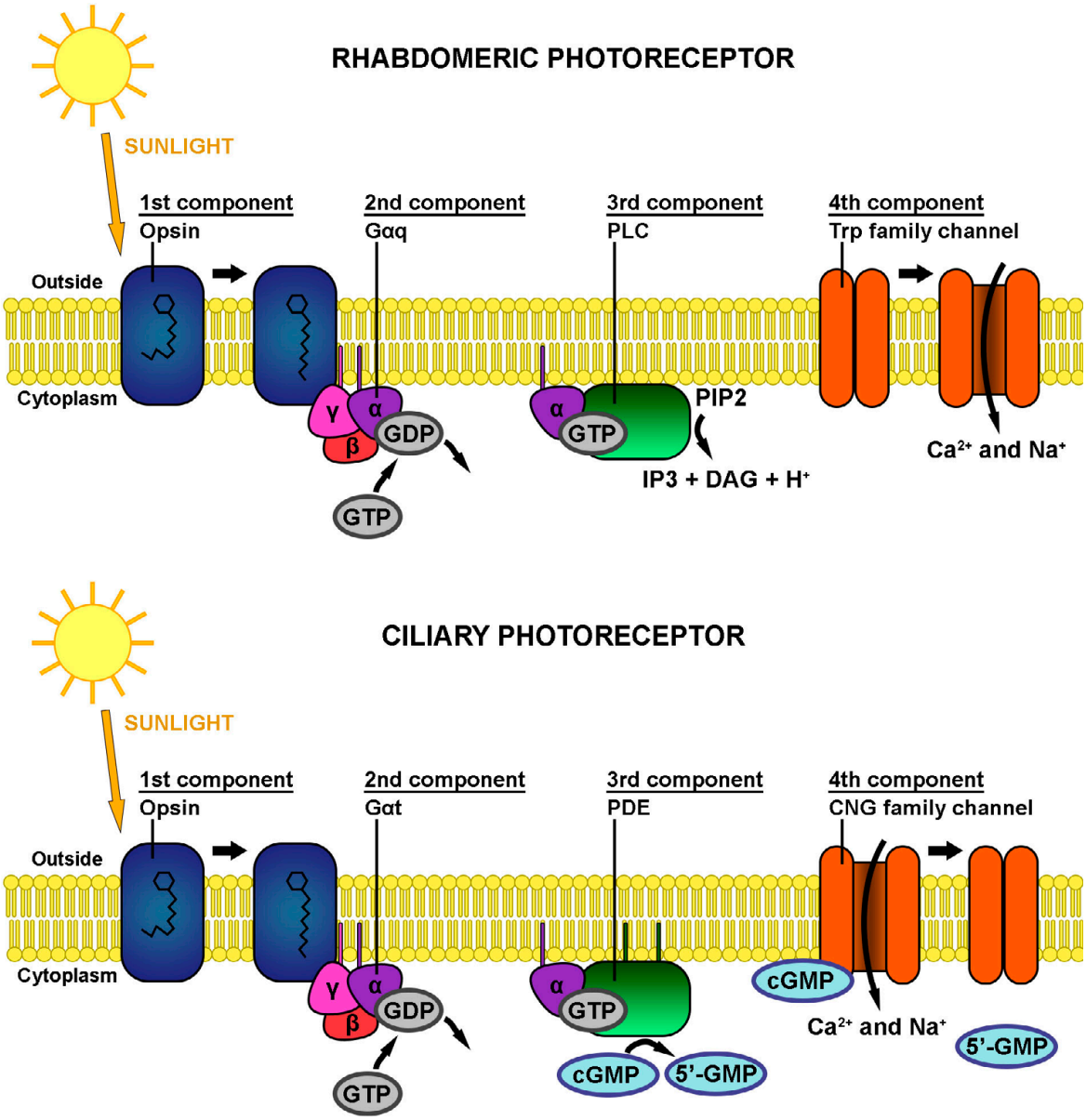

Fig. 1.3: The phototransduction cascade. Rhabdomeric and ciliary PRs employ two different mechanisms to detect light. In both cases, light induces a change in the spatial conformation of opsins, which activate heterotrimeric G proteins. In the case of rhabdomeric PRs, Gαq signals through PLC, leading to the opening of cation channels in the cytoplasmic membranes, which causes the depolarisation of the membrane (Hardie and Juusola, 2015; Montell, 2012). In the case of ciliary PRs, Gαt signals through PDE, which induces the closure of cation channels, and thus causes the hyperpolarisation of the cytoplasmic membrane (Fu and Yau, 2007; Purves et al., 2004b).

In the case of ciliary PRs, PDE consists of a complex formed by four subunits. Reportedly, two of these subunits are catalytic and can be post-translationallymodified through prenylation (in particular, geranylgeranylation) of their C-terminal region. This causes PDE to be primarily located at the cytoplasmic membrane (Catty and Deterre, 1991; Catty et al., 1992; Cote, 2006; Francis et al., 2011). During phototransduction, Gαt (also called transducin) activates PDE, which

changes its spatial conformation without any of its four subunits being dissociated from the complex (Berger et al., 1997; Cote, 2006; Kroll et al., 1989), and thus PDE is able to catalyse the conversion of cGMP into 5'-GMP. Importantly, cation channels in the cytoplasmic membrane of ciliary PRs are cGMP-gated. Therefore, while cGMP molecules keep these channels open in dark conditions, light stimulation induces a PDE-dependent decrease of cGMP levels, which causes cation channels to close, and thus modifies the membrane potential (Fu and Yau, 2007; Nelson and Cox, 2013; Purves et al., 2004b).

1.3.4. Effects of the cation channels on the membrane potential

Both rhabdomeric and ciliary PRs possess cation channels (permeable to Ca^{2+} and Na^{+}) in their cytoplasmic membrane (Fain et al., 2010). However, the subunits that form these channels are not homologues: in the case of rhabdomeric PRs they are Transient receptor potential family members and, in *Drosophila*, they include Transient receptor potential (Trp) and Transient receptor potential-like (Trpl), both of which assemble as homotetrameric complexes. By contrast, in ciliary PRs cation channels belong to the cyclic nucleotide-gated (CNG) family, and they form heterotetrameric complexes (Katz et al., 2013; Kaupp and Seifert, 2002; Venkatachalam and Montell, 2007).

In dark conditions, channels of the Trp family are closed in rhabdomeric PRs, and the resting potential of their cytoplasmic membrane is about −70 mV, which is similar to that of most other neurons (Hardie, 2001; Hardie, 2012; Heimonen et al., 2012; Leung et al., 2000). By contrast, CNG channels are open in ciliary PRs, and their resting membrane potential is approximately −40 mV, higher (more depolarised) than in most neurons (Fu and Yau, 2007; Nelson and Cox, 2013; Purves et al., 2004b).

After light stimulation, PLC and PDE cause opposite effects on the membrane potential of rhabdomeric and ciliary PRs, respectively (Fain et al., 2010). In the case of rhabdomeric PRs, PLC activation induces the opening of the cation channels. This allows the entrance of Ca^{2+} and Na^{+}, leading to the depolarisation of the cytoplasmic membrane, which may reach a maximum value of approximately 0 mV in *Drosophila* (Hardie, 2001; Hardie, 2012; Heimonen et al., 2012; Leung et al., 2000). In the case of ciliary PRs, PDE activation causes the closing of cation channels, which stops the entrance of Ca^{2+} and Na^{+}, and thus hyperpolarises the cytoplasmic membrane, down to a minimum value around −60 mV in vertebrate

rods and cones (Fig. 1.4) (Fu and Yau, 2007; Nelson and Cox, 2013; Purves et al., 2004b).

Changes in the membrane potential of PRs are propagated through their axons, and affect synaptic activity. Thus, light-related information is transmitted to the target cells of PRs. For example, in *Drosophila* rhabdomeric PRs, depolarisation of their cytoplasmic membrane causes the release of the neurotransmitter histamine, which induces an excitatory response in postsynaptic neurons (Hardie, 1987; Pollack and Hofbauer, 1991). In the case of vertebrate ciliary PRs – rods and cones – membrane hyperpolarisation inhibits the spontaneous release of the neurotransmitter glutamate, which has both excitatory and inhibitory effects on the postsynaptic targets of PRs, depending on which receptors they express (Purves et al., 2004b).

How is the membrane potential of PRs restored to its normal value? On the one hand, the Na^+/K^+ pump, which is present in the cytoplasmic membrane of PRs (and in that of all neurons), can restore the correct ionic charges on both sides of the membrane after the phototransduction cascade stops (which, as explained above, happens when light does no longer stimulate opsins, and after GTP–Gαq is

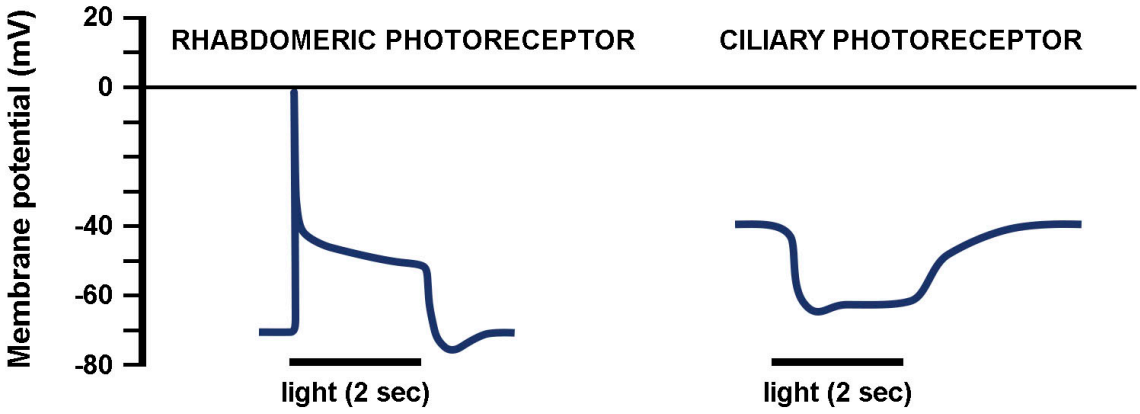

Fig. 1.4: Changes in the membrane potential of PRs during phototransduction. Upon light stimulation, activation of the phototransduction cascade modifies the bioelectrical properties of the cytoplasmic membrane of rhabdomeric and ciliary PRs in a different manner. In the case of rhabdomeric PRs, these cells undergo depolarisation, e.g. in darkness, the resting potential of *Drosophila* compound eye PRs is similar to that of most neurons, around −70 mV, and this value quickly increases to a maximum of 0 mV during phototransduction. Then, the activity of the phototransduction cascade is readjusted and the membrane potential of these cells reaches a plateau around −40 mV, which lasts for as long as there is light stimulation (Hardie and Juusola, 2015; Montell, 2012). In the case of ciliary PRs, phototransduction causes them to become hyperpolarised, e.g. the resting potential is about –40 mV in vertebrate rods and cones, which is higher than that of most neurons. During phototransduction, this value drops to a minimum of −60 mV (Fu and Yau, 2007; Purves et al., 2004b).

converted to GDP–Gαq and thus stops activating either PLC or PDE) (Purves et al., 2004a; Purves et al., 2004c). On the other hand, there are additional feedback mechanisms that negatively regulate phototransduction. These mechanisms could be important for the rapid termination of this cascade after the stimulus has passed, as well as for the adaptation of PRs to light. For example, in *Drosophila* rhabdomeric PRs, Ca^{2+} negatively regulates the opening of cation channels, but it is unknown how this mechanism works (Gu et al., 2005; Hardie and Juusola, 2015). In the case of vertebrate ciliary PRs, guanylate cyclase-activating protein (GCAP), a calcium binding protein, activates guanylate cyclase upon a reduction in the levels of intracellular Ca^{2+}, which happens during light stimulation. Thus, Guanylate Cyclase starts transforming GTP to cGMP, causing the reopening of cation channels (which were closed during phototransduction) (Palczewski et al., 2004; Potter, 2011).

1.4. Mechanisms of eye and photoreceptor formation

1.4.1. Eye development is associated with photoreceptor formation and with the expression of phototransduction proteins

PR differentiation requires that the components of the phototransduction cascade are expressed in these cells. Interestingly, each individual phototransduction component is primarily expressed in PRs, and not in other cell types (Fu and Yau, 2007; Montell, 2012). Also in the case of those phototransduction proteins that belong to broadly expressed families, which have multiple functions in different parts of the body, such as arrestins, PLC or PDE, typically there are homologues that are mainly expressed in PRs (Francis et al., 2011; Gurevich et al., 2011; Kaupp and Seifert, 2002; Kim et al., 1995; Rebecchi and Pentyala, 2000). Thus, most members of the phototransduction cascade are highly specialised.

Importantly, PRs normally differentiate in the eye, and not in other parts of the body. Therefore, it would be possible that the mechanisms underlying eye formation also play a later role for PR differentiation.

1.4.2. Eye development starts similarly in most metazoans

It was initially shown that the transcription factor Eyeless (Ey) plays an essential role during early eye development in *Drosophila*. This protein takes its name

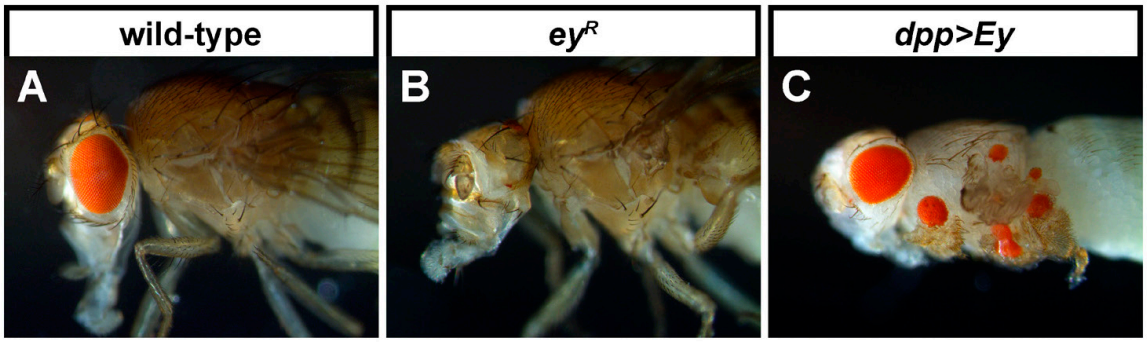

Fig. 1.5: Effects of genetic manipulations affecting RDN transcription factors. RDN genes are fundamental for eye development. Flies possessing mutant alleles for them, like, for example, *ey*^{R} (a hypomorph *eyeless* mutant) can completely lack eyes (Hoge, 1915) (B). Conversely, misexpressing these transcription factors typically causes the formation of ectopic eyes, such as in *dpp>Ey* animals, which die during pupation (C) (Halder et al., 1995).

because those flies in which Ey is selectively knocked-down from the developing eye lack or have strongly reduced eyes. Also, misexpressing Ey is sufficient to induce the formation of ectopic eyes, which are structurally similar to the 'normal' eyes of *Drosophila* (Fig. 1.5) (Halder et al., 1995; Hoge, 1915). More recently, other works have identified additional transcription factors that show phenotypes which are similar to those of Ey, such as Sine oculis (So) or Eyes absent (Eya) (Bonini et al., 1993; Cheyette et al., 1994; Pignoni et al., 1997). These proteins, which are collectively called the 'retinal determination network' (RDN), seem to work together, establishing complex epistatic interactions with each other. RDN members have homologues in most metazoans, and their role during eye development seems to be evolutionarily conserved. In particular, Ey, So and Eya are expressed during the process of eye field specification in the eye primordium (called eye disc in *Drosophila* and optic vesicle in vertebrates), which consists of a pseudostratified epithelium formed by a homogeneous pool of proliferating, multipotent cells (Chow et al., 1999; Loosli et al., 1999; Quiring et al., 1994; Silver and Rebay, 2005; Zuber et al., 2003).

It is still a matter of debate whether the eye, as an organ, is a homologous structure among different phyla or not. Given that there are species with more than one eye-type (such as the ocelli and the compound eyes in *Drosophila*, or the dorsal and ventral eyes in *Platynereis*), it seems necessary that eyes have appeared multiple times in evolution. However, it is believed that all eye-types require the participation of RDN genes for their development, and, as a consequence, it is generally assumed that at least some elements of the mechanisms leading to eye formation have been evolutionarily conserved among different phyla and eye-types

(Arendt, 2003; Arendt et al., 2002; Blanco et al., 2010; Gehring and Ikeo, 1999; von Salvini-Plawen, 2008).

1.4.3. Neurogenesis in the retina

After eye field specification, neurogenesis results in the commitment of some of the cells in the developing eye to become PRs. Both in *Drosophila* and in vertebrates, neurogenesis is initiated by cells of the optic stalk (a constricted region of the eye primordium that connects it to the brain) (Jarman, 2000; Neumann, 2001; Neumann and Nuesslein-Volhard, 2000). In the case of *Drosophila*, the optic stalk is located posteriorly in the eye disc, and it induces adjacent cells to transiently express the proneural transcription factor *atonal* (*ato*) (Hsiung and Moses, 2002; Jarman et al., 1994; Treisman, 2013; Tsachaki and Sprecher, 2012). *ato* expression requires the presence of RDN transcription factors, which directly bind to its promoter (Zhang et al., 2006). Lateral inhibition ensures that Ato expression is progressively restricted to a group of evenly spaced cells that become specified as PR precursors of the R8 subtype (Baker et al., 1996). Each R8 cell is the first element to be specified in an ommatidium. R8 cells secrete Spitz, which causes adjacent cells to be sequentially recruited into the developing ommatidia. Thus, additional PR precursors of different subtypes become specified: first R2/R5, then R3/R4, R1/R6 and finally, R7 (Ready et al., 1976; Tomlinson and Ready, 1987; Treisman, 2013). Newly specified PR precursors produce Hedgehog, a secreted morphogen, which induces the transient expression of Ato in cells located more anteriorly within the eye disc. As a consequence, more cells become R8 PR precursors and initiate the formation of additional ommatidia, progressively more and more anteriorly in the eye disc (Freeman, 1994; Freeman, 1996; Heberlein et al., 1995; Hsiung and Moses, 2002; Jarman et al., 1994; Ma et al., 1993; Strutt and Mlodzik, 1997; Treisman, 2013; Tsachaki and Sprecher, 2012). Therefore, a loop of signalling is established between Ato and Hedgehog: Ato induces the specification of PR precursors, which secrete Hedgehog, and Hedgehog induces the expression of Ato in more anterior cells. In this way, neurogenesis spreads as a wave throughout the eye disc. At the front of this wave, where Ato is expressed, cells contract apically, forming a transient groove that is called the 'morphogenetic furrow'.

In the case of vertebrates, the early events of PR neurogenesis have been investigated mainly in zebrafish. At this stage, the optic vesicle (formed by a pseudostratified epithelium) has already developed into the optic cup, a three-

layered structure. Similar to *Drosophila*, neurogenesis in zebrafish is initiated by the optic stalk, and it propagates through the innermost layer of the optic cup as a wave. The expression of the zebrafish homologues of Hedgehog and Ato, abbreviated Shh and Ath5, spreads in close association with the front of this wave. However, the mechanism that drives the propagation of neurogenesis is not yet understood in detail (Jarman, 2000; Neumann, 2001; Neumann and Nuesslein-Volhard, 2000). Loss of function analysis have shown that Ath5 is absolutely necessary for the formation of all retinal ganglion cells in zebrafish, some of which (the ipRGC subpopulation) might be the vertebrate homologues of *Drosophila* rhabdomeric PRs (Arendt, 2003; Kay et al., 2001). Similarly, in the case of the mammalian retina, null mutants for the mouse Ato homologue (called MATH5) possess 80% less retinal ganglion cells than wild-type animals. Other cell types are not lost in the retina of *Math5* mutants (rather, the number of amacrine cells is increased) (Wang et al., 2001). However, this transcription factor is only transiently expressed at the onset of neurogenesis. It is primarily expressed in those retinal progenitors that will later differentiate into retinal ganglion cells, but not in all of them: only 55% of the retinal ganglion cells in the adult retina descend from MATH5-expressing progenitors (Brzezinski et al., 2012). This expression pattern resembles that of *Drosophila*, and it suggests that Ato homologues may not directly instruct any specific cell identity. Rather, these orthologues seem to act primarily in a non-cell autonomous manner (Brzezinski et al., 2012; Jarman et al., 1994; Jarman et al., 1995; Treisman, 2013; Wang et al., 2001). Later, a second and a third wave of neurogenesis spread through the other two layers of the optic cup, and thus induce the differentiation of all types of neurons and glial cells in the retina (Neumann, 2001). Cell fate determination in the vertebrate retina has been well studied in *Xenopus*. Similar to *Drosophila*, initially, all cells in the eye primordium of the frogs are equally competent to become any of the cell types that compose the adult retina. Later, their competence becomes restricted, and thus different cell types are recruited sequentially: first the retinal ganglion cells, then horizontal cells, cones, rods, amacrine cells, bipolar cells, and finally Muller cells (Wong and Rapaport, 2009). It has been proposed that a similar process is probably happening also in other vertebrates (Brzezinski and Reh, 2015).

1.4.4. Terminal photoreceptor differentiation. Why did we start investigating Glass?

By the end of their development, PRs differentiate morphologically and tend to form either a rhabdomere (in most types of rhabdomeric PRs) or an outer segment (in

ciliary PRs). Also, they initiate the expression of the proteins that participate in the phototransduction cascade, which allows them to detect light. This process – which is the subject of this Ph.D. thesis – culminates the differentiation of PRs and, therefore, it is of great importance for understanding how functional PRs are made. Particularly, we know very little about how the earlier processes of eye field specification and neurogenesis lead to the formation of mature PRs.

In the case of vertebrate rhabdomeric PRs – the ipRGCs – these cells are important for non-image forming visual functions, such as irradiance detection (Münch et al., 2015; Pickard and Sollars, 2012). However, we know virtually nothing about how ipRGCs differentiate. It has been described that mutants for the T-box transcription factor 2 (*Tbr2*) lack this type of PRs, indicating that TBR2 plays a role during their development. However, TBR2 is similarly expressed in other subpopulations of retinal ganglion cells, which are also defective in *Tbr2* mutants, and only 18% of all TBR2-positive cells co-express melanopsin, the opsin orthologue that characterise ipRGCs (Mao et al., 2014; Sweeney et al., 2014).

In contrast to ipRGCs, vertebrate ciliary PRs – rods and cones – are specialised in image-forming vision (Purves et al., 2004b), and it is generally accepted that the cone-rod homeobox protein (CRX) is the main transcription factor regulating their differentiation and maintenance (Brzezinski and Reh, 2015; Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). CRX is primarily expressed in rods and cones, where it directly binds to the promoters of many PR genes, including phototransduction proteins (Furukawa et al., 1997; Peng and Chen, 2005). Also, *Crx* mutants fail to form functional rods and cones: these cells are still present, but the outer segment does not develop and they lack the expression of several phototransduction components (Furukawa et al., 1999).

Unlike the case of vertebrates, *Drosophila* mutants for the insect homologue of *Tbr2*, called *bifid*, do not lack rhabdomeric PRs (Tsai et al., 2015). However, the fly homologue of *Crx*, which is called *orthodenticle* (*otd*), does play a comparatively small function during the development of *Drosophila* rhabdomeric PRs. Flies lacking Otd in PRs present morphological defects in their rhabdomeres, and they do not express a subset of opsin orthologues (Rhodopsins 3, 5 and 6). Nevertheless, these mutants are still attracted towards visible light, indicating that they possess functional rhabdomeric PRs (Tahayato et al., 2003; Vandendries et al., 1996). It should be noted that Otd is broadly expressed throughout different regions of the *Drosophila* brain, and not particularly enriched in the eye (Hirth et al., 2003). Apart

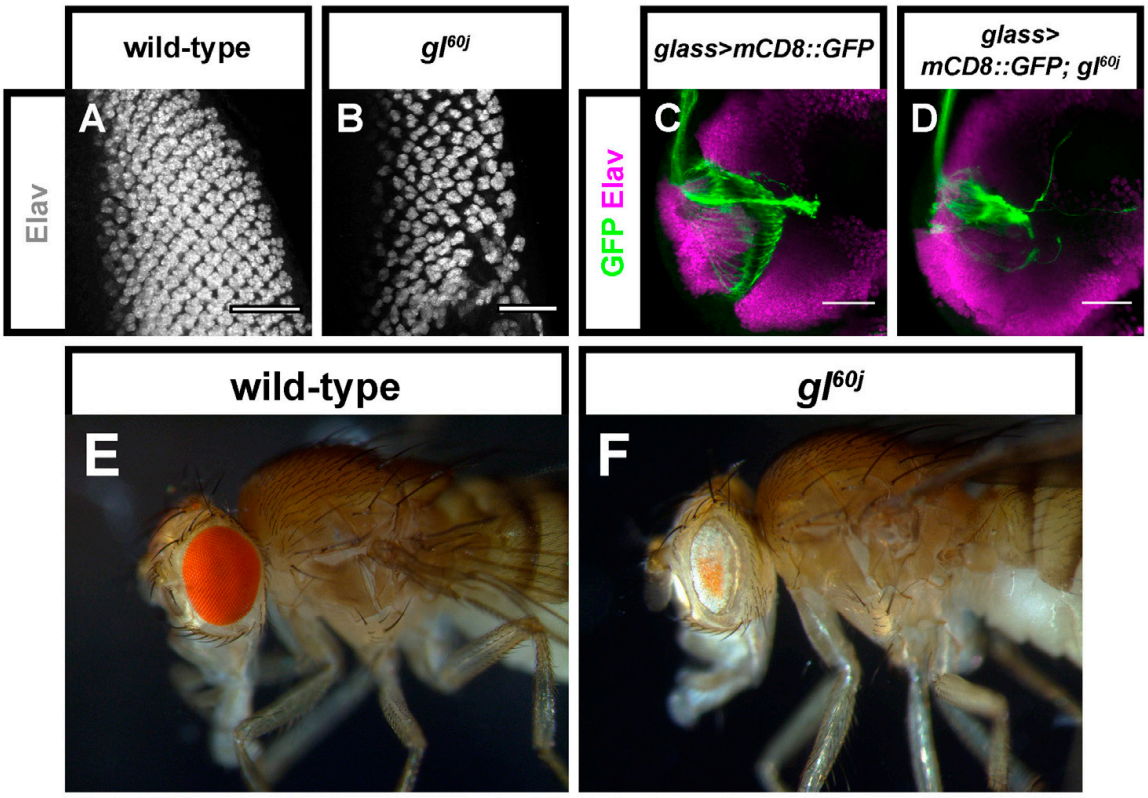

Fig. 1.6: *glass* mutant PRs develop abnormally. PR precursors in the eye discs of wild-type larvae are orderly recruited into the developing ommatidia, forming a regular array of rows and columns. This can be observed when PRs are stained using an antibody against the neuronal marker Embryonic lethal abnormal vision (Elav) (A). By contrast, PR precursors look disorganised in the eye discs of gl^{60j} larvae (which possess an amorphic mutation for *glass*) (B) (Moses et al., 1989; Treisman and Rubin, 1996). Similarly, wild-type PR precursors project their axons orderly into the optic neuropil (C), whereas those of *glass* mutants appear misrouted (D) (Selleck and Steller, 1991). It has been suggested that, in contrast to wild-type, *glass* mutant PR precursors die during metamorphosis and are not present in the adult retina (E, F) (Moses et al., 1989; Ready et al., 1986; Stark et al., 1984).

of *otd*, it has been shown that another homeodomain transcription factor, called *hazy*, partly regulates the differentiation of rhabdomeric PRs. *hazy* mutant PRs also present morphological defects in their rhabdomere, and they lack a subset of phototransduction proteins (Mishra et al., 2010; Zelhof et al., 2003). Importantly, in contrast to Otd, Hazy seems to be expressed exclusively in rhabdomeric PRs in arthropods (Mahato et al., 2014).

In addition to Otd and Hazy, the transcription factor Glass is also involved in the formation of rhabdomeric PRs. It has been shown that it is primarily expressed in all visual organs in *Drosophila*, including the Bolwig organ, the ocelli, and the compound eye. Particularly, in the case of the compound eye, its expression starts right after the passing of the morphogenetic furrow, at a very early stage during PR development, and it is also present in adult PRs (Bridges and Morgan, 1923; Ellis et

al., 1993; Johannsen, 1924; Moses et al., 1989; Moses and Rubin, 1991; Stark et al., 1989; Stark et al., 1984). The RDN member So directly binds to the *glass* promoter and, therefore, it could be that Glass is the missing link between early-acting processes – such as eye field specification and neurogenesis – and the terminal differentiation of PRs (Jusiak et al., 2014). However, this hypothesis has not been tested because it was previously suggested that *glass* mutant PR precursors die during metamorphosis. As a consequence, the *glass* mutant phenotype was investigated in the third instar eye imaginal disc, but not in adult PRs (Moses et al., 1989; Ready et al., 1986; Stark et al., 1984; Treisman and Rubin, 1996) (Fig. 1.6).

In the present Ph.D. thesis we show that, indeed, Glass links the action of the RDN with the formation of functional, light-sensing PRs. The transcription factor So is important for directly activating the *glass* promoter, and Glass is key for initiating the expression of virtually all the phototransduction proteins. Given the importance of Glass in *Drosophila* we have also analysed its expression pattern in *Platynereis*, a marine annelid, and we discuss the possibility that it plays an evolutionarily conserved role during PR development.

2. THE TRANSCRIPTION FACTOR GLASS LINKS EYE FIELD SPECIFICATION WITH PHOTORECEPTOR DIFFERENTIATION IN *DROSOPHILA*

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2.1. 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.

2.2. 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 (Bonini et al., 1993; Cheyette

et al., 1994; Hoge, 1915). 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; Treisman, 2013; Tsachaki and Sprecher, 2012).

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 (Mishra et al., 2010; Tahayato et al., 2003; Vandendries et al., 1996; Zelhof et al., 2003). 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 (Ellis et al., 1993; Moses and Rubin, 1991). It has been suggested that *glass* mutant PR precursors die during metamorphosis. Therefore, its role in PR differentiation has not been assessed (Moses et al., 1989; Ready et al., 1986; Stark et al., 1984).

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.

2.3. Results

2.3.1. 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 (Moses et al., 1989; Ready et al., 1986; Stark et al., 1984). 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. 2.1A). PR precursors can be distinguished from cone cells by their position and the expression of the pan-neuronal protein Elav (Cagan and Ready, 1989; Robinow and White, 1991; Tomlinson and Ready, 1987). This pattern of H2B::YFP expression was maintained during pupation and in the adult retina (Figs. 2.1B–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. 2.1E, Fig. S1). H2B::YFP was expressed in some, but not all PR precursors and in presumptive cone cells, comparable to expression in wild-type

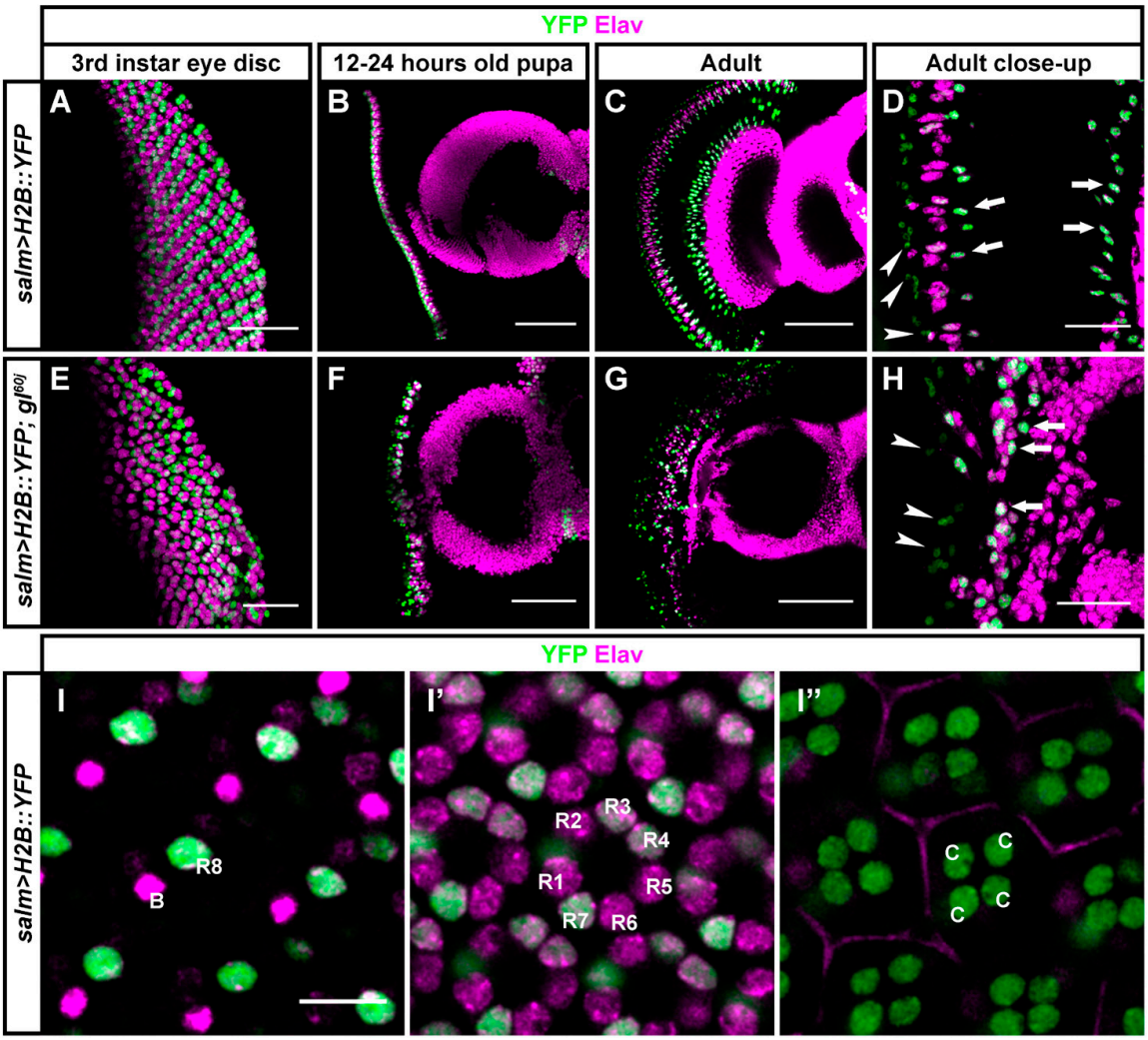


Fig. 2.1: *glass* mutant PR precursors survive metamorphosis and are still present in the adult retina. (A–H) Expression of YFP (green) and Elav (magenta) in a *salm>H2B::YFP* reporter line at different developmental time points in control and *glass* mutant background. The *salm>H2B::YFP* reporter is 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 hours 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.

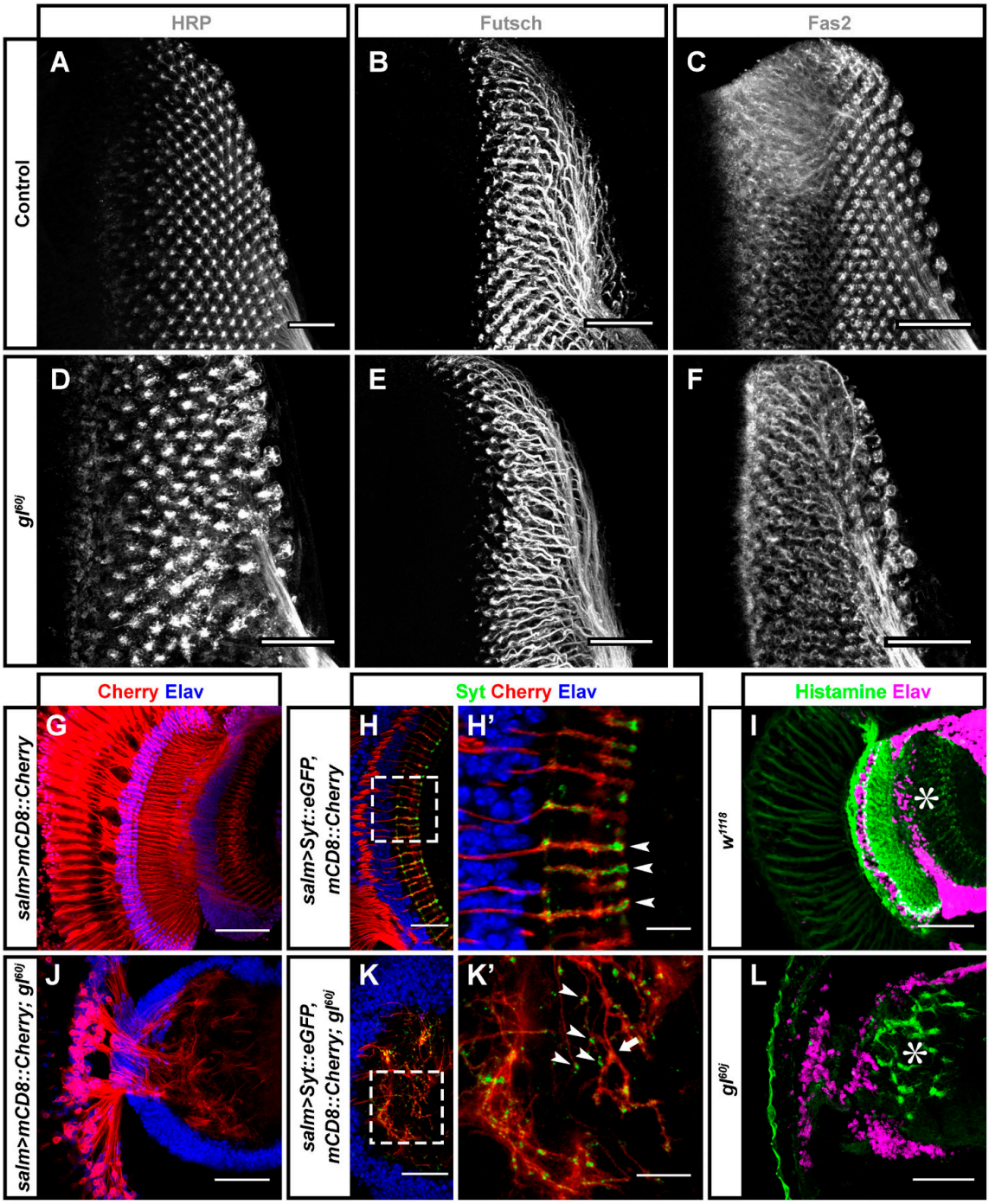
discs. We followed the expression of H2B::YFP during metamorphosis and found that although the regular organisation of the retina is severely compromised, double-positive cells for H2B::YFP and Elav are maintained into the adult stage of gl^{60j} mutant flies (Fig. 2.1F–H). We obtained similar results 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 relocalised to the basal side of the retina (Fig. S3). Thus, we conclude that, in contrast to previous reports, Glass is not required for the survival of PR precursors, and that presumptive PRs are still present in the adult *glass* mutant retina.

2.3.2. *glass* mutant PR precursors differentiate as neurons

PR precursors in the eye discs of *glass* mutant larvae express neuronal markers and project axons (Kunes et al., 1993; Moses et al., 1989; Selleck and Steller, 1991; Treisman and Rubin, 1996). We confirmed these results using antibodies against Elav (Fig. 2.1E), Futsch, Fasciclin 2 (Fas2) and horseradish peroxidase (HRP) (Figs. 2.2A–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*. At 50–60 hours 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. 2.2G). *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. 2.2J). 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 (Chen et al., 2014; Sánchez-Soriano et al., 2005; Zhang et al., 2002). In control pupae, Syt::eGFP



accumulated at the distal tips of PR axons (Chen et al., 2014) (Figs. 2.2H, H'). In *gl*^{60j} mutants, Syt::eGFP also accumulated at distinct foci, but these were spread along the length of the axons (Figs. 2.2K, 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. 2.2I). Histaminergic projections are still present in the optic

⟶ **Fig 2.2: *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 hours after pupation were labelled by the expression of *salm>mCD8::Cherry* (red) and brains were 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 hours 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.

lobe of *glass* mutant flies (Fig. 2.2L). 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. 2.2J). 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.

2.3.3. *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 (Hayashi et al., 2008; Jarman et al., 1995; Lim and Choi, 2004; Naval-Sánchez et al., 2013; Treisman and Rubin, 1996). 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. 2.2J) 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) (Figs. 2.3A–D) (Chou et al., 1999; de Couet and Tanimura, 1987); 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) (Figs. 2.3E, K–O) (Dolph et al., 1993;

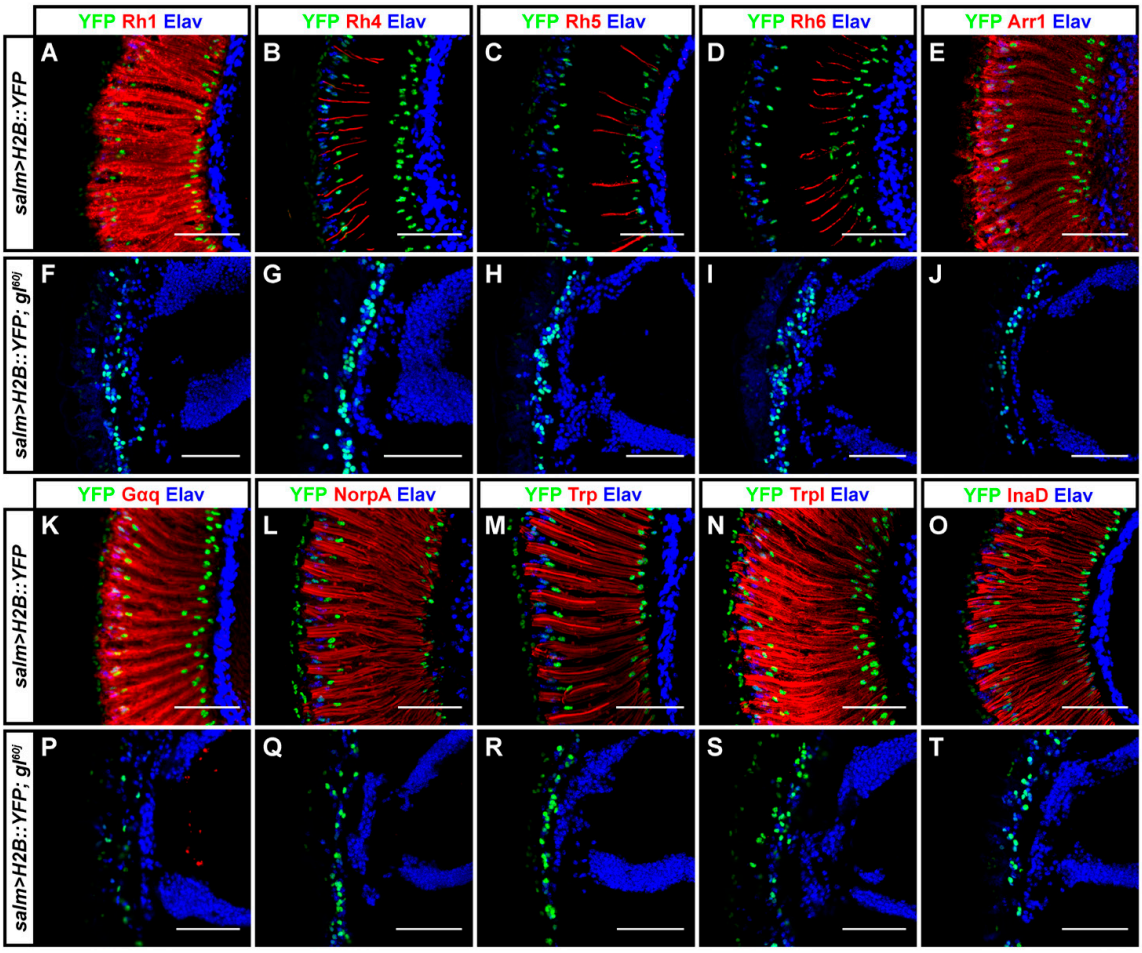


Fig 2.3: Glass is required for acquisition of the phototransduction machinery. (A–T) Expression of proteins involved in the phototransduction cascade in the adult retina of *salm>H2B::YFP* (used as control) and *salm>H2B::YFP; gl60j* flies, which were stained against YFP (green), different phototransduction 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.

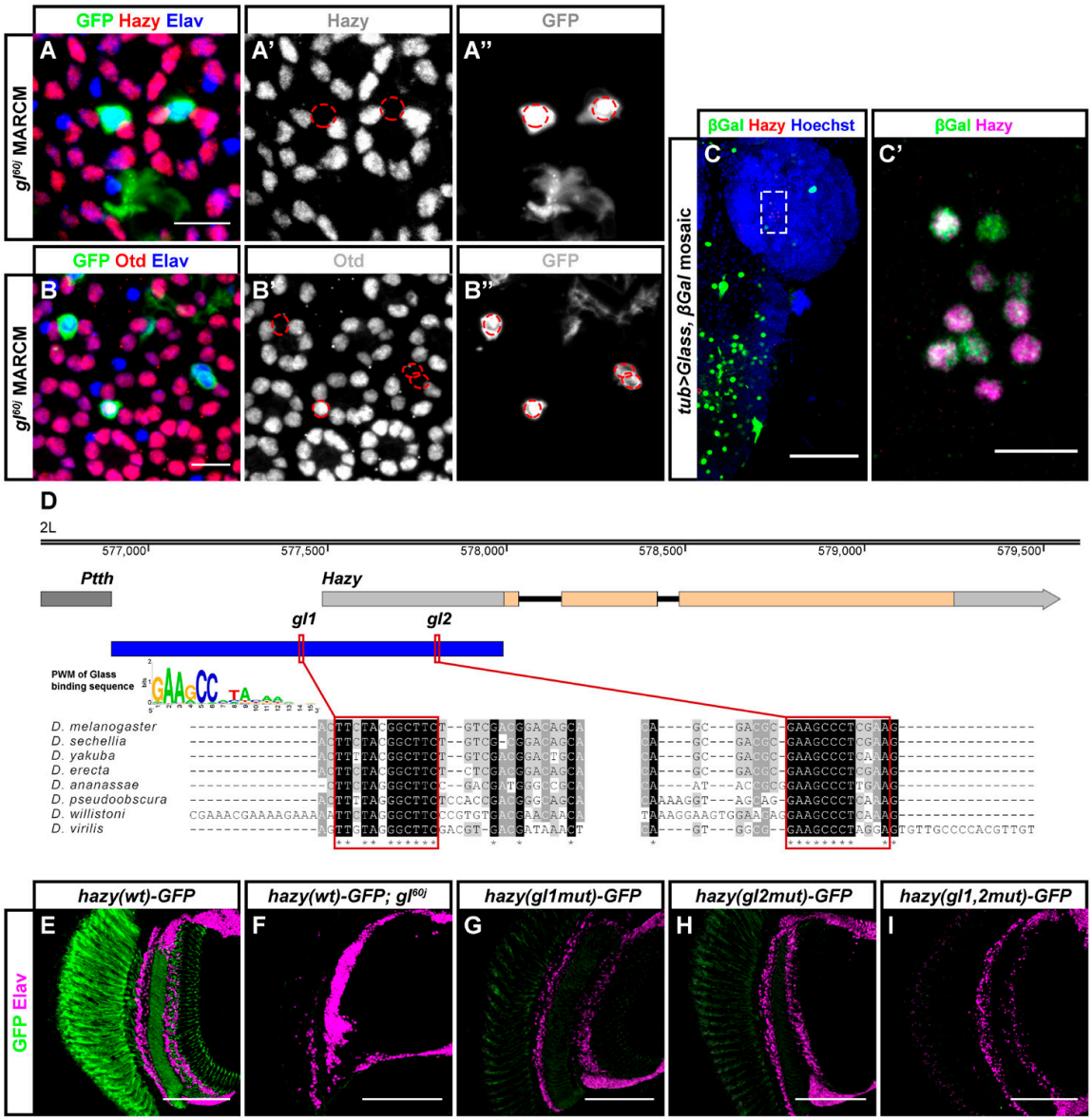
Lee et al., 1994; Montell, 2012; Niemeyer et al., 1996; Shieh and Niemeyer, 1995; Wong et al., 1989; Zhu et al., 1993). In all cases, these proteins were expressed in the retinas of control flies, but were absent in the retinas of *glass* mutant flies (Figs. 2.3F–J, P–T; Fig. S4). The ocelli-specific 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.

2.3.4. 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* (Mishra et al., 2010; Tahayato et al., 2003; Vandendries et al., 1996; Zelhof et al., 2003). 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 (Figs. S6A, B). By clonal analysis, we found that Glass is required in a cell-autonomous manner for the expression of Hazy (Fig. 2.4A). 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; Figs. 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. 2.4B).

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 (Figs. 2.4C, 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. 2.4D). We first generated flies containing a *hazy(wt)-GFP* reporter construct, which expressed GFP specifically in PRs, reflecting the expression pattern of Hazy (Fig. 2.4E). When this reporter was introduced into



glass mutant background, GFP expression was completely lost (Fig. 2.4F). 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. 2.4D). Expression of the GFP reporter was reduced when either *gl1* or *gl2* were mutated alone (Figs. 2.4G, H), and lost when both *gl1* and *gl2* were mutated (Fig. 2.4I). 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

⟶ **Fig. 2.4: 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 hours 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.

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, 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.

2.3.5. 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 (Figs. 2.5A, A'), Arr1 (Figs. 2.5B, B'), NorpA (Figs. 2.5C, C'), Trpl (Figs. 2.5D, D') and InaD (Figs. 2.5E, E'). These results demonstrate that Hazy can partially rescue the *glass* mutant phenotype. It should be noted that,

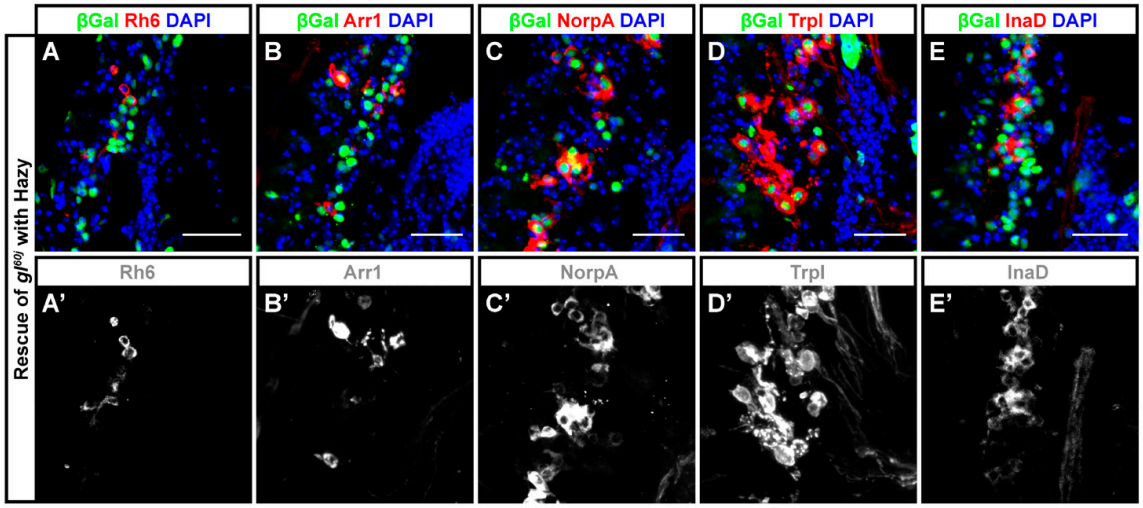


Fig. 2.5: Hazy expression can partially rescue the *glass* mutant phenotype. (A–E') Hazy was expressed in the adult *glass* mutant retina in clones labelled with nuclear βGal. Samples were stained for βGal (green), different proteins involved in the phototransduction cascade (red) and with DAPI (used 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.

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.

2.3.6. 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 (Figs. 2.6A–G').

Chp is an early PR marker known to require expression of Glass (Moses et al., 1989; Naval-Sánchez et al., 2013; Zipursky et al., 1984). Our finding that Glass can broadly drive Chp expression across the CNS of larvae (Figs. 2.6A, 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) (Figs. 2.6B, B'), whereas Rh2 is primarily expressed in the VNC (Figs. 2.6C, 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 (Figs. 2.6H–N'). Trpl was broadly expressed in the CNS (Figs. 2.6I, 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 (Figs. 2.6O–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,

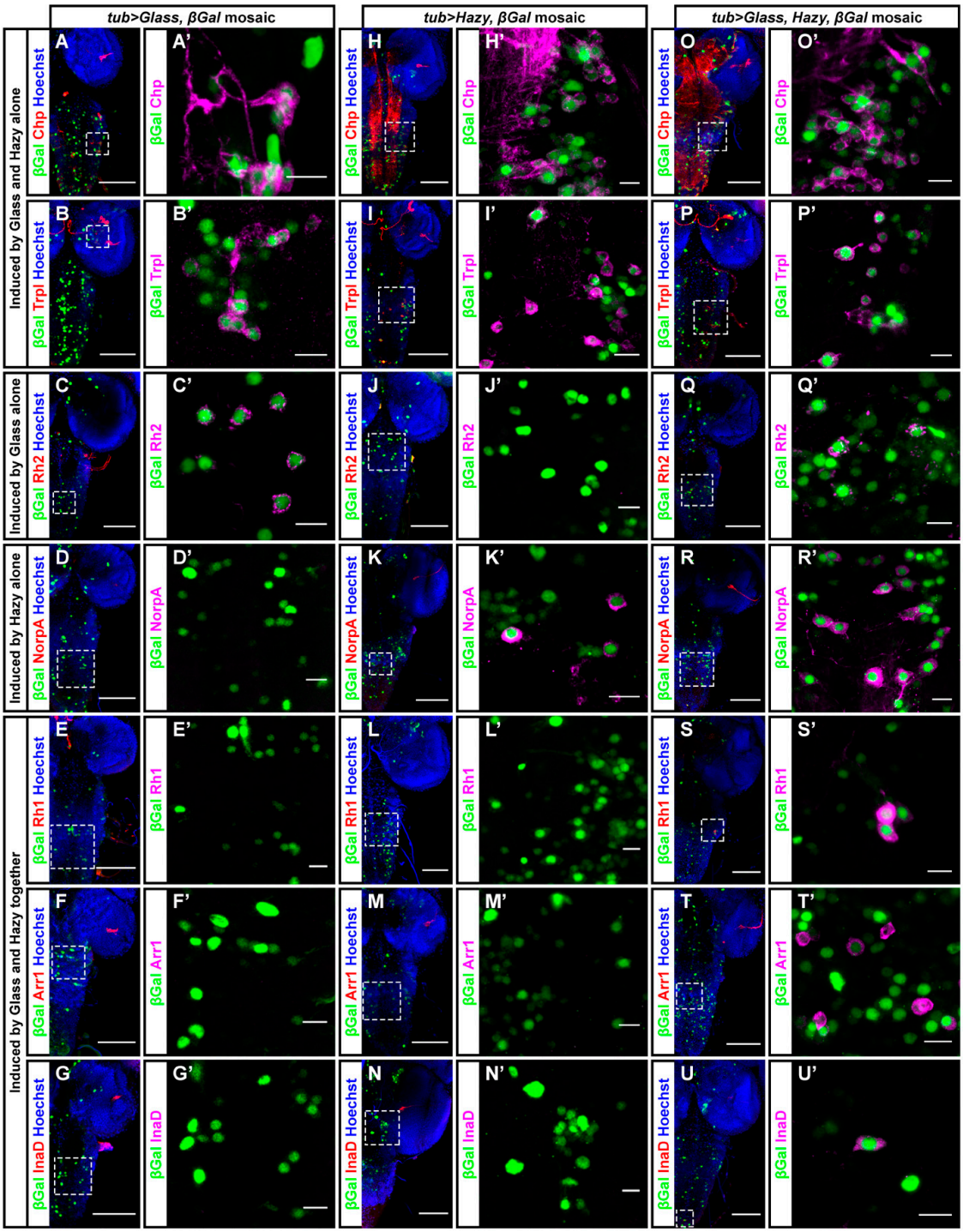


Fig. 2.6: Glass and Hazy can ectopically induce expression of phototransduction proteins. (A–U') The CNS of third instar larvae, which ectopically express combinations of Glass and Hazy in clones labelled with nuclear βGal, were stained with antibodies against βGal (green), different PR proteins (red/magenta) 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.

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.

2.3.7. 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 (Aerts et al., 2010; Jusiak et al., 2014; Tanaka-Matakatsu and Du, 2008; Treisman, 2013; Zhang et al., 2006), 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 (Figs. 2.7A–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. 2.7C) (Jemc and Rebay, 2007; Liu et al., 1996). 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 (Figs. 2.7D, D'). After mutating the three So binding sites, GFP was no longer expressed (Figs. 2.7E, 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*

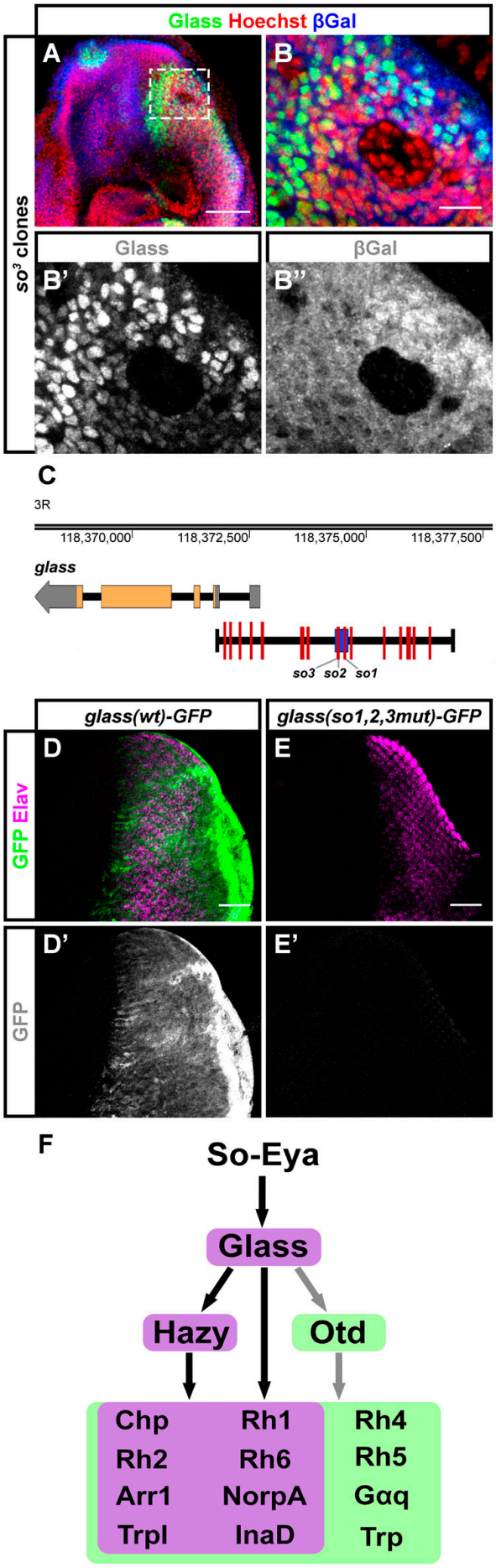


Fig. 2.7: Glass transcriptionally links the RDN with the expression of proteins involved in PR terminal differentiation. (A–B'') The RDN member So is required for expression of Glass. Third instar eye discs carrying *so*^{3} mutant clones were stained for Glass (green), βGal (blue) and with Hoechst 33258 (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 labelled 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 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) (Aerts et al., 2010; Jarman et al., 1994; 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).

2.4. Discussion

RDN genes have a key function in eye field specification both in vertebrates and invertebrates (Chow et al., 1999; Halder et al., 1998; Halder et al., 1995; Hoge, 1915; Loosli et al., 1999; Pignoni et al., 1997; Quiring et al., 1994; 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. 2.7F). 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* 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 (Mishra et al., 2010; Tahayato et al., 2003; Vandendries et al., 1996; Zelhof et al., 2003). 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 (Mishra et al.,

2016; Rister et al., 2015; Zelhof et al., 2003). 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-Sánchez 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 feed-forward mechanisms (Alon, 2007; Baumgardt et al., 2009; Baumgardt et al., 2007; Etchberger et al., 2009; Etchberger et al., 2007). 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 (Cheyette et al., 1994; Moses and Rubin, 1991; 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 (Chow et al., 1999; Loosli et al., 1999; Quiring et al., 1994; 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 (Etchberger et al., 2007; Liu and Friedrich, 2004). 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 PRs (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 (Etchberger et al., 2007; Tursun et al., 2011; Uchida et al., 2003). 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.

2.5. Materials and methods

2.5.1. 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.

2.5.2. Generation and analysis of clones

Mutant and overexpression clones were induced and analysed as described in supplementary Materials and Methods.

2.5.3. Generation of transgenic flies

To make *hazy*, *otd* and *glass* reporters we amplified their enhancers by PCR and cloned them into a GFP vector as described in supplementary Materials and Methods. For a list of primers see Table S1. To generate *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), as described in the supplementary Materials and Methods.

2.5.4. Immunohistochemistry and imaging

Samples were dissected and fixed at room temperature for 20 minutes 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 minutes with 4% ethyldimethylaminopropyl carbodiimide (EDAC) in PB. After fixation, we followed previously described methods (Wolff, 2000a; Wolff, 2000b). 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.

2.6. Acknowledgements

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2.7. Competing interests

The authors declare no competing or financial interests.

2.8. 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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2.10. Supplementary material

2.10.1. Supplementary methods

2.10.1.1. 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: gl^{60j} (no. 509); *FRT82B,* gl^{60j} (no. 6333); gl^2 , e^4 (no. 507) and gl^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).

2.10.1.2. 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-Gαq (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).

Secondary antibodies were conjugated with Alexa Fluor fluorescent proteins (405, 488, 546, 568 or 647) and we used them in a 1:200 dilution (Molecular Probes; no. A-11029, A11001, A-11031, A-21235, A-31553, A-11006, A-11077, A-21247, A-11008, A-11011, A-21244, A- 11039, A-11041, A-11073, A-11074, A-21450, A-10037, A-31571, A-21206, A-10042 and A-31573). We also used Hoechst 33258 (1:100, Sigma, no. 94403) and DAPI (in Vectashield mounting medium, Vector, H-1200) as fluorescent markers of cell nuclei, and phalloidin conjugated with ATTO 647N to label actin microfilaments (no. 65906).

2.10.1.3. Generation and analysis of clones

MARCM analysis of glass mutant clones was performed in *hsFLP; tub-Gal4, UAS-mCD8::GFP; FRT82B, tub-Gal80/FRT82B, gl*^{60j} 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 *gl*^{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; tub(FRT cassette)Gal4, UAS-lacZ.nls; gl*^{60j} flies with others carrying combinations of the *UAS-hazy* and *UAS-Otd* constructs with the *gl*^{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; tub(FRT cassette)Gal4, 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 *tub(FRT 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 *eyFLP; FRT42D, arm-lacZ/FRT42D,* so^3 larvae, and could be identified as groups of cells negatively labelled for βGal.

ato^w mutant clones were generated in the eye discs of *eyFLP;; FRT82B, ubiGFP/FRT82B,* ato^w larvae, and we could recognise them as groups of cells negatively labelled for GFP.

2.10.1.4. 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-GFPhsp70 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 NcoI (*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.

hazy 1.1 Kpn fw	ctggtaccACATGTGTGCAGAGGCAAAGGG
hazy noStart Not re	aagcggccgcGCGAATCCTGAGCTTCCTGTTGG
hazy gl1 site mut Sph fw	GGGCGACTTCTACgcatgcTGTCGACGGACAGCACG
hazy gl1 site mut Sph re	CGTGCTGTCCGTCGACAgcatgcGTAGAAGTCGCCC
hazy *gl2* site mut RV fw	GAAGAAGCAGCGACGCgatatcCTCGAAGTGTCGACG
hazy gl2 site mut RV re	CGTCGACACTTCGAGgatatcGCGTCGCTGCTTCTTC
otd PRenh Kpn fw	cggagcgttGGTACCtcgtc
otd PRenh BamHI re	ggccagaccatcGGATCCcc
otd PRenh gl mut Xho fw	agCTCGAGcctgcagtggtcggctcc
otd PRenh *glass* mut Xho re	ggCTCGAGtccttaatcgctgttgctttttacggc
glass 5′ UTR BamH fw	gaggatCCTCGCCAAAAGTCGCTTCTTG
glass exon 4 re	ccccgactgcgaaaatCTGAGCAGGCAGAGCTTGCAC
glass exon 5 fw	gctctgcctgctcagATTTTCGCAGTCGGGGAACTTG
glass Stop Xho re	ggctcgaGTCATGTGAGCAGGCTGTTGCC
glass Start+Kozak attB1 fw	ggggacaagtttgtacaaaaaagcaggcttcaaCATGGGATTGTTATATAAGGGTTCCAAACT
glass Stop attB2 re	ggggaccactttgtacaagaaagctgggtcgTCATGTGAGCAGGCTGTTGCC
glass BRenh *so1* mut Spe fw	acACTAGTttgaagcgaagtaaaaaaaaaaagaaatataaaaattgaaaactgg
glass BRenh *so1* mut Spe re	ttTGATCAgtttcatgtcaacaacttggctaaggac
glass BRenh *so2+so3* mut Nco fw	ggGACGCtgggggatatagctCCATGGgtatgcgatcactgcaagcc
glass BRenh *so2+so3* mut Nco re	acCCATGGagctatatcccccaGCGTCccttaccttatcgatgggaatttcagg

Table S1: Primer sequences.

2.10.1.5. 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.

2.10.2. Supplementary figures

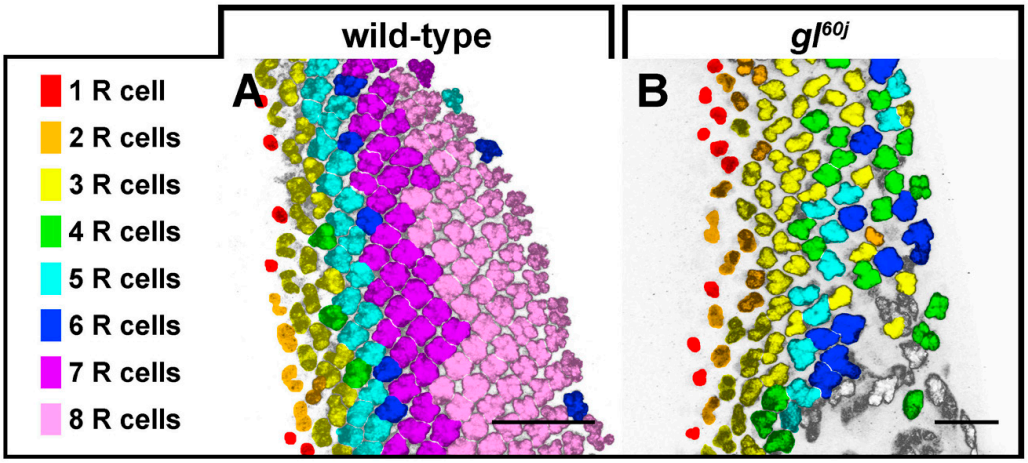

Fig. S1: *glass* mutant PR precursors are not correctly recruited into the developing ommatidia in the third instar eye disc. It has been reported that, at this stage, *glass* mutant PR precursors fail to acquire a correct subtype identity, based on the expression of subtype specific PR markers (Hayashi 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 gl^{60j} 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: 30 μm.

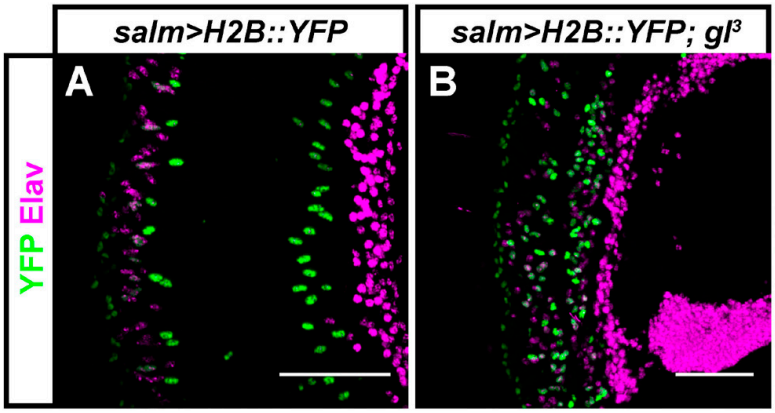

Fig. S2: *glass* mutant PR precursors survive metamorphosis and are still present in the adult retina. Expression of YFP (green) and Elav (magenta) in the *salm>H2B::YFP* reporter line in the adult retina of control (A) and gl^{3} mutant flies (B). In both cases, a subset of presumptive PRs can be identified by the co-expression of YFP and Elav, while cone cells express YFP but not Elav. Scale bars: 40 μm.

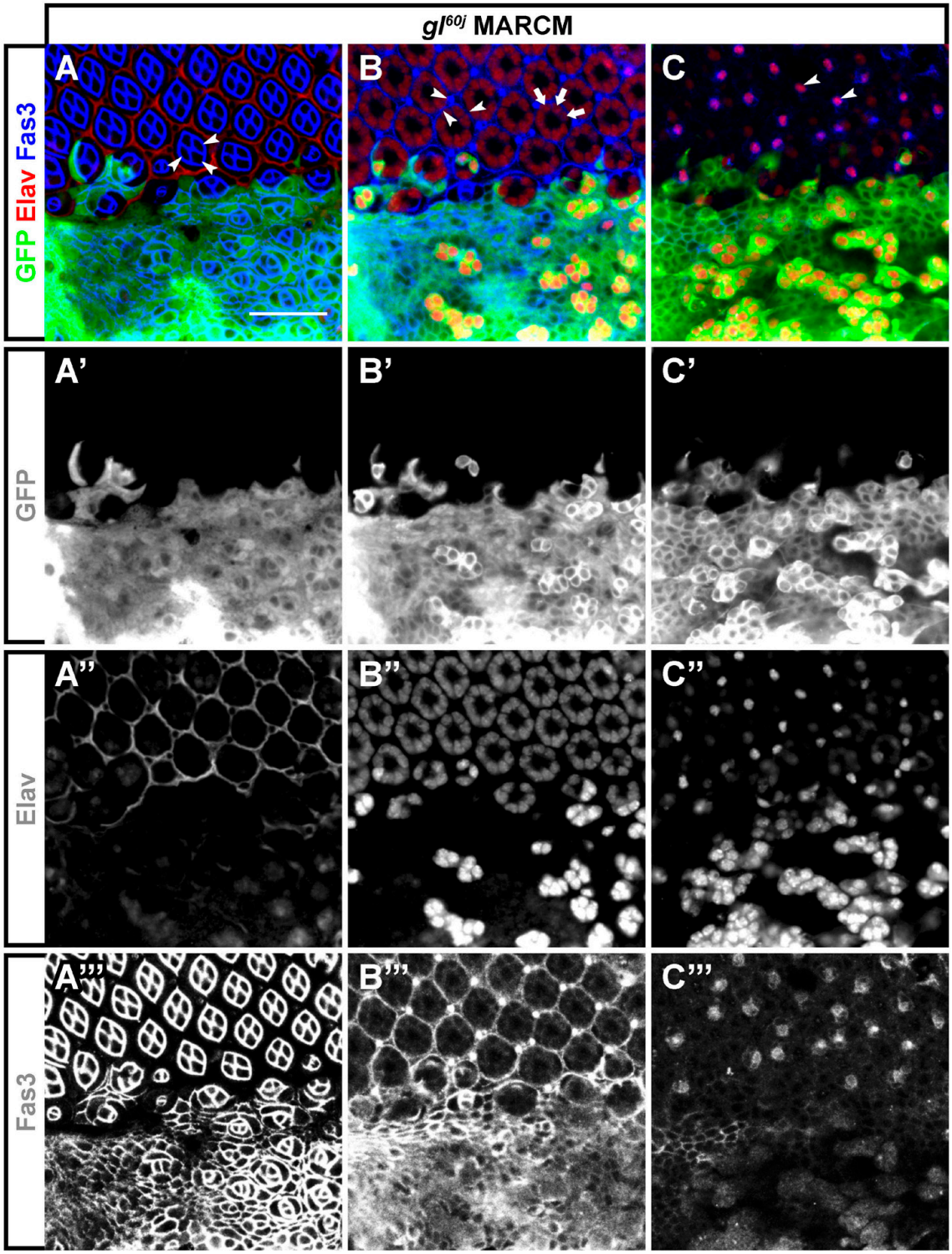

Fig. S3: *glass* mutant PR precursors are irregularly distributed in the retina. (A–C''') We used MARCM analysis to induce the formation of gl^{60j} mutant clones, labelled with the expression of *UAS-mCD8::GFP*. Retinas were dissected ~50 hours after pupation, and stained against GFP (green), Elav (used 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, 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^{60j} 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-labelled *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 (Moses et al., 1989; Treisman and Rubin, 1996) (Fig. 2.1E, Fig. S1). 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: 30 μm.

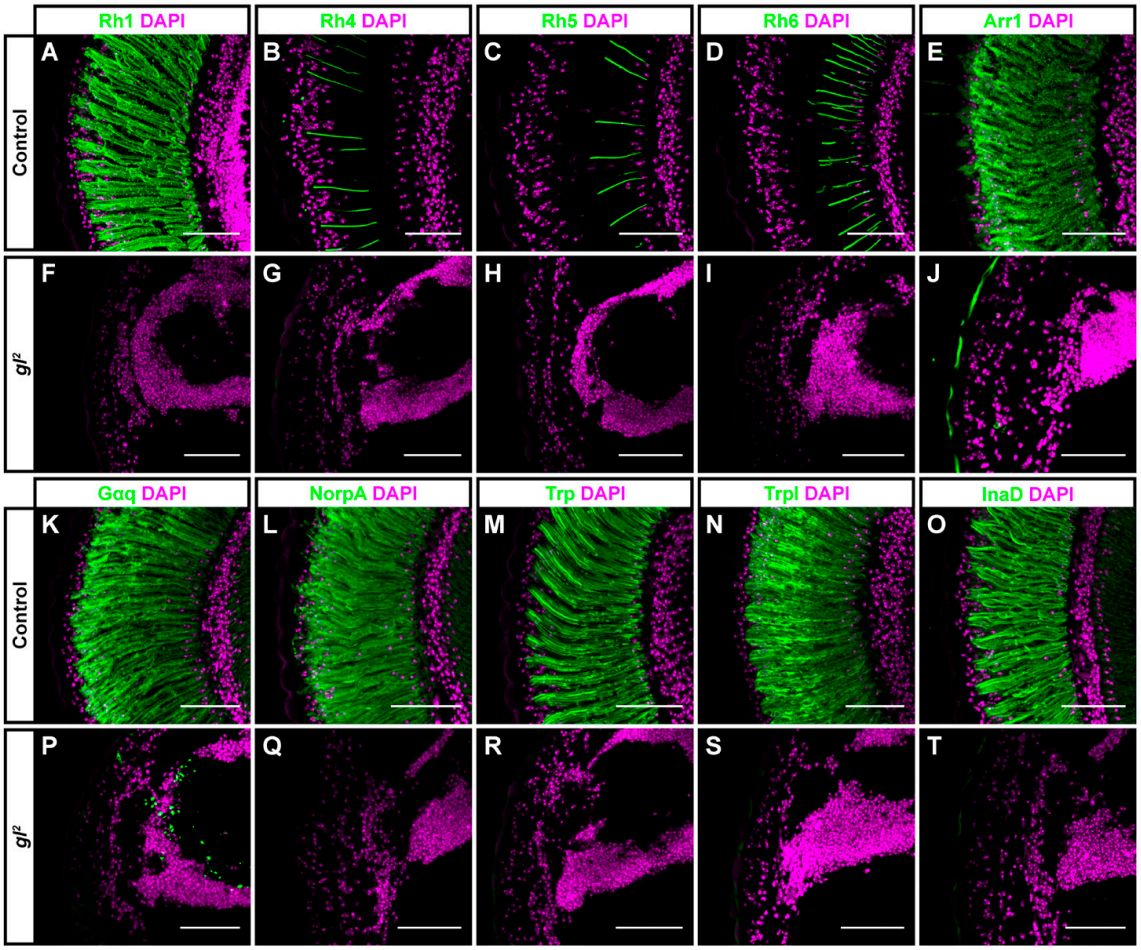

Fig. S4: Glass is required for the acquisition of the phototransduction machinery. (A–T) Expression of different proteins involved in the phototransduction cascade in the adult retina of control (*salm>H2B::YFP*) and gl^2 mutant flies. Samples were stained against phototransduction proteins (green) and 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 gl^2 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), Gαq (K), NorpA (L), Trp (M), Trpl (N) and InaD (O). These proteins are not expressed in the retina of gl^2 mutant flies: Arr1 (J), Gαq (P), NorpA (Q), Trp (R), Trpl (S), or InaD (T). Scale bars: 40 μm.

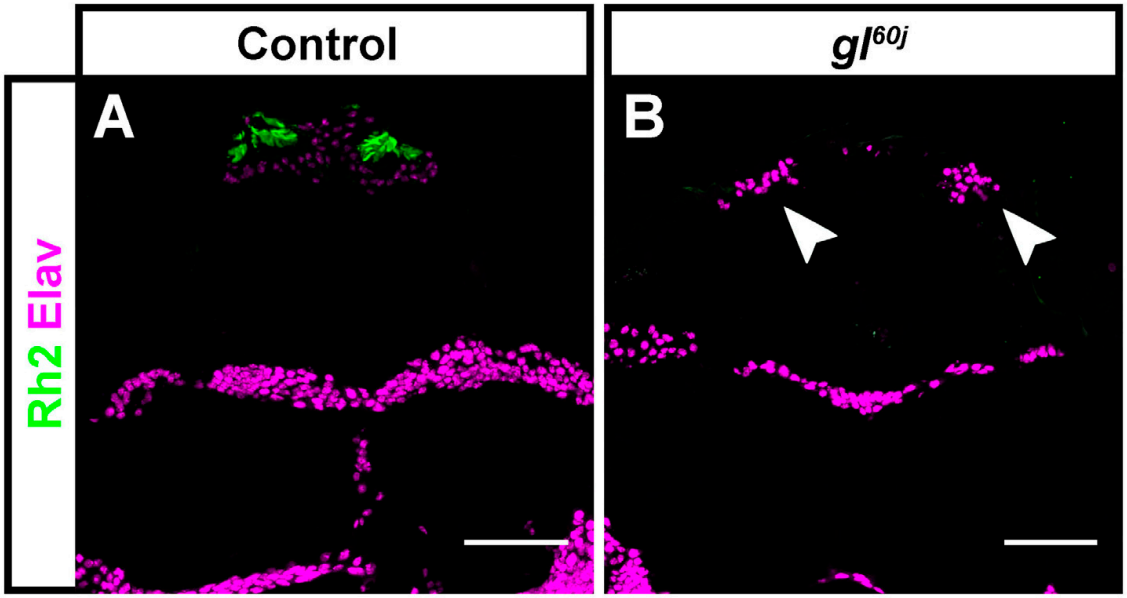

Fig. S5: Glass is required for Rh2 expression. Both control and *glass* mutant flies were stained against Rh2 (green) and counterstained with Elav (magenta). Rh2 is expressed in ocellar PRs in control flies (A). In *glass* mutant, there is no expression of Rh2 in the presumptive ocelli PRs (arrowheads, B). Scale bars: 40 µm.

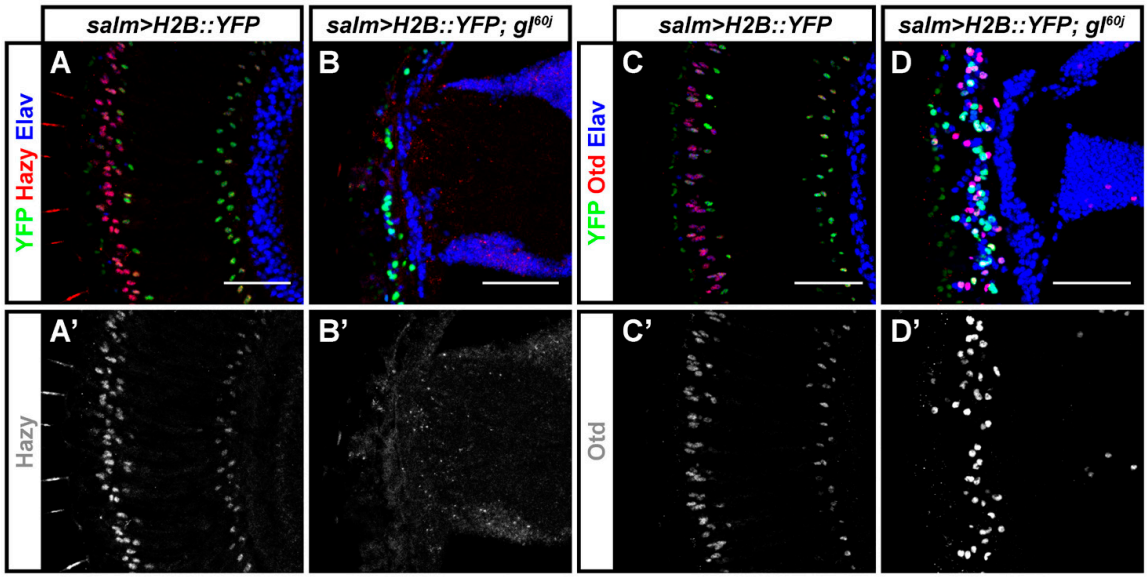

Fig. S6: Glass is required for the correct expression of Hazy and Otd. (A–D') We used the *salm>H2B::YFP* reporter to label the retina of adult flies, and stained against GFP (green), either Hazy or Otd (red), and the neuronal marker Elav (Blue). For each image, the red channel is shown below in greyscale. 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: 50 µm.

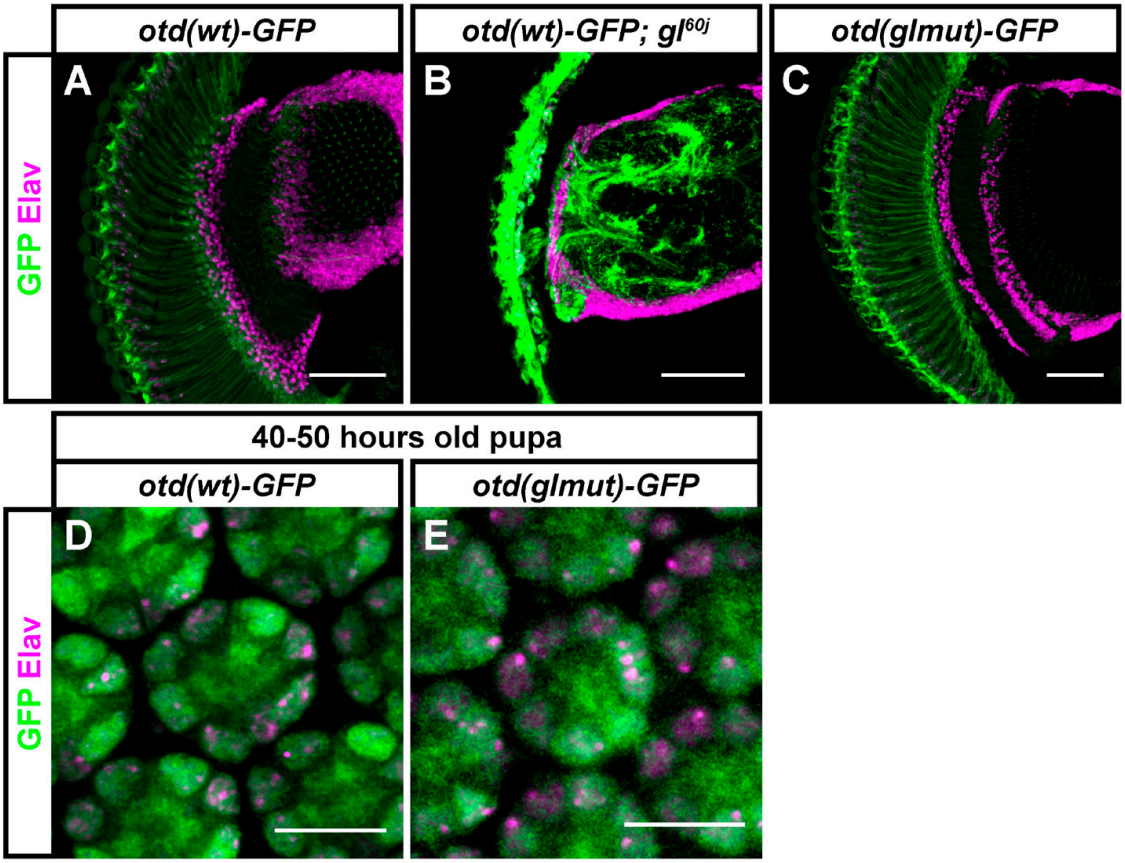

Fig. S7: Expression of the *otd(wt)-GFP* reporter is independent of Glass. Samples were stained against GFP (green) and against the neuronal marker Elav (magenta). (A–C) In adult flies, *otd(wt)-GFP* is expressed both in control (A) and *glass* mutant background (B). This reporter is also expressed when 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: 10 µm in D, E; 50 µm in A–C.

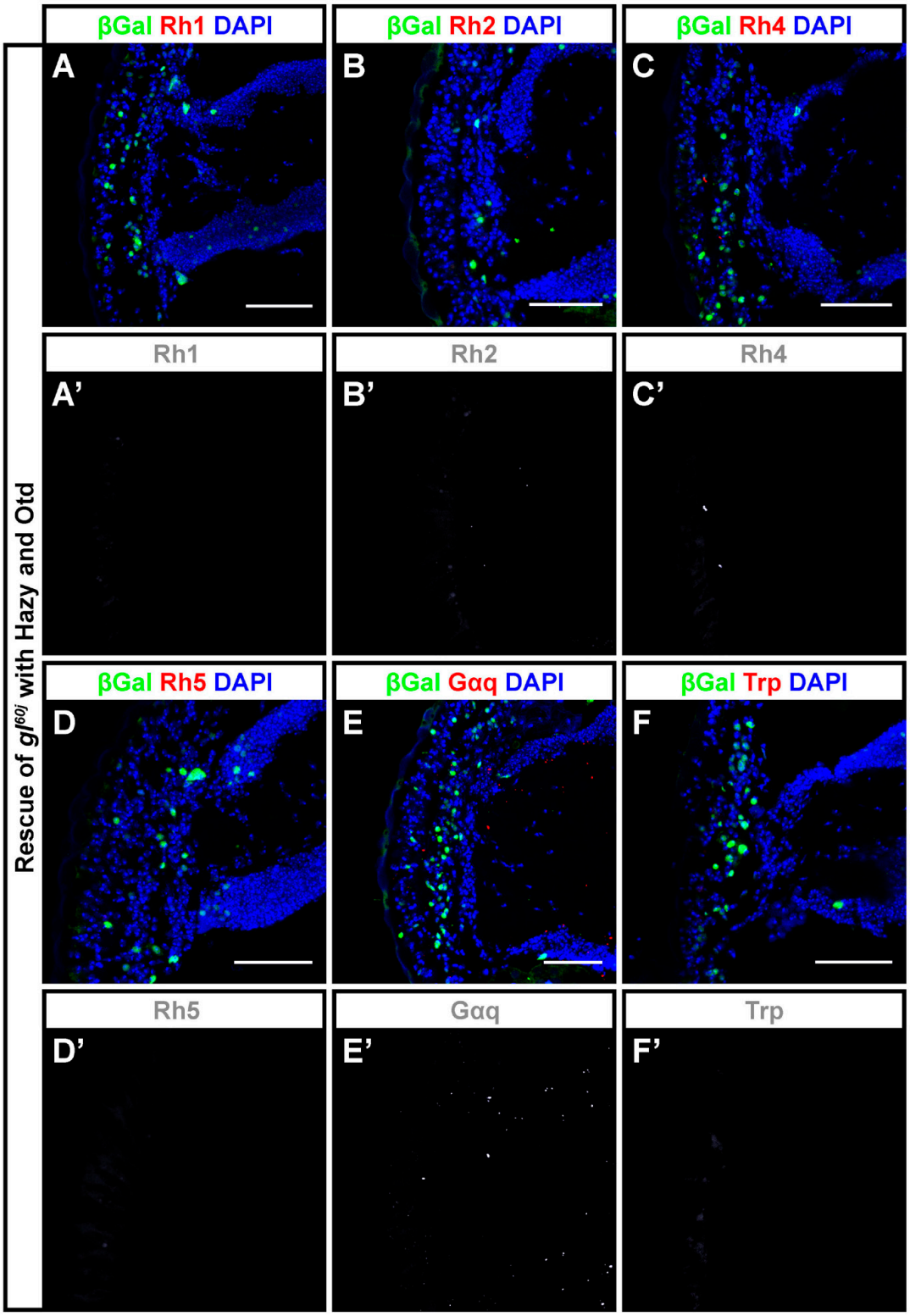

Fig. S8: Induced co-expression of Hazy and Otd does not rescue the *glass* mutant phenotype better than Hazy alone. (A–F') Hazy and Otd were expressed in the *glass* mutant retina in clones labelled with nuclear βGal. Samples were stained against βGal (green), different proteins involved in the phototransduction 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. 2.4), namely the expression of Rh1 (A, A'), Rh2 (B, B'), Rh4 (C, C'), Rh5 (D, D'), Gαq (E, E') and Trp (F, F') was not induced in Hazy–Otd-expressing clones. Scale bars: 40 μm.

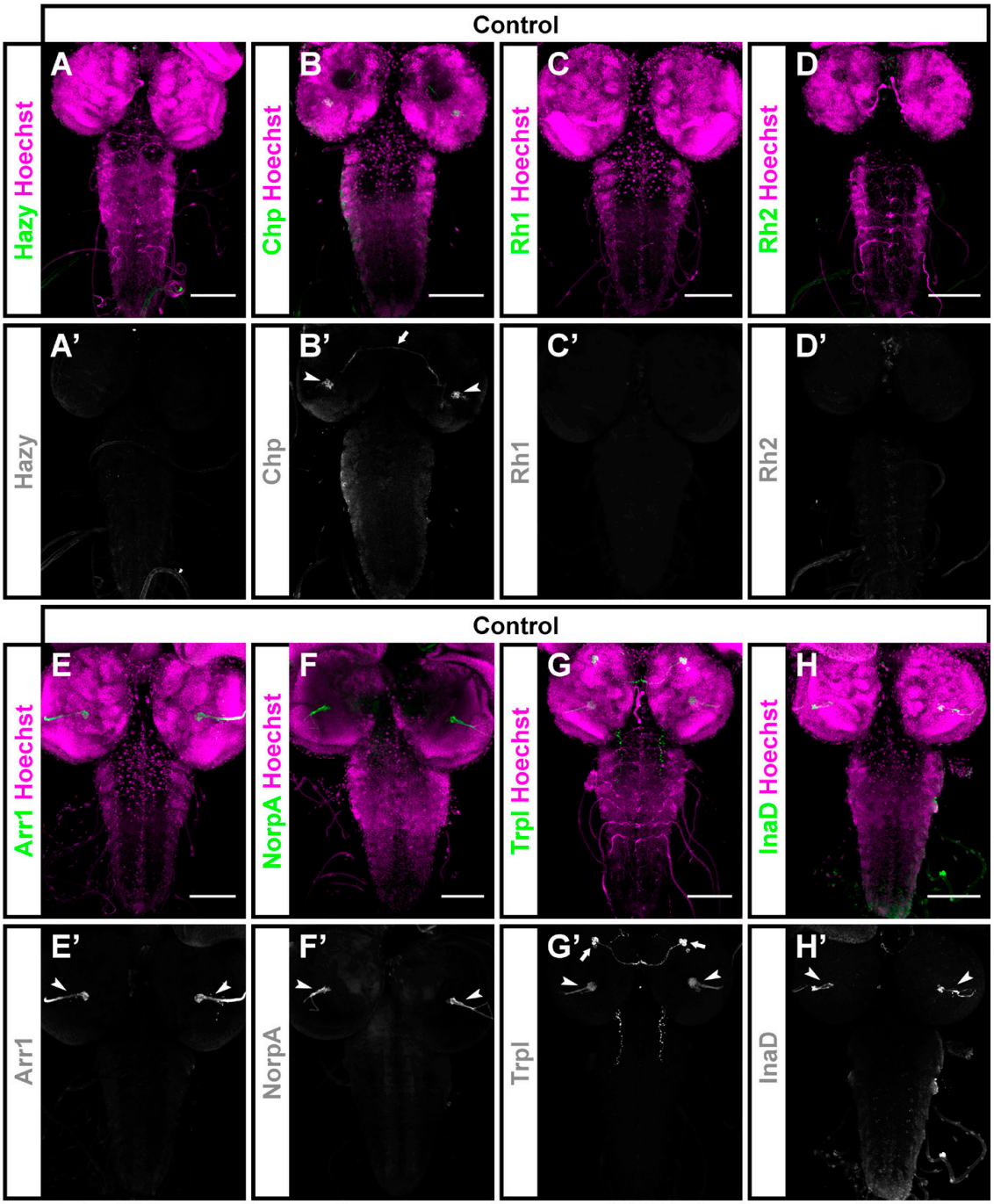

Fig. S9: Expression of PR markers in the CNS of third instar larvae. (A–H') The CNS of control (Canton-S) animals was stained with antibodies against different PR proteins (green) and counterstained with Hoechst (used to label cell nuclei, magenta). For each image, the green channel is shown below in 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'). Trpl 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: 80 µm.

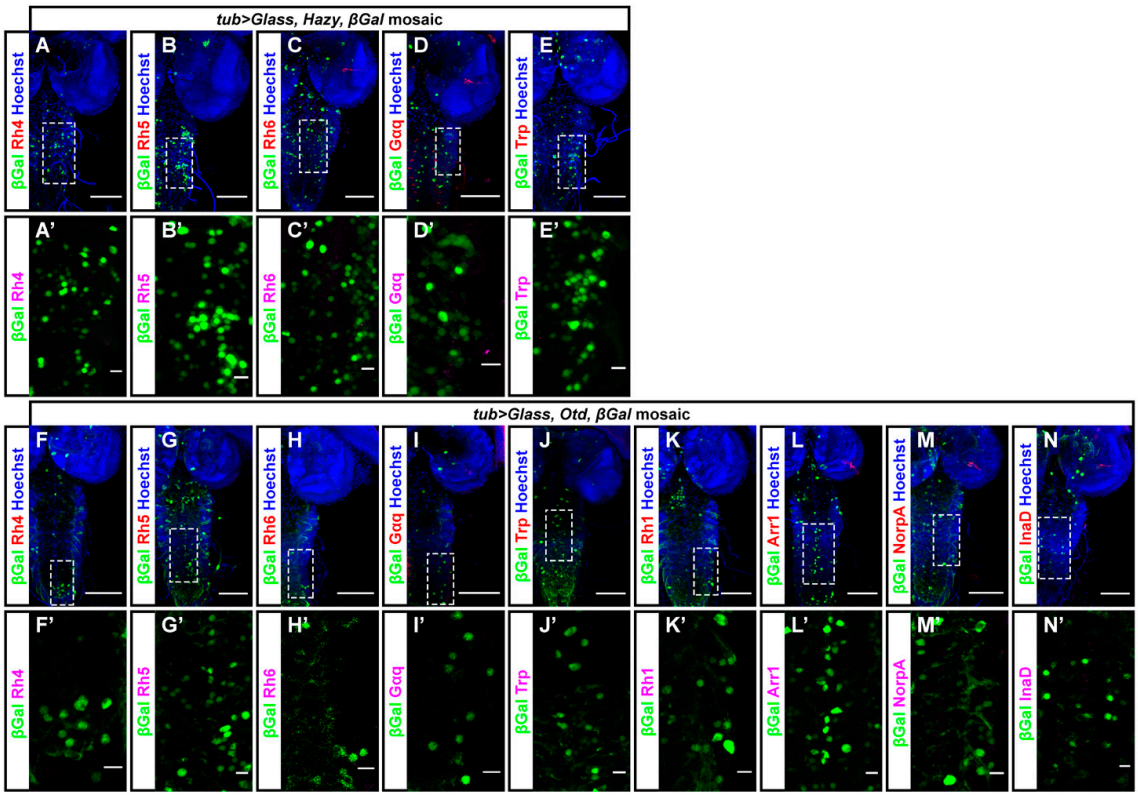

Fig. S10: Co-misexpression of Glass and Hazy is not sufficient to ectopically induce all the phototransduction proteins that we have tested, and co-misexpression of Glass and Otd does not ectopically induce more phototransduction proteins than Glass alone (for a comparison, see Fig. 2.6). Misexpression 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 (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'), Gαq (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'), Gαq (I, I'), Trp (J, J'), Rh1 (K, K'), Arr1 (L, L'), NorpA (M, M') nor InaD (N, N'). Scale bars: 10 μm in A'–N'; 80 μm in A–N.

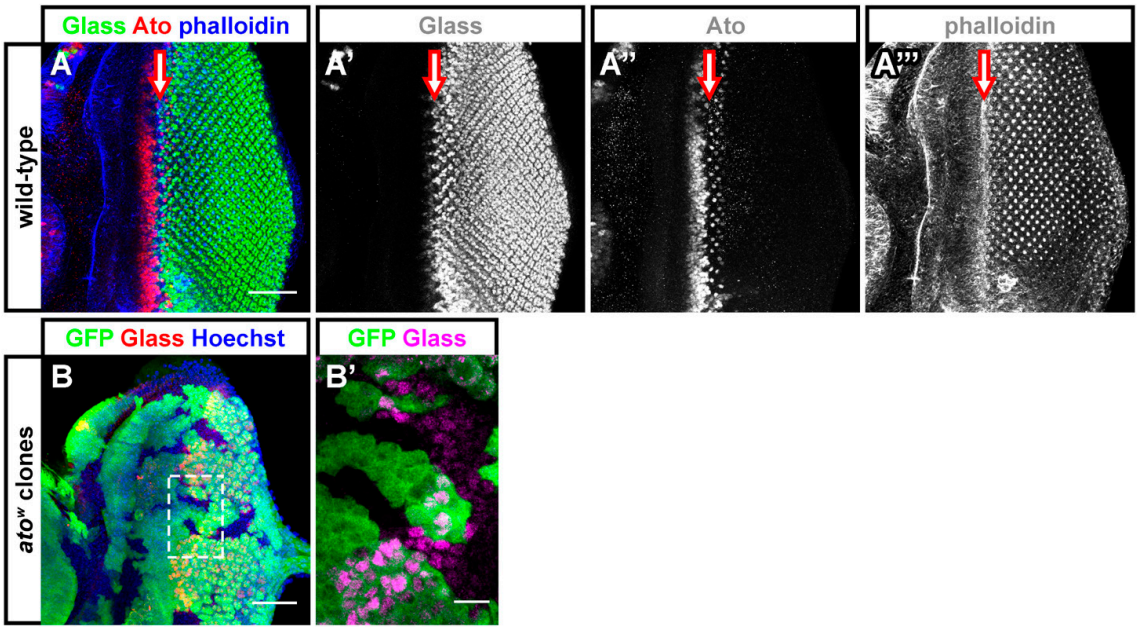

Fig. S11: The proneural transcription factor Ato is not required for *glass* expression. (A) During the development of the third instar eye disc, Ato expression (red) precedes that of Glass (green). Counterstaining with phalloidin (blue) serves to locate the position of the morphogenetic furrow (arrow), 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*^{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 (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: 10 µm in B'; 40 µm in A and B.

3. SUCCESSIVE REQUIREMENT OF GLASS AND HAZY FOR PHOTORECEPTOR SPECIFICATION AND MAINTENANCE IN *DROSOPHILA*

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3.1. Abstract

Development of the insect compound eye requires a highly controlled interplay between transcription factors. However, the genetic mechanisms that link early eye field specification to photoreceptor terminal differentiation and fate maintenance remain largely unknown. Here, we decipher the function of two transcription factors, Glass and Hazy, which play a central role during photoreceptor development. The regulatory interactions between Glass and Hazy suggest that they function together in a coherent feed-forward loop in all types of *Drosophila* photoreceptors. While the *glass* mutant eye lacks the expression of virtually all photoreceptor genes, young *hazy* mutants correctly express most phototransduction genes. Interestingly, the expression of these genes is drastically reduced in old *hazy* mutants. This age-dependent loss of the phototransduction cascade correlates with a loss of phototaxis in old *hazy* mutant flies. We conclude that Glass can either directly or indirectly initiate the expression of most phototransduction proteins in a Hazy-independent manner, and that Hazy is mainly required for the maintenance of functional photoreceptors in adult flies.

3.2. Introduction

Cell differentiation is typically controlled by networks of transcription factors, which gradually shift during development from specifying organ and cellular identity to activating terminal gene expression in mature cells. Such a network acts during the formation of photoreceptors (PRs) in *Drosophila* (Potier et al., 2014; Treisman, 2013; Tsachaki and Sprecher, 2012). During early eye development, an evolutionarily conserved set of transcription factors, called the 'retinal determination network' (RDN), specifies eye field identity in the eye imaginal disc. RDN genes are both necessary and sufficient for eye formation, and thus can induce

the formation of ectopic eyes when misexpressed in other imaginal discs (Cheyette et al., 1994; Halder et al., 1995; Hoge, 1915; Pignoni et al., 1997; Silver and Rebay, 2005).

Recently, we have shown that PR differentiation is critically regulated by the zinc finger transcription factor Glass. Glass provides a genetic link between the early-acting RDN, terminal differentiation transcription factors, and genes functioning in mature PRs, such as those involved in the phototransduction cascade (Bernardo-Garcia et al., 2016). In the absence of Glass, PR precursors retain a neuronal identity but fail to express any PR markers. Therefore, PR precursors require Glass for differentiating into functional light-sensing cells.

A direct target of Glass is the homeobox transcription factor Hazy. Ectopic expression of Glass is sufficient to induce expression of Hazy and some phototransduction proteins. Similarly, ectopic expression of Hazy can only induce a subset of phototransduction components. However, when co-expressed, Glass and Hazy can ectopically induce most of the phototransduction cascade, suggesting that both Glass and Hazy act synergistically during PR development (Bernardo-Garcia et al., 2016). Thus, the combinatorial action of these two transcription factors appears to play a central role in directing PR precursors toward a terminal differentiation program.

Here, we investigate the regulatory interaction between Glass and Hazy. We found that, while Glass is able to activate its own promoter, Hazy does not appear to auto-activate its own expression nor that of Glass. Also, by analysing *hazy* mutants we disentangle the individual roles of Glass and Hazy in regulating the expression of phototransduction genes. Interestingly, we found that Hazy is particularly relevant to ensure the continued expression of phototransduction proteins in adult PRs. Young *hazy* mutants correctly express most of the phototransduction components, and show a similar attraction to white light as wild-type flies. By contrast, the expression of most phototransduction proteins is reduced in old *hazy* mutants, and they fail to show phototaxis. Together with previous results, our data suggest that Glass and Hazy are required for different tasks and at different steps in PR development. During early eye development, Glass contributes to specifying PR identity (Bernardo-Garcia et al., 2016; Liang et al., 2016; Moses et al., 1989; Naval-Sánchez et al., 2013). Later, during terminal differentiation, both Glass and Hazy activate genes that are required for the maturation of functional PRs (Bernardo-Garcia et al., 2016; Liang et al., 2016; Moses et al., 1989; Naval-

Sánchez et al., 2013; Zelhof et al., 2003). Finally, Hazy is required for maintaining the expression of phototransduction genes, and thus ensures the continued functionality of adult PRs.

3.3. Results

3.3.1. *hazy* is a direct target of Glass in all visual organs in the fly

PR development in the eye imaginal disc starts after the passage of the so-called 'morphogenetic furrow' (MF), which sweeps across the disc from the posterior edge toward anterior, initiating the formation of ommatidia. RDN genes are present in the eye disc prior to the MF, while the proneural gene *atonal* is transiently expressed at the MF (Treisman, 2013; Tsachaki and Sprecher, 2012). Glass expression is initiated at the MF, and maintained in differentiating cells in the retina (Ellis et al., 1993; Moses and Rubin, 1991). In contrast, the expression of Hazy starts later during pupation, after all PRs have been specified (Potier et al., 2014; Zelhof et al., 2003). We and others have shown that the expression of a *hazy(wt)-GFP* reporter in compound eye PRs depends on Glass binding to two sites in the *hazy* promoter (Bernardo-Garcia et al., 2016; Liang et al., 2016). In addition to the compound eye, flies also have PRs in the ocelli (three separate eyes located at the top of the adult head) and in the larval eye, also termed Bolwig organ. Since both Glass and Hazy also play a role in the development of these organs (Bernardo-Garcia et al., 2016; Mishra et al., 2016; Mishra et al., 2013; Mishra et al., 2010; Moses et al., 1989; Moses and Rubin, 1991; Stark et al., 1989) we hypothesised that Glass might similarly activate *hazy* in PRs outside the compound eye.

The *hazy(wt)-GFP* reporter, which we have previously used to study the expression of *hazy* in the compound eye, was also expressed in PRs of the ocelli and Bolwig organ (Figs. 3.1A, A', D–D'''), reflecting the expression pattern of the endogenous Hazy protein (Mishra et al., 2016; Zelhof et al., 2003). When the *hazy(wt)-GFP* transgene was placed in *glass* mutant background, GFP expression was lost in the ocelli and the Bolwig organ (Figs. 3.1B, E–E'''). The wild-type embryonic Bolwig organ consists of 12 PRs, and can be identified in stage 14 embryos because of the co-expression of Kruppel and Fasciclin 2 (Fas2) (Mishra et al., 2016; Schmucker et al., 1992). Interestingly, in *glass* mutants we only found 4 Kruppel-positive cells in the Bolwig organ (Fig. 3.1E'''), indicating an early defect in larval eye formation. In addition, a *hazy(gl1,2mut)-GFP* reporter in which the Glass binding sites were mutated was not expressed in the ocelli nor in the Bolwig organ (Figs. 3.1C, F–F''').

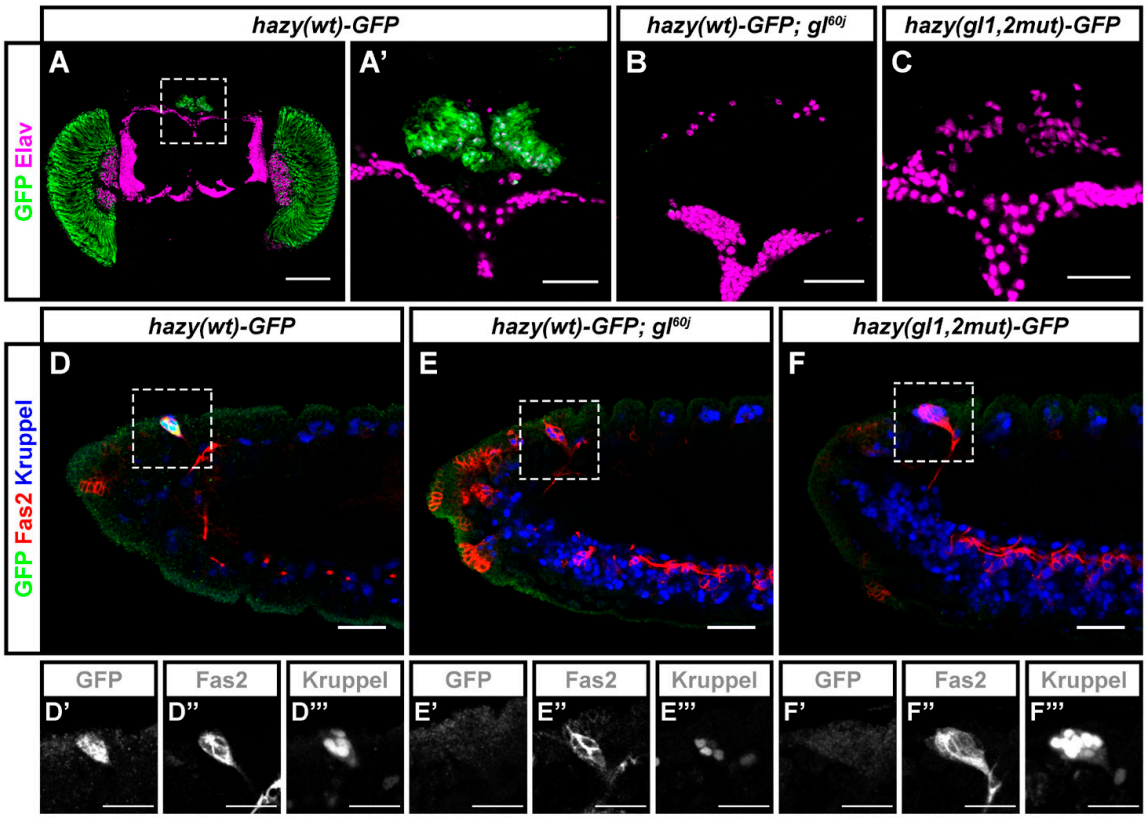


Fig. 3.1: Expression analysis of the *hazy(wt)-GFP* reporter in the ocelli and Bolwig organ PRs. (A–C) In the case of the ocelli, these are 3 visual organs located dorsally on the head of adult flies (A). Samples were stained with antibodies against GFP (green), and Elav (used as a neuronal marker, magenta). The *hazy(wt)-GFP* reporter was expressed in the ocelli in wild-type (A, A'), but not *glass* mutant background (B). A *hazy(gl1,2mut)-GFP* reporter in which the 2 Glass binding sites were mutated was not expressed in the ocelli (C). (D–F) In the case of the Bolwig organ, this is a larval eye that develops from the optic placode during embryogenesis. Stage 14 embryos were stained with antibodies against GFP (green), Fas2 (red) and Kruppel (blue). At this stage, the developing Bolwig organ is located dorsally, still in contact with the surface, and can be identified both because of its position and the co-expression of Fas2 and Kruppel (Campos-Ortega and Hartenstein, 1985; Mishra et al., 2016; Schmucker et al., 1992). Similar to the ocelli, the *hazy(wt)-GFP* reporter was expressed in the Bolwig organ in wild-type (D), but not *glass* mutant background (E). Also, *hazy(gl1,2mut)-GFP* was not expressed in the Bolwig organ (F). For each image, the 3 channels from a close-up of the Bolwig organ were separated and are shown below in greyscale (D'–F'''). Scale bars: 20 μm in D'–F'''; 30 μm in A', B–F; and 100 μm in A.

Together, these results corroborate our findings for the compound eye (Bernardo-Garcia et al., 2016). We conclude that the *hazy* promoter is directly bound and activated by Glass in all PRs in the three visual organs of *Drosophila*.

3.3.2. Glass can auto-activate its own expression

We have previously shown that ectopically expressing Glass or Hazy induces the expression of some phototransduction proteins in the central nervous system (CNS). Co-expressing Glass and Hazy displays a synergistic effect on the induction

of phototransduction components. Not only the genes that are activated by either Glass or Hazy alone become ectopically expressed, but also additional phototransduction proteins are induced, suggesting that Glass and Hazy function together in a coherent feed-forward loop. Glass activates the expression of Hazy and together they are able to activate the expression of more target genes than either Glass or Hazy could activate on their own (Bernardo-Garcia et al., 2016). Here we tested additional regulatory interactions between Glass and Hazy.

For this, we used a *glass-DsRed* and a *hazy(wt)-GFP* reporter (Bernardo-Garcia et al., 2016; Park et al., 2011). *glass-DsRed* larvae expressed nuclear DsRed in the Bolwig organ and in Glass-expressing cells in the brain (Figs. 3.2A–A'''), while *hazy(wt)-GFP* was expressed exclusively in the Bolwig organ PRs (Fig. 3.1D, Fig. 3.2B), whose axons could be seen projecting into the brain (Fig. 3.2B'). Thus, both reporters faithfully reflect the expression patterns of Glass and Hazy (Moses and Rubin, 1991; Zelhof et al., 2003). We performed flip-out experiments in which we

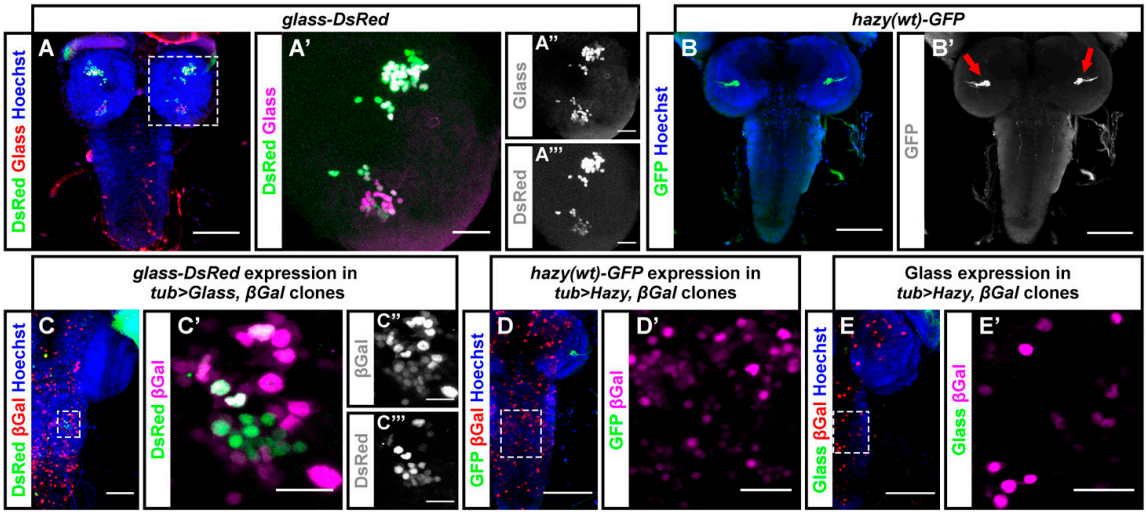


Fig. 3.2: Test for additional regulatory interactions between Glass and Hazy. (A, B) We used Hoechst (blue), which labels cell nuclei, as a counterstain to analyse the expression pattern of the *glass-DsRed* and *hazy(wt)-GFP* reporters in the CNS of third instar larvae. The *glass-DsRed* reporter was expressed the nuclei of some cells in the brain (green, A). A close-up to the right shows that those neurons endogenously expressing Glass (red/magenta) also co-express the reporter (green, A'). These two channels are shown separately to the right in greyscale (A'', A'''). The *hazy(wt)-GFP* reporter is exclusively expressed in PRs (green, B). A greyscale image to the right shows GFP labelling the axonal projections of the PRs in the brain (arrows, B'). (C–E) In flip-out experiments we ectopically induced either Glass or Hazy expression in clones labelled with nuclear β-galactosidase (βGal). We stained the CNS of third instar larvae with antibodies against βGal (red/magenta); either DsRed, GFP or Glass (green) and with Hoechst (blue). We found that Glass ectopically induced the expression of the *glass-DsRed* reporter in the ventral nerve cord (C, C'; channels are also shown separately in greyscale in C'', C'''). By contrast, Hazy did not ectopically induce the *hazy(wt)-GFP* reporter (D, D') nor Glass (E, E'). Scale bars: 20 µm in A', C'–E'; 80 µm in A–E.

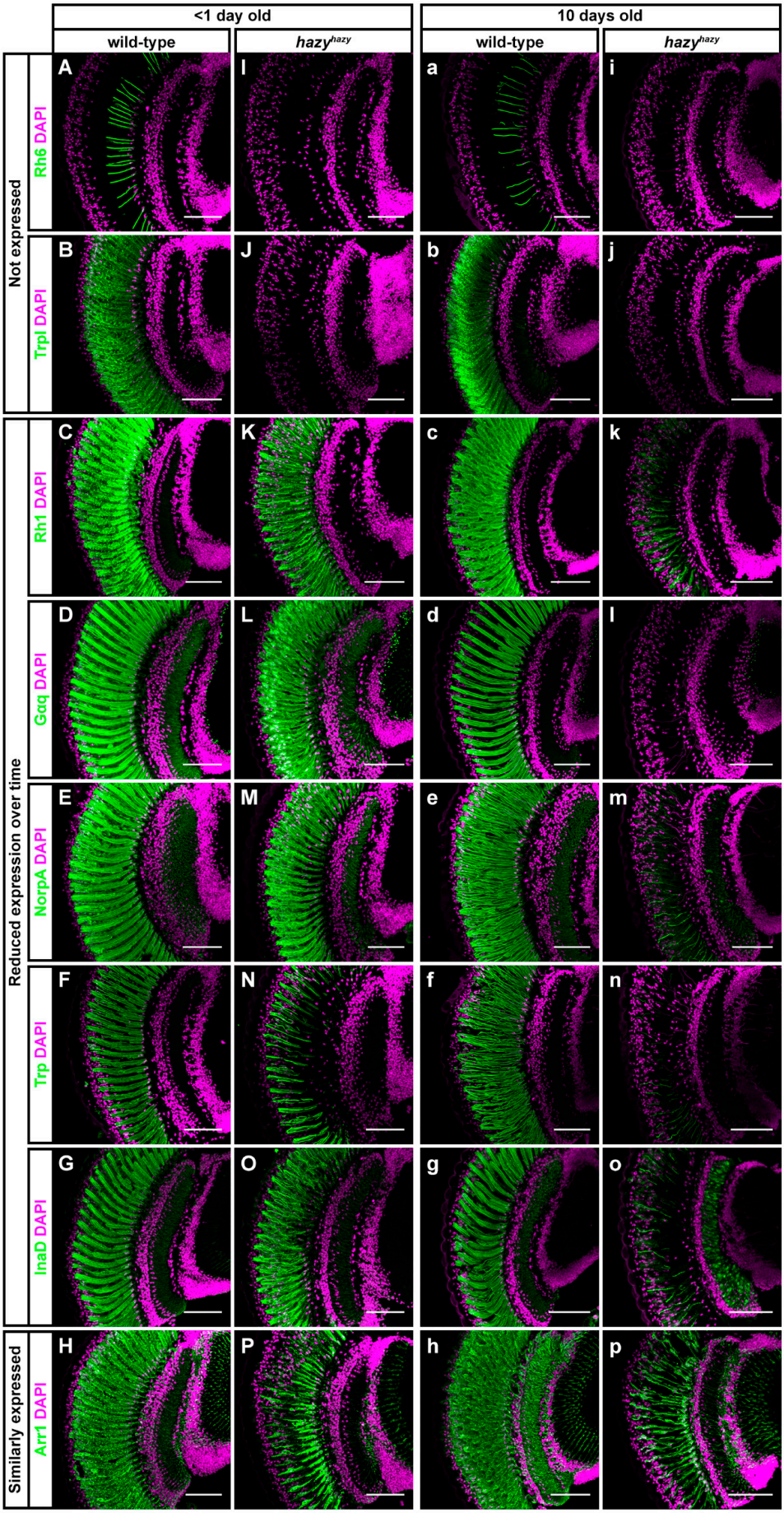
ectopically induced either Glass or Hazy in the CNS of third instar larvae, and found that Glass was able to activate the *glass-DsRed* reporter in a subset of cells (Figs. 3.2C–C'''). Therefore, Glass may be able to maintain its own expression by auto-regulation. In contrast, ectopic expression of Hazy did not induce the expression of the *hazy(wt)-GFP* reporter (Figs. 3.2D, D'), suggesting that Hazy cannot activate its own expression. Similarly, we did not observe ectopic expression of Glass in the CNS after ectopically expressing Hazy (Figs. 3.2E, E'), suggesting that Glass expression is not activated by Hazy.

3.3.3. Glass can initiate the expression of most phototransduction proteins independently of Hazy

Both Glass and Hazy are required for the expression of PR genes (Bernardo-Garcia et al., 2016; Zelhof et al., 2003). Since we have shown that Glass directly activates *hazy*, and that inducing the expression of Hazy partly rescues the *glass* mutant phenotype (Bernardo-Garcia et al., 2016), it would be possible that Glass mainly relies on Hazy for activating the expression of phototransduction proteins. To test this, we examined the individual role of Hazy.

We found that young *hazy* mutant flies – less than one day old – failed to express Rhodopsin 6 (Rh6) and Transient receptor potential-like (Trpl), but did express correctly most of the phototransduction proteins that we tested: Rhodopsin 1 (Rh1), G protein α q subunit (Gαq), No receptor potential A (NorpA), Transient receptor potential (Trp), Inactivation no afterpotential D (InaD) and Arrestin 1 (Arr1) (Figs. 3.3A–P) (Chou et al., 1999; de Couet and Tanimura, 1987; Dolph et al., 1993; Lee et al., 1994; Montell, 2012; Niemeyer et al., 1996; Shieh and Niemeyer, 1995; Wong et al., 1989; Zhu et al., 1993). Of these, after 10 days the levels of Rh1, Gαq, NorpA, Trp and InaD were strongly reduced, and only Arr1 expression appeared unchanged (Figs. 3.3a–p). Thus, our results indicate that Hazy is not required for initiating the expression of most phototransduction proteins, but that it plays an important role in maintaining the differentiated state of PRs. These results contrast with an earlier description of the *hazy* mutant phenotype (Zelhof et al., 2003). The differences between our findings and this report may be explained because we analysed young and old flies separately.

Since most phototransduction proteins are expressed in the retina of young *hazy* mutants, we infer that Glass does not mainly act via Hazy for initiating the


<1 day old
10 days old
wild-type
hazy^hazy
wild-type
hazy^hazy
Not expressed
Rh6 DAPI
Trpl DAPI
Reduced expression over time
Rh1 DAPI
Gαq DAPI
NorpA DAPI
Trp DAPI
InaD DAPI
Similarly expressed
Arr1 DAPI
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a
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Fig. 3.3: Expression of phototransduction proteins in the *hazy* mutant retina. Head sections were taken of control and *hazy*^{hazy} flies, and stained with antibodies against different phototransduction proteins (green) and with Hoechst (used to label cell nuclei, magenta). (A–P) One group of flies was dissected on the day they eclosed. At this age we did not detect neither Rh6 (I) nor Trpl (J) in the retina of *hazy* mutants, but most of the phototransduction proteins that we tested were correctly expressed: Rh1 (K), Gαq (L), NorpA (M), Trp (N), InaD (O) and Arr1 (P). (a–p) A second group of flies were dissected 10 days after eclosion. Neither Rh6 (i) nor Trpl (j) were expressed in the retina of these older *hazy* mutants, and most phototransduction proteins showed decreased expression levels: Rh1 (k), Gαq (l), NorpA (m), Trp (n) and InaD (o). Only Arr1 (p) expression did not seem reduced over time in the *hazy* mutant retina. Scale bars: 50 μm.

expression of most phototransduction proteins. In the case of Rh6, Trpl, Rh1, Gαq, NorpA, Trp, InaD and Arr1, we found that all these genes contain putative Glass binding sites in their regulatory sequences (the GAARCC motif, which is present in either their promoter or their introns) (Enuameh et al., 2013). Therefore, it would be possible that Glass either directly or indirectly activates the expression of these phototransduction components.

3.3.4. Hazy is not required for white light detection in young flies

To further assess the impact of their age-dependent loss of phototransduction genes we analysed the phototactic behaviour of young versus old *hazy* mutant flies. It has been previously reported that *hazy* mutants fail to detect light due to the absence of many phototransduction proteins (Mishra et al., 2010; Zelhof et al., 2003). However, our finding suggests that young *hazy* mutant flies express a set of genes sufficient for the phototransduction machinery to detect white light.

Adult wild-type flies display a positive phototactic behaviour. In a two-choice assay, they move toward the light. This preference for light is very high in newly eclosed flies and shows some reduction when the flies get older (Le Bourg and Badia, 1995). 10 day old flies are still able to distinguish between light and darkness (Fig. 3.4). In contrast, *glass* mutant flies do not show phototaxis from the day they eclose (Fig. 3.4) (Pak et al., 1969). This is in agreement with our previous finding that *glass* mutants do not express any of the proteins in the phototransduction cascade (Bernardo-Garcia et al., 2016). In the case of *hazy* mutants, we observed normal phototactic behaviour in young flies, comparable with that of wild-type. However, at the age of 10 days they differed from wild-type and displayed the same disability to distinguish between light and darkness as *glass* mutants (Fig. 3.4). These experiments are in agreement with our antibody analysis above, and

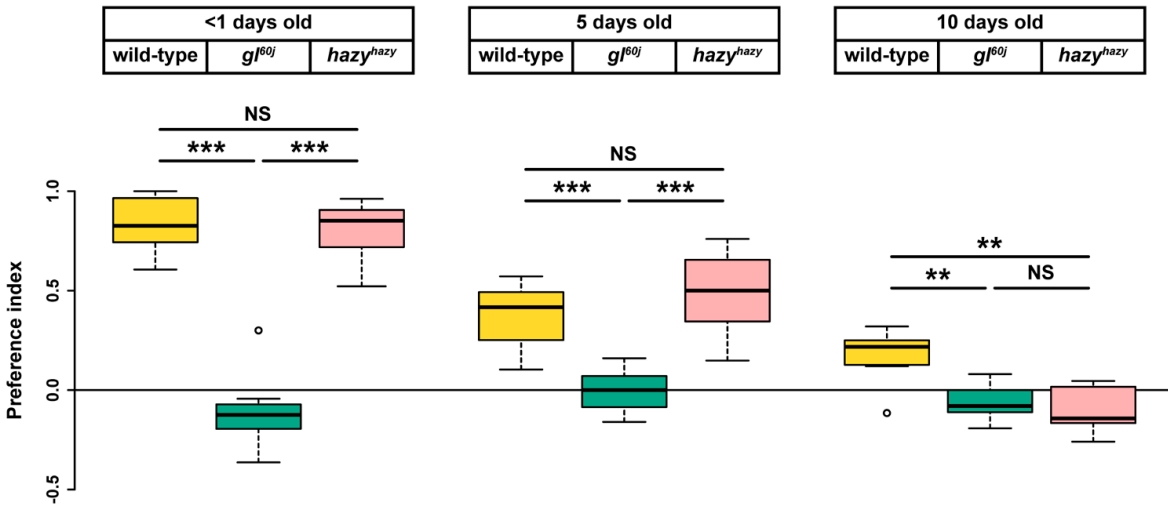

Fig. 3.4: Age-related changes in the phototaxis of wild-type, *glass* and *hazy* mutant flies. Box plots show the light preference indices (PIs) of wild-type (yellow), *glass* (cyan) and *hazy* mutants (pink) of different ages. Bold lines represent medians. The upper and lower quartiles are represented by the top and the bottom of each box. Whisker lines indicate the maximum and minimum data points that are closer than 1.5 interquartile range of their nearest quartiles. Circles indicate outliers. We used Welch's *t*-test for comparing the PIs between groups (n = 7 per age and genotype) and to zero. Significance levels represent $p > 0.05$ (not significant, NS), $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). In a 2-choice assay, groups of wild-type flies of every age showed positive phototaxis, which decreases with age (indicated by positive PI values, which were significantly different from zero). *glass* mutants were photoneutral at all ages (their PIs were not significantly different from zero). Newly eclosed *hazy* mutants showed positive phototaxis, not different from that of wild-type flies ($p = 0.67$, median wild-type PI = 0.83). Five day old *hazy* mutants and wild-type flies show a decreased positive phototaxis, but their PIs are not different from each other ($p = 0.30$, median wild-type PI = 0.42). Ten day old *hazy* mutants were photoneutral, with their PIs comparable to zero ($p = 0.08$) or to *glass* mutants ($p = 0.56$), and significantly different from wild-type (median wild-type PI = 0.22).

show that young *hazy* mutants are able to detect white light, but lose this ability over time. Thus, Hazy is required for the maintenance of PR functionality.

3.4. Discussion

Glass and Hazy play important roles in PR specification and maintenance (Bernardo-Garcia et al., 2016; Moses et al., 1989; Rister et al., 2015; Zelhof et al., 2003). Here we have shown that Glass activates the expression of *hazy* in all PRs in the three visual organs of *Drosophila* – the compound eye, the ocelli and the Bolwig organ – by binding to the same sites in the *hazy* promoter. Also, in agreement with a previous report, Glass is able to auto-activate its own expression (Moses and Rubin, 1991), but we found no evidence that Hazy either activates *glass* or auto-activates its own expression. Together, our data favour a feed-forward mechanism in which Hazy acts downstream of Glass (Alon, 2007; Bernardo-Garcia et al., 2016).

The notion that Glass and Hazy function through a coherent feed-forward loop to activate PR genes is further supported by our previous findings that inducing the expression of Hazy partly rescues the *glass* mutant phenotype, and also that co-expressing Glass and Hazy together ectopically induces more phototransduction proteins than either Glass or Hazy alone (Bernardo-Garcia et al., 2016). However, these findings were based on ectopic expression of both transcription factors in CNS cells in which they are normally not expressed and where we were not able to control their expression levels. Our analysis of the *hazy* mutant phenotype rather suggests that, by the end of PR terminal differentiation, Glass can induce the expression of most phototransduction components even in the absence of Hazy. Hazy itself is one of the targets of Glass and is required to maintain the expression of most phototransduction components throughout adult life. Thus, for the initial induction, Glass either directly activates PR-specific genes, or it interacts with other transcription factors to induce them. Hazy might be required for the initial induction of only a few genes, such as Rh6 or Trpl, but it is essential for the maintenance of most phototransduction components. Therefore, we anticipate that further research on the direct targets of Glass will reveal novel mechanisms for activating the expression of PR genes. Also, it will be interesting to explore further how Hazy functions in PR fate maintenance. Particularly, it remains unresolved whether Hazy is only required for maintaining PR gene expression or if it also functions to suppress PR dedifferentiation or degeneration.

3.5. Materials and methods

3.5.1. Fly stocks

In the present work w^{1118} (courtesy of R. Stocker) was used as wild-type control, and w^{1118}; $hazy^{hazy}$ was used to study the *hazy* mutant phenotype. The $hazy^{hazy}$ mutant allele was provided by C. Desplan (Zelhof et al., 2003), and was isogenised by crossing it to Canton-S flies for 6 generations. Other stocks that we used are: *hazy(wt)-GFP* (Bernardo-Garcia et al., 2016), *hazy(gl1,2mut)-GFP* (Bernardo-Garcia et al., 2016), gl^{60j} (Bloomington Stock Center, no. 509) (Moses et al., 1989), *UAS-glass* (Bernardo-Garcia et al., 2016) and *UAS-hazy* (courtesy of J. Bischof) (Bischof et al., 2013). As a reporter for *glass* we used *glass-DsRed* flies (courtesy of S. Kim), which are also called *glass5.2-RHS* (Park et al., 2011). Flip-out misexpression experiments were performed as described previously (Bernardo-

Garcia et al., 2016) by using *hsFLP; tub(FRTcassette)Gal4, UAS-lacZ.nls* (courtesy of E. Piddini).

Flies were raised at 25 °C in a 12:12 hour light–dark cycle on cornmeal medium supplemented with molasses, fructose and yeast.

3.5.2. Antibody stainings

Immunohistochemistry was performed as previously described (Bernardo-Garcia et al., 2016; Mishra et al., 2016; Rothwell and Sullivan, 2000). Antibodies against proteins in the phototransduction cascade were kindly provided by N. Colley and S. Britt. To compare the expression of phototransduction proteins in control and *hazy* mutants, head sections from both genotypes were taken simultaneously, stained together on the same slide, and imaged with identical settings on a Leica SP5 confocal microscope.

3.5.3. Phototaxis assay

Our phototaxis analysis was based on a previous protocol (Le Bourg and Badia, 1995). Briefly, newly eclosed flies were transferred each day to vials containing fresh food, which we used to stage them. For each experiment, we tested an average of 25.2 flies (ranging from 20 to 33). These flies were kept for 10 minutes in darkness, and then placed without anaesthesia into a T-maze under red light conditions. Our set-up consisted of two tubes connected to each other, where a single LED illuminated from the end of one of the tubes with white light (SOLAROX, Germany, no. 50008300001). Light intensity was moderate: we measured 418.0739 $\mu W/cm^2$ with a spectrometer (the emitted light spectrum possessed two intensity peaks: the first peak was at 444 nm with an intensity of 1.494 $\mu W/cm^2/nm$ and half-widths of 16 nm, and the second peak was at 585 nm with an intensity of 2.768 $\mu W/cm^2/nm$ and half-widths of 61.5 nm). We allowed the flies to move freely between both tubes for 2 minutes. Then, we counted the flies in the illuminated tube (L), in the dark tube (D), and those in the intersection between the two tubes (M). The preference index (PI) was calculated from the formula $PI = (L - D)/(D + L + M)$. Data were analysed in R with Welch's *t*-test.

3.6. Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

3.7. Acknowledgments

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4. A DISSIMILAR TRANSCRIPTIONAL PATHWAY REGULATES RHABDOMERIC PHOTORECEPTOR DIFFERENTIATION IN *DROSOPHILA* AND *PLATYNEREIS*

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This chapter is currently being adapted for publication in a scientific journal

4.1. Abstract

Across metazoans, visual systems employ different types of photoreceptor neurons (PRs) to detect light. These include rhabdomeric PRs, which exist in distantly related phyla and have diverse functions (from image-formation in insects to irradiance detection in vertebrates). However, very little is known about how rhabdomeric PRs develop in most animals. To investigate this question, we tested whether the transcription factor Glass, which is crucial for the differentiation of rhabdomeric PRs in *Drosophila*, has a similar role in other species. We were able to identify multiple, widely distributed Glass homologues, indicating that this protein appeared early during the evolution of metazoans. However, we could not find it in vertebrates. Also, very unexpectedly, we detected *glass* expression in a subset of neurons in the head of the marine ragworm *Platynereis*, but not in rhabdomeric PRs. We conclude that rhabdomeric PRs develop both through Glass-dependent and Glass-independent mechanisms in different species.

4.2. Introduction

Most animals are able to sense visual cues, which provide them with detailed information about their environment. This information may include the shape of nearby objects, colours, movements, the day–night cycle... and it is relevant for surviving. As a consequence, animals have evolved various types of photoreceptor neurons (PRs) such as ciliary and rhabdomeric PRs (Fain et al., 2010; Nilsson, 2005), which have different functions in different species. In the case of rhabdomeric PRs these cells are important, for example, for image-forming vision (e.g. in *Drosophila*, due to compound eye PRs), for identifying the direction of a light source (e.g. in the marine ragworm *Platynereis*), or for sensing irradiance

(e.g. in vertebrates, owing to the 'intrinsically photosensitive retinal ganglion cells', ipRGCs) (Borst, 2009; Hankins and Hughes, 2014; Randel et al., 2014). However, we know little of how rhabdomeric PRs form in most metazoans.

Interestingly, all known rhabdomeric PRs appear to use a similar assortment of phototransduction proteins. These cells possess rhabdomeric opsins that can modify their spatial conformation upon light stimulation, which allows them to activate Gαq. Then, Gαq signals through phospholipase C (PLC), causing the opening of cation channels on the cytoplasmic membrane of PRs, and thus leads to the formation of action potentials. This light-sensing machinery is present in distantly related animal phyla (Fain et al., 2010; Montell, 2012; Provencio and Warthen, 2012), which poses the question of to what degree the development of rhabdomeric PRs is evolutionarily conserved. Is the acquisition of the rhabdomeric phototransduction cascade regulated by a similar set of transcription factors across metazoans? Recently, we have shown that the zinc finger transcription factor Glass is essential for activating the expression of virtually all phototransduction proteins in *Drosophila* (Bernardo-Garcia et al., 2016), where it acts in all types of rhabdomeric PRs (including those in the Bolwig organ, the ocelli, and the compound eye) (Bernardo-Garcia et al., 2017; Ellis et al., 1993; Moses et al., 1989). For this reason, here we have investigated whether Glass also controls rhabdomeric PR differentiation outside *Drosophila*.

Unexpectedly, our findings indicate that Glass is not expressed in *Platynereis* rhabdomeric PRs, and it does not seem to have a homologue in vertebrates. This indicates that metazoans must have evolved at least two different transcriptional pathways leading to the differentiation of rhabdomeric PRs: one of them strongly requires Glass (e.g. in *Drosophila*) while the other is a Glass-independent process (e.g. in *Platynereis*).

4.3. Results

4.3.1. Most metazoans possess clear Glass homologues

Glass plays a fundamental role for the differentiation of rhabdomeric PRs in fruit flies (Bernardo-Garcia et al., 2016; Liang et al., 2016; Moses et al., 1989). To investigate if it has a similar function in other animals, we decided to search for Glass-like sequences outside *Drosophila* by using BLAST.

Intriguingly, we did not find any protein clearly similar to Glass in choanoflagellates, nor in vertebrates. However, we were able to retrieve multiple NCBI-deposited sequences belonging to clear Glass homologues (https://blast.ncbi.nlm.nih.gov/). These were distributed across distantly related genera, including *Amphimedon* (Porifera), *Nematostella* (Cnidaria), *Aplysia* (Mollusca), *Caenorhabditis* (Nematoda), *Strongylocentrotus* (Echinodermata) and *Branchiostoma* (Cephalochordata). Also, we found one single Glass homologue in the marine ragworm *Platynereis* (Annelida) (http://jekely-lab.tuebingen.mpg.de/blast/) (Fig. 4.1, Table 4.1). Therefore, based on sequence alignment, Glass is broadly distributed across metazoans.

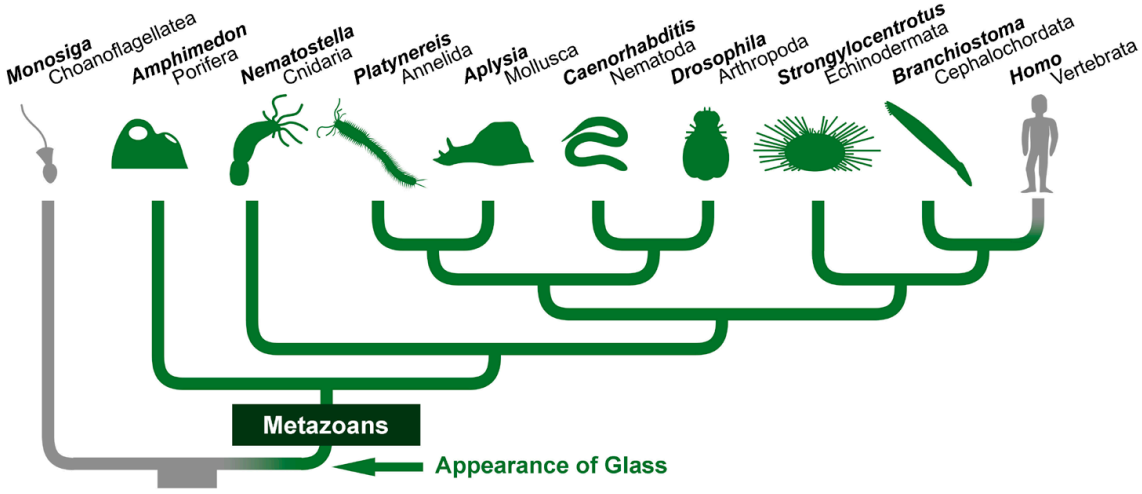
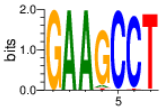
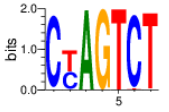
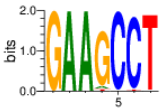
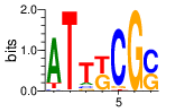
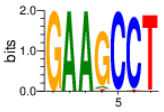
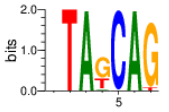
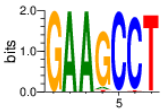
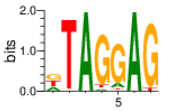
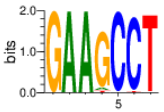
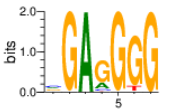
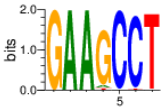
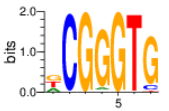
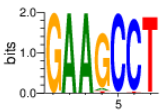
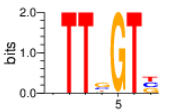
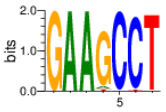
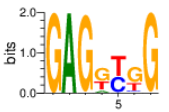


Fig. 4.1: Glass homologues exist in most animal groups. Based on sequence comparison (see Table 4.1), we infer that *glass* appeared in the common ancestor of all metazoans, and that this gene has been transmitted to most present-day animals (shown in green on the phylogenetic tree). However, we were not able to identify it in vertebrates.

4.3.2. Neither vertebrates nor choanoflagellates have clear Glass homologues

Given that Glass exists in most animals, we were curious about why we could not find a vertebrate homologue for this protein. Some species have fully sequenced, well annotated genomes, like zebrafish, mice, or humans (http://www.ensembl.org/) (Howe et al., 2013; Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002). Therefore, we decided to further investigate the evolutionary conservation of Glass by scrutinising its sequence.

Glass homologues share a characteristic cluster of five Cys_2His_2 zinc fingers in different species (except in *Caenorhabditis*, which only has four zinc fingers

Glass homologues		Glass-like proteins (vertebrates and choanoflagellates)	
>*Amphimedon*, https://blast.ncbi.nlm.nih.gov/ XP_019864430.1 CDYCGKCFADTSTLTKHKRIHSGEKPYRCKICNLGFSQSGNLHRHMKTH		>Human, https://blast.ncbi.nlm.nih.gov/ XP_011525220.1, ZSCAN22 CDACGRAFSDCSALIRHLRIHSGEKPYQCKVCPKAFAQSSSLIEHQRIH	
>*Nematostella*, https://blast.ncbi.nlm.nih.gov/ XP_001639755.1 CKVCNKAFADSSTLTKHVRTHTGEKPYQCEICHQRFSQSGNMNRHKRIH		>Human, https://blast.ncbi.nlm.nih.gov/ EAW84863.1, ZNF253 CEECGKAFNRSTDLTTHKIVHTGEKPYKCEECGKAFNQPSTLSKFESLY	
>*Platynereis*, http://jekely-lab.tuebingen.mpg.de/blast/ comp416373_c0_seq8 CRMCKKAFSDSSTLTKHLRIHSGEKPYQCKICLLRFSQSGNLNRHMRVH		>Human, https://blast.ncbi.nlm.nih.gov/ CAA36583.1, KOX 26 CKECGEAFSQSSTLTKHIKVYTGEKPYTCKDCRKAFSQSSSLTQHQRVH	
>*Aplysia*, https://blast.ncbi.nlm.nih.gov/ XP_005091155.1 CRMCKKAFSDSSTLTKHLRIHSGEKPYQCKICLLRFSQSGNLNRHMRVH		>Human, http://www.uniprot.org/ ZNF764 CPDCGRCFRQSSEMAAHRRTHSGEKPYPCPQCGRRFGQKSAVAKHQWVH	
>*Caenorhabditis*, https://blast.ncbi.nlm.nih.gov/ NP_001076597.1, CHE-1 CAQCEKAFTDSSTLTKHLRTHTGHKPYVCSICMMKFTQSGNLHRHMKTH		>Human, http://www.uniprot.org/ ZNF683 REVCHKRFSSSSNLKTHLRLHSGARPFQCSVCRSRFTQHIHLKLHHRLH	
>*Drosophila*, http://flybase.org/ Glass CSSCKKSFSDSSTLTKHLRIHSGEKPYQCKICLLRFSQSGNLNRHMRVH		>Human, http://www.uniprot.org/ ZNF500 CTQCGKRFNNSSHFSAHRRTHTGEKPYTCPACGRGFRRGTDLHKHQRTH	
>*Strongylocentrotus*, https://blast.ncbi.nlm.nih.gov/ XP_790671.3 CAYCRKAFADSSTLTKHLRIHSGEKPYQCKICLLRFSQSGNLTRHMKVH		>*Salpingoeca*, https://blast.ncbi.nlm.nih.gov/ XP_004993762.1 CSICGKSFITSSHVRMHERTHDDARHFSCPLCSKPFKTKPGLKQHLKTG	
>*Branchiostoma*, https://blast.ncbi.nlm.nih.gov/ XP_019644085.1 CTVCPRAFADASTLTKHVRTHTNEKPYQCSECGQSFTQSGNLKRHLKVH		>*Salpingoeca*, https://blast.ncbi.nlm.nih.gov/ XP_004989424.1 CDQCSFKTAYKHILTTHKRIHTGEKPYKCDQCEYKTAYKRNMPRHKRIH	

⟶ **Table 4.1: Analysis of the Glass zinc fingers**. Generally, Glass homologues possess a cluster of five Cys_2His_2 zinc fingers, each of them containing the following motif: Cys-$X_{2,4}$-Cys-X_{12}-His-$X_{3,4,5}$-His. Of these, we compared the sequences of the 4th and the 5th, which are the main responsible zinc fingers for binding to the DNA (Enuameh et al., 2013; Etchberger et al., 2007; Naval-Sánchez et al., 2013; O'Neill et al., 1995). In the table, those aminoacids that match the Glass consensus sequence (deduced by aligning the homologues of different species, on the first column) appear on black background. We also aligned Glass-like proteins found either in vertebrates (e.g. human) or in choanoflagellates (e.g. *Salpingoeca*) with BLAST (https://blast.ncbi.nlm.nih.gov/) and MUSCLE (Edgar, 2004), but they did not entirely match the Glass consensus sequence (shown on the second column). Importantly, the 3D structure of the DNA-bound Cys_2His_2 domain has been resolved (Pavletich and Pabo, 1991), and, accordingly, it is expected that four aminoacids per zinc finger – located at specific positions – directly recognise three base pairs. These aminoacids are well evolutionarily conserved across different homologues and, in the sequences that we show, they are no. 10 (D), 12 (S), 13 (T), and 16 (K) in the 4th zinc finger, and no. 38 (Q), 40 (G), 41 (N), and 44 (R) in the 5th zinc finger. Other residues and neighbouring zinc fingers are also expected to contribute to the DNA binding specificity of Glass (Garton et al., 2015). Furthermore, specialised software has been developed to predict the binding motif of Cys_2His_2 proteins based on their sequence (http://zf.princeton.edu/) (Persikov and Singh, 2014). This software indicates that all Glass homologues (on the first column) bind to the same DNA motif: GAARCC, which was expected from experimental works in *Drosophila* and *Caenorhabditis* (Etchberger et al., 2007; Naval-Sánchez et al., 2013). By contrast, it predicts that Glass-like proteins of vertebrates and choanoflagellates (on the second column) are not able to recognise this motif. Full details on the proteins that we tested can be found by following the hyperlinks provided (underlined text).

because the first one is missing). This is important for the function of Glass and, particularly, the 4th and the 5th zinc fingers are responsible for guiding this transcription factor towards its targets, given that they recognise the Glass binding motif *in vivo*, GAARCC (Enuameh et al., 2013; Etchberger et al., 2007; Naval-Sánchez et al., 2013; O'Neill et al., 1995). Therefore, we modified our bait by using the consensus sequence of either the full cluster of five zinc fingers, or only the 4th and 5th zinc fingers. Then, we repeated our BLAST search against vertebrates and choanoflagellates (the sister group of metazoans), and obtained results like, for example, ZSCAN22, ZNF253, or KOX 26 in humans, which showed less similarity to Glass than any of the homologues that we have identified in other species (Table 4.1). We also considered the human candidates that appear annotated as putative Glass orthologues in Flybase via DIOPT (http://flybase.org/) (Hu et al., 2011), including ZNF764, ZNF683, or ZNF500, but, likewise, they aligned poorly with the consensus sequence of the Glass zinc fingers (Table 4.1). Moreover, we analysed if any of these proteins would be able to substitute Glass functionally by recognising its binding motif, GAARCC (Enuameh et al., 2013; Etchberger et al., 2007; Naval-Sánchez et al., 2013). For this, we used software that predicts the DNA binding behaviour of zinc finger proteins (http://zf.princeton.edu/) (Persikov and Singh, 2014). This analysis indicated that those Glass-like proteins that exist in vertebrates and choanoflagellates cannot recognise the GAARCC motif, in contrast to the clear Glass homologues that we found in other animals (i.e. in *Amphimedon*,

Nematostella, *Platynereis*, *Aplysia*, *Caenorhabditis*, *Strongylocentrotus* and *Branchiostoma*) (Table 4.1).

As a consequence, it remains unclear what happened to Glass during the evolution of vertebrates. It could be that Glass changed its DNA binding motif, or that it was lost. Nevertheless, similar to *Drosophila*, vertebrates also have rhabdomeric PRs in their retina – called ipRGCs – and, therefore, we conclude that these cells must develop through different mechanisms in *Drosophila* and in vertebrates.

4.3.3. Glass is not expressed in *Platynereis* rhabdomeric PRs

We next tested whether one of our newly identified Glass homologues is present in rhabdomeric PRs. For this we used *Platynereis*, which is an emergent model organism that presents some advantages: first, we only found one Glass homologue in *Platynereis*. Second, its visual system has been well studied, both from a molecular and a functional point of view. *Platynereis* possesses two types of bilateral eyes containing rhabdomeric PRs, called the dorsal and ventral eyes (also known as adult and larval eyes, respectively). These two eye types are able to detect the direction of light, thus mediating phototaxis (Arendt et al., 2004; Backfisch et al., 2013; Jékely et al., 2008; Randel et al., 2014; Randel et al., 2013).

glass is expressed in all rhabdomeric PRs in the three visual organs of *Drosophila*, that is: in the Bolwig organ, the ocelli, and the compound eye (Ellis et al., 1993; Moses and Rubin, 1991). As an example, here we show that it is possible to detect *glass* expression in the compound eye of adult flies both with *in situ* hybridisation and with a *glass-Gal4* line crossed to *UAS-mCD8::RFP* (Figs. 4.2A–B'). By contrast, in the case of *Platynereis*, *in situ* hybridisations performed in 3–5 day old larvae did not show co-expression of the *glass* transcript with *rhabdomeric opsin 1* (*r-opsin1*), which is a marker for rhabdomeric PRs in both the dorsal and the ventral eyes (Backfisch et al., 2013; Randel et al., 2013), indicating that *glass* is not present in these cells (Figs. 4.2C–C''''). In addition, we also generated a *Platynereis glass* reporter by cloning 5,789 bp of its upstream sequence into a plasmid, where the *glass* start codon was in frame with *Tomato*. We used this plasmid for transient transgenesis, by injecting it in 1-cell embryos containing a stable *r-opsin1-GFP* insertion (Backfisch et al., 2013). *r-opsin1-GFP* animals consistently showed strong GFP signal in their dorsal eye PRs, and this signal was weaker in the ventral eyes.

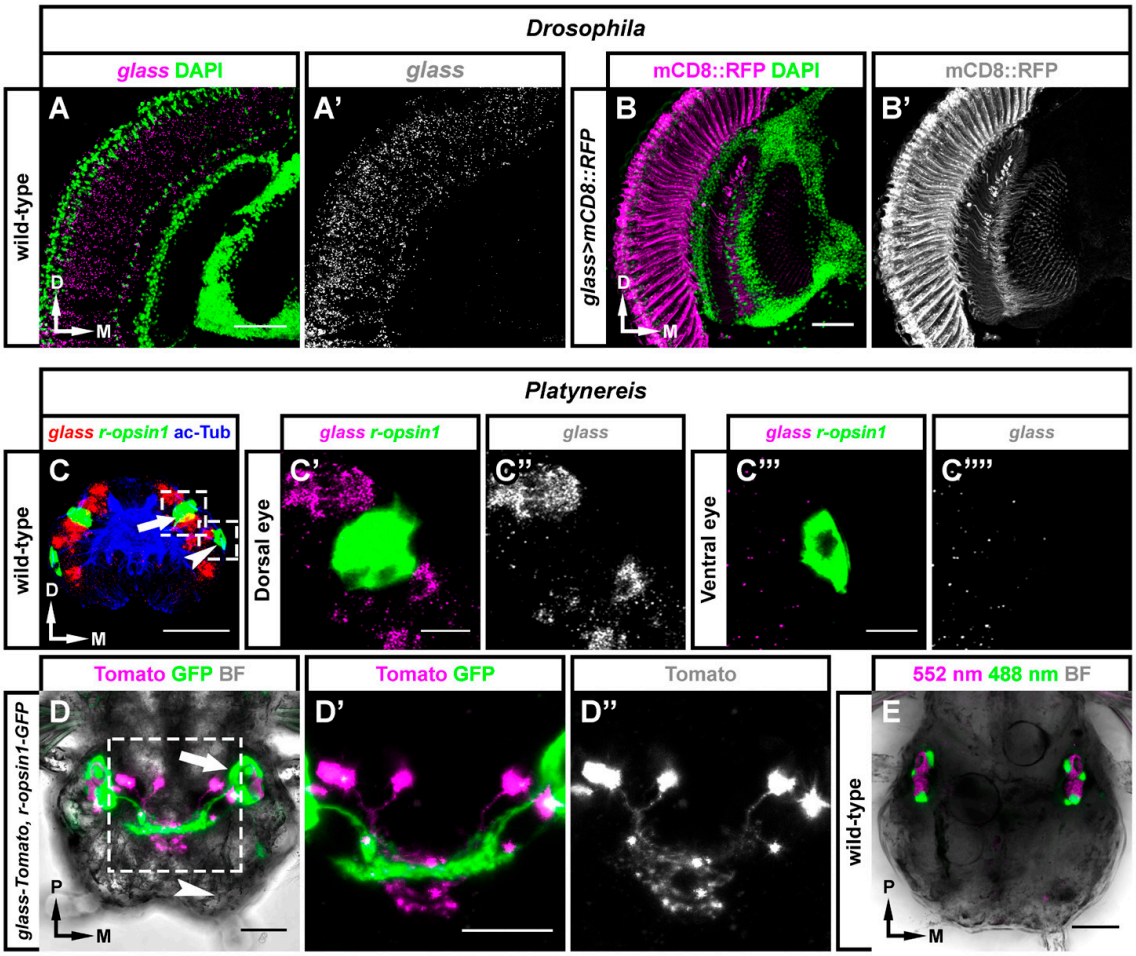


Fig. 4.2: Glass is present in rhabdomeric PRs in *Drosophila*, but not in *Platynereis*. (A, B) *glass* is expressed in all *Drosophila* rhabdomeric PRs, including those in the compound eye (Ellis et al., 1993; Moses and Rubin, 1991). This can be observed in head cryosections, either by using *in situ* hybridisation (magenta in A, greyscale in A') or with *glass>mCD8::RFP* flies (magenta in B, greyscale in B'). In both cases, samples were counterstained with DAPI (green) to clearly see the positions of the retina and the optic lobe. (C–E) By contrast, in *Platynereis*, *glass* is not expressed in *r-opsin1*-positive cells, which are rhabdomeric PRs. We performed double *in situ* hybridisations and, as an example, we show a transversal view of a whole-mounted, 5 day old larva. This animal was tested for the *glass* (red) and *r-opsin1* (green) transcripts, and counterstained with antibodies against acetylated Tubulin (ac-Tub, blue), which is a neuropil marker (C). To the right, close-ups of the dorsal (arrow in C; C',C'') and ventral eyes (arrowhead in C; C''', C'''') are shown, demonstrating that *glass* (in magenta/greyscale) is not expressed in either of these visual organs. Similarly, we also found that a microinjected *glass-Tomato* reporter (magenta/greyscale) was not co-expressed with a stable *r-opsin1-GFP* insertion (green). For instance, we present a dorsal view of a whole mounted, 8 day old larva, where brightfield (BF, greyscale) was imaged as a reference (D–D''). The positions of the dorsal and ventral eyes are shown with an arrow and an arrowhead, respectively. Close-ups to the right show how the axons of Tomato and GFP-positive neurons project into two different areas in the brain (D', D''). In addition, we also imaged an 8 day old, wild-type, uninjected larva as an autofluorescence control (we used a 552 nm laser wavelength for Tomato, and 488 nm for GFP). Scale bars: 10 µm in C', C'''; 20 µm in D–E; and 50 µm in A, B. Axes: D, dorsal; M, medial; P, posterior.

In the case of the dorsal eyes, all PRs project their rhabdomeres into a pigment cup, and their axons form four nerves that innervate the optic neuropil in the brain (Backfisch et al., 2013; Randel et al., 2014; Randel et al., 2013). After microinjections, we tested 3–8 day old larvae (slightly older than those that we used for *in situ*, to guarantee that positive cells had enough fluorescence to distinguish them) but we did not observe co-expression of GFP and Tomato. *glass-Tomato*-expressing neurons were consistently located in the head of *Platynereis*, distant from the ventral eyes. Some of these Tomato-positive cells appeared close to the dorsal eyes, but they did not project any rhabdomere-like extension into the pigment cup, and their axons did not innervate the optic neuropil (Figs. 4.2D–E''), indicating that they were not part of the eye rhabdomeric PRs.

We conclude that, while Glass is expressed in all types of rhabdomeric PRs in *Drosophila*, it is not present in known rhabdomeric PRs in *Platynereis*.

4.3.4. Glass is expressed in *Platynereis* sensory neurons

In *Drosophila*, *glass* is primarily expressed in PRs. Therefore, if *glass*-expressing cells in *Platynereis* are not rhabdomeric PRs, what is their function?

Most of the neurons that we saw labelled with the *glass-Tomato* reporter innervated the neurosecretory neuropil, a region located posterior to the optic neuropil (also, some Tomato-positive axons innervated a slightly more antero-ventral region, forming a ring) (Figs. 4.2D–D''). Interestingly, an ongoing electron microscopy (EM) reconstruction of the *Platynereis* connectome shows that most

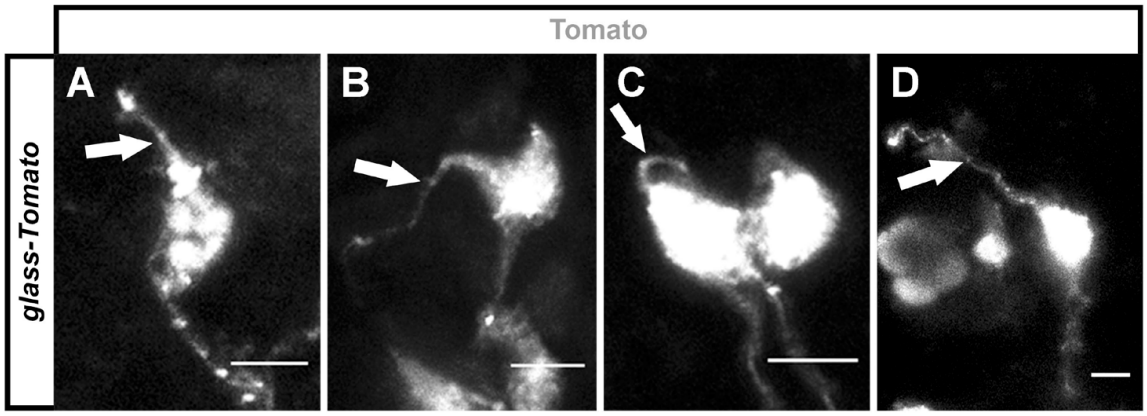


Fig. 4.3: Glass-expressing cells in *Platynereis* include sensory neurons. When we injected our *glass-Tomato* reporter, we observed that many of the neurons that appeared labelled in the *Platynereis* head were close to the surface, and they often possessed membranous specialisations resembling sensory dendrites (arrows) (A–D). Scale bars: 5 µm.

sensory neurons in the head of *Platynereis* synapse on interneurons located in the neurosecretory neuropil, suggesting that *glass* is likely expressed in sensory neurons (Williams et al., 2017). To test this hypothesis, we examined the morphology of the cells that were labelled with the *glass-Tomato* reporter. In EM reconstructions, sensory neurons are identified because they possess membranous specialisations that project towards the surface, called sensory dendrites, and we also see this type of structures on some *glass-Tomato*-expressing neurons (Fig. 4.3).

Notably, the *Caenorhabditis* homologue of Glass (called CHE-1) is expressed in ASE chemosensory neurons, and it regulates their development (Etchberger et al., 2007; Uchida et al., 2003). Therefore, it could be possible that Glass plays a similar function in *Platynereis*.

4.4. Discussion

Remarkably, the earliest steps of eye development are controlled by a group of transcription factors, called the 'retinal determination network' (RDN), which is both required and sufficient for eye formation in distantly related species (Chow et al., 1999; Halder et al., 1998; Halder et al., 1995; Hoge, 1915; Loosli et al., 1999; Pignoni et al., 1997; Quiring et al., 1994; Silver and Rebay, 2005). RDN members, such as Eyeless, Sine oculis, or Eyes absent are important for inducing eye field specification, and, to do this, they establish complex epistatic interactions with each other. These interactions occur similarly across model organisms, suggesting that this is an evolutionarily conserved process (Donner and Maas, 2004; Silver and Rebay, 2005). In contrast to eye field specification, our present work reveals that, at a later time point during eye development, rhabdomeric PRs mature through different mechanisms in different species.

Very little is known about how the RDN instructs eye formation in most animals. However, in *Drosophila*, we have recently shown that Sine oculis (an RDN component) directly activates the expression of the transcription factor *glass*, which is crucial for activating the expression of virtually all the phototransduction proteins in all types of *Drosophila* PRs (Bernardo-Garcia et al., 2016; Bernardo-Garcia et al., 2017; Jusiak et al., 2014). Based on their similar light-sensing machinery, *Drosophila* PRs are considered homologous to the ipRGCs of vertebrates, and also to the rhabdomeric PRs that exist in the dorsal and ventral eyes of *Platynereis* (Arendt et al., 2004; Fain et al., 2010; Montell, 2012; Provencio and Warthen,

2012). Intriguingly, we did not find a Glass homologue in vertebrates, nor any Glass-like protein that can bind to the same DNA sequence as the *Drosophila* Glass. Also, while we did identify clear Glass homologues in most metazoans, we did not see *glass* expression in the rhabdomeric PRs of *Platynereis*. These two lines of evidence indicate that metazoans must have evolved alternative transcriptional pathways to direct the formation of rhabdomeric PRs. One of these pathways requires Glass (e.g. in *Drosophila*), while others do not (e.g. in vertebrates or in *Platynereis*).

Therefore, if we aim at comparing the differentiation of Glass-expressing and non-Glass-expressing rhabdomeric PRs, it will be essential to identify additional transcription factors capable of activating the expression of phototransduction proteins. Indeed, our data reveal a complex scenario for the evolution of rhabdomeric PRs, but future works on the targets of the RDN may help to better understand how rhabdomeric PR identity is regulated.

4.5. Materials and Methods

4.5.1. Glass sequence conservation analysis

We used BLAST (https://blast.ncbi.nlm.nih.gov/) to retrieve NCBI-deposited sequences belonging to clear Glass homologues from several species, including *Amphimedon queenslandica*, *Nematostella vectensis*, *Aplysia californica*, *Caenorhabditis elegans*, *Strongylocentrotus purpuratus*, and *Branchiostoma belcheri*. Also, we found a Glass homologue in the transcriptome of *Platynereis dumerilii* (http://jekely-lab.tuebingen.mpg.de/blast/). For more information on these sequences, see Table 4.1 (also, for the *Platynereis* version of Glass, see supplementary Materials and Methods). Glass-like proteins from both vertebrates and choanoflagellates were also investigated (see Table 4.1).

We used either BLAST (https://blast.ncbi.nlm.nih.gov/) or MUSCLE (Edgar, 2004) to align protein sequences. To investigate the DNA binding specificity of those transcription factors that we tested in Table 4.1, we used specialised software (http://zf.princeton.edu/) (Persikov and Singh, 2014). Particularly, we generated a sequence logo for each candidate by pasting its full aminoacid sequence as input in http://zf.princeton.edu/. Then, the software searched for Cys_2His_2 domains, and we asked it to predict the binding site for the region that best aligned with the 4th and the 5th zinc fingers of Glass, which are responsible for recognising its targets *in*

vivo (Enuameh et al., 2013; Etchberger et al., 2007; Naval-Sánchez et al., 2013; O'Neill et al., 1995). We used 'expanded linear SVM' as prediction model.

4.5.2. Animal caretaking

Drosophila melanogaster stocks were cultured at 25 °C in a 12:12 hour light–dark cycle, and we fed them with cornmeal medium (which was supplemented with molasses, fructose and yeast). We used Canton-S as a wild-type strain (courtesy of R. Stocker), *glass-Gal4* (courtesy of S. Kim) (Park et al., 2011) and *UAS-mCD8::RFP* (Bloomington Stock Center, no. 32219).

Our wild-type *Platynereis dumerilii* were a mixed population of worms, originally captured in the sea in Naples (Italy) and Arcachon (France). We also used *r-opsin1-GFP* worms (courtesy of F. Raible) (Backfisch et al., 2013). These animals were kept in sea water at 22 °C, in a 16:8 hours light–dark cycle. We maintained them synchronised to an artificial moon cycle, induced by slightly increasing the light intensity at night for 1 week per month (using a 10 W light bulb, to simulate the full moon). *Platynereis* had a varied diet that included *Artemia salina*, *Tetraselmis marina*, fish food and spinach leaves. For our experiments (i.e. *in situ* hybridisation and microinjections) we crossed males and females and collected the fertilised eggs, as previously described (Hauenschild and Fischer, 1969). The larvae that hatched from these eggs were kept at 18 °C.

4.5.3. Immunohistochemistry and in *situ* hybridisation

In the case of *Drosophila* antibody stainings, these were performed on cryosections of *glass>mCD8::RFP* flies, as previously described (Bernardo-Garcia et al., 2016; Wolff, 2000b). Basically, we dissected heads (removing the proboscis to improve the penetration of our reagents) and fixed them for 20 minutes with 3.7% formaldehyde in 0.01 M phosphate buffer (PB; pH 7.4). Then, we washed our samples with PBT (Triton X-100 0.3% in PB) and incubated them with a cryoprotectant solution (sucrose 25% in PB) overnight, at 4 °C. The following day, we embedded the fly heads in OCT, froze them with liquid nitrogen, and cut 14 µm cryosections in the transverse plane. Once the samples were dry, we proceeded to immunostain them. For this, we washed the slides with PBT (this buffer was also used in subsequent washing steps) and incubated them in primary antibody (rabbit anti-DsRed, 1:100, Clontech, no. 632496) at 4 °C overnight. Then, we washed the cryosections and incubated them in secondary antibody (goat anti-rabbit

conjugated to Alexa Fluor 568, 1:200, Molecular Probes, no. A-11011) at 4 °C overnight, and washed again the next day. We covered our samples with Vectashield containing DAPI (Vector, H-1200).

To detect the *glass* transcript in *Drosophila*, we used the ViewRNA *in situ* hybridisation kit of Affimetrix (no. QVT0012) – which is a proprietary method – and proceeded according to the instructions of the company. Briefly, we took head cryosections (as described in the previous paragraph for antibody stainings) and ordered a mix of labelled RNA probes against *glass* from Affimetrix. Then, we prepared these samples by digesting with protease QF and washing with PB and with various commercial solutions included in kit. We incubated our cryosections with the *glass* probes for 2 hours, at 40 °C. After this, we continued with a series of washing and signal amplification steps, followed by a colour reaction (we used Fast Red as a fluorophore). We finished by washing the samples with PB, and used Vectashield containing DAPI (Vector, H-1200) to cover the slides.

To perform double *in situ* hybridisation in *Platynereis*, we followed – with few modifications – a protocol that has been used for characterising the expression pattern of *r-opsin1* (Randel et al., 2013; Tessmar-Raible et al., 2005). In the present work we used an RNA probe against the *glass* transcript (for details on the *glass* probe, see supplementary Materials and Methods). Briefly, we fixed 3–5 day old larvae in 4% formaldehyde, and we subjected them to a mild proteinase K digestion to improve the penetration of our reagents. We prehybridised the larvae at 65 °C by using a hybridisation mix (Hyb-Mix), containing 50% formamide, 5x saline-sodium citrate buffer (SSC), 50 µg/ml heparin, 0.1% Tween 20, and 5 mg/ml torula RNA. Then, we dissolved the probes against *r-opsin1* and *glass* (labelled with either fluorescein-UTP or digoxigenin-UTP) in Hyb-Mix, denatured them at 80 °C for 10 minutes, and added this solution to our samples. We hybridised both probes simultaneously by incubating at 65 °C overnight. Then, we washed the samples at 65 °C with a solution that initially contained 50% formamide and 2x SSCT (obtained from a stock solution with Tween 20 0.1% in 4x SSC), and we progressively decreased the concentration of both formamide and SSCT throughout successive washes. After washing, we placed the larvae at room temperature and proceeded to immunostaining them. We detected the two probes sequentially, by using peroxidase-conjugated primary antibodies against fluorescein (1:250, Roche) and digoxigenin (1:50, Roche). Basically, first we incubated our samples overnight at 4 °C with in one of these antibodies, washed them with Tris NaCl Tween 20 buffer (TNT; 0.1 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20; pH 7.5), and started the

colour reaction by adding a solution that contained fluorescent tyramide (conjugated to either Cy3 or fluorescein). We controlled the development of the signal with a fluorescent microscope and, when it was ready, we washed in TNT and stopped the peroxidase activity with H_2O_2. To detect the second probe, we repeated these immunostaining steps similarly. We mounted our samples with 90% glycerol, and scanned them in a confocal microscope.

4.5.4. Microinjection of *glass-Tomato*

To make a *glass-Tomato* reporter for *Platynereis*, we PCR-amplified a fragment of the *glass* promoter, and cloned it into a plasmid containing a tandem dimer version of *Tomato* (courtesy of L. A. Bezares-Calderón) by using ApaI and SgsI as restriction enzymes. The fragment that we cloned included a 5,789 bp long upstream sequence, and also the *glass* start codon (predicted both with http://atgpr.dbcls.jp/ and by aligning the *Platynereis* version of Glass to the Glass homologues of other species). We placed the *glass* start codon in frame with *Tomato*. To do this, we used the following primers (oriented 5' to 3'):

- *Platynereis glass* ApaI fw `aagggcccGCATCCGTGCTTGACAGAATG`
- *Platynereis glass* SgsI re `aaggcgcgccTCCACAGTCATCCGAACGTTTAGC`

These primers were designed with the help of an unpublished reconstruction of the *Platynereis* genome. For details on the sequence that we cloned, see supplementary Materials and Methods.

For microinjections, we collected freshly fertilised *Platynereis* eggs and proceeded as previously described (Backfisch et al., 2013). Briefly, we removed the jelly of the eggs by digesting with proteinase K and washing with abundant sea water, using a sieve. We diluted the *glass-Tomato* plasmid to a final concentration of about 200 ng/µl, and delivered it into 1-cell embryos with a microinjection set-up, by using Femtotip II microcapillaries (Eppendorf). Larvae were kept at 18 °C, and we imaged them with a confocal microscope to study the expression of the reporter.

4.6. Acknowledgements

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4.7. Competing interests

The authors do not declare competing or financial interests.

4.8. Author contributions

F.J.B.-G., G.J., and S.G.S. conceived the study. F.J.B.-G., G.J. and M.S. performed the experiments. F.J.B.-G. wrote this manuscript.

4.9. Supplementary material

To find a Glass homologue in *Platynereis*, we searched a transcriptome database (http://jekely-lab.tuebingen.mpg.de/blast/) (see Table 4.1). We also had available an unpublished reconstruction of the *Platynereis* genome. Both sources indicated that *Platynereis* possesses one single Glass homologue, and that two different Glass isoforms can be produced by alternative splicing. These are:

>Glass isoform 1, *Platynereis dumerilii*
MLNVRMTVDVPLCAKNTSYQKPQARMESCYLSAGGHSHGGHHGHGSHGGGGPGGHCGGGGGGSPGPSYYT
SSAAAAVAAAGELWRSSPLKPGSPASASEVCGPPRSSVDLSVNTFAMPPLDIDPLSNFFSFSSPAYKELS
VFKEKGPQDIADALLSLKHAVVHPGMNGQLSPLSPGLPPHLPQSISSAMAPSSSLSSYPMSHQHSQLQSY
GMSSQYGSPPPPPPPAPQYGETGSGQASPCHPQAPQHHSMFPAMSVNVSMNMNVAMGNQYNNSMDNWGHH
PTHQPSAQYSPAAAAAAAQMTSQYPSYGHHHHHHHHHHQNHSYSASYAFSPELRSSSSVSVRDMMYPHPH
KPSSASDSYKDVSSKLYSLSAMRRSPRGSCSPVGLGPPPGLRSHHPSGHLGSASSVDSKVNLCRICGKTY
ARPSTLKTHMRTHSGEKPYRCQTCSKSFSQAANLTAHLRTHSGEKPFRCPMCDRRFSQSSSVTTHMRTHS
GERPYRCRMCKKAFSDSSTLTKHLRIHSGEKPYQCKLCLLRFSQSGNLNRHMRVHANNA

>Glass isoform 2, *Platynereis dumerilii*
MESCYLSAGGHSHGGHHGHGSHGGGGPGGHCGGGGGGSPGPSYYTSSAAAAVAAAGELWRSSPLKPGSPA
SASEVCGPPRSSVDLSVNTFAMPPLDIDPLSNFFSFSSPAYKELSVFKEKGPQDIADALLSLKHAVVHPG
MNGQLSPLSPGLPPHLPQSISSAMAPSSSLSSYPMSHQHSQLQSYGMSSQYGSPPPPPPPAPQYGETGSG
QASPCHPQAPQHHSMFPAMSVNVSMNMNVAMGNQYNNSMDNWGHHPTHQPSAQYSPAAAAAAAQMTSQYP
SYGHHHHHHHHHHQNHSYSASYAFSPELRSSSSVSVRDMMYPHPHKPSSASDSYKDVSSKLYSLSAMRRS
PRGSCSPVGLGPPPGLRSHHPSGHLGSASSVDSKVNLCRICGKTYARPSTLKTHMRTHSGEKPYRCQTCS
KSFSQAANLTAHLRTHSGEKPFRCPMCDRRFSQSSSVTTHMRTHSGERPYRCRMCKKAFSDSSTLTKHLR
IHSGEKPYQCKLCLLRFSQSGNLNRHMRVHANNA

To do *in situ* hybridisation against Glass, we obtained a plasmid from a *Platynereis* EST library, and we generated an RNA probe by using T7 RNA polymerase. We checked the size of our probe by running it in an agarose gel next to an RNA ladder. The plasmid contained the following sequence:

>DNA template for *glass* RNA probe
CTTGCTCCAAATCTTTCTCTCAAGCTGCTAATCTCACTGCACACTTGCGCACCCACTCTGGCGAGAAACC
TTTCAGATGTCCGATGTGTGACCGCCGTTTTTCCCAGTCGTCTTCGGTGACCACTCACATGAGAACACAT
AGCGGAGAACGGCCTTACAGATGTCGGATGTGCAAGAAGGCCTTCTCCGACAGCTCAACCCTCACCAAGC
ACCTAAGAATCCACAGTGGCGAAAAACCTTACCAGTGCAAACTCTGTTTATTAAGATTTTCTCAGTCTGG

AAATCTAAATAGGCACATGCGAGTACATGCTAACAATGCCTGATTATATTCTGAGCTATTCTGCTACTTC
ATTCATTCTGTGGACTGTGAACAATACCATAATGATCTGACAAGTTGTGACAGTTGTGAATTTACTGCTG
TAGTCTGTGGAATACAGCAATGTGAACTATGGACAGTTTCTTTTTAAATGTTGACAATGGACAATGTCTA
TTATGACTGTAACATTCGTGACATGAATGTGTTGTTTAATGATCAAAACATGTCATAGTTAATATATTTC
TTCATGACAACAAAATCTTGGTAATACACTTTCTTCTTTCCGCCTTGAAATTATTGTAGATTCAATAGTT
ATACCAATCCAATTGTTTTCATAATGATTTTTTCCCTTGCCTGTTGTTTTTTCAAATAAATTCCTTGATC
AATTCCTTCTATTTCTTTTTTACTTCCTTCACTTCTTTTTTTTCTCCATTTCGCTTTCCTTCCATAGGCC
TAATCCTTCTTTCCTTGCTTCCTTCATTGCCTGATTCACATTCCTTCTGTCTTCCTTCTTGTTGCATCCT
CTCCTTGCTTCTTTGTCTATCACTCATTCCTTTCTTCCTCGCTTCTTTCTATTTTTCCTTCCTTCCTTCC
TTTGCTTTCTCGTAACAATTGCTTGCTTGCTTCATTACTTTCTTCCTTCACCACTTGCTTCCTTCCTTGC
TCACTTTCTTGCCTCCTTCCTTTCTTGTTTCCTTCTCTTCTTCCTTCTGTGCTTCCTATTCTGCTTCATT
CCTTGCTTCCTACCTTGTTTGATTCCATAATCTTTTTCCTTCCTTGCTTTCTTGATAGTTTCTTCCTTGC
TTGCTTTCTTGAAAGTGTCTTGCTTCCTTGATTCATAAAAACTATAAAACAAATTTATACATACACACAA
CTTCAGATTCTTTTTGCTACACATGATTTGAATGTGAAACCATTTGTGCATAATATGTTGATATACATTT
GTTCTACTGGTGCTATTGACTATGATGTTTTATTCTGTCTTTACACTTAATTTCTTTACATCTCTTCTTT
ATTGATATGTAAAATTGTTCATTGCTGTTATTTTTGTTTTGTATATATAATAAACATTTTGTCTTGACTG
TGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAA

To generate a *glass-Tomato* reporter, we amplified the following sequence of the *glass* promoter by using *Platynereis* genomic DNA as a template (restriction enzymes are shown in lowercase letters, primer sequences are underlined, start codon appears in green and italics):

>Fragment of the *glass* promoter
aagggcccGCATCCGTGCTTGACAGAATGTGGAGTCAGGTGTTTGCACATCTTCATCACAATACAACAGC
TCCTATAAGGCTCATCCAATGGAAGGTCATGATGCCAAGTTTTGCATAAAAGGTTCTAGCCTGTTCCACT
GACGGTTATTGCCTGCATGGGTTCCAACCCGGAATGGGGAACCCACAATTTACAGCCACTGTGGACCTTC
ATGAGAAAAAATCCAGCTGGATGTCGTTCGGATAAGATGTTTATAATTGGAGGTCCCTTGCATCAGTGAC
TTTCAGTTTCTGTGCTGGGCAAGTAAAATATCCCTCACATGGCGATGATGGCGTAATATAAATGTGTACC
GGTAATGCCTAGATCACACGAGAGTGGTTAAGTGACGGCGAAGTTGTAGAGGTGCCAGGAACTCAAGTTT
CGCCTACTTTCGGCGTTTCAAGCCATTTTTGGACAATTATATAATCAATTAGAGCTTATAAATCGTCGTT
GATGGTTCCTATTACGACGAGGCACTCGTTTTTTACGCCGAATCGCTCGTAATTACGGACGAGGCAAGAA
GAGACTTGTGTCGGCGAGTTGTCAGGAGCCTCGACGCAGTTCCATTGCTTCAATGTTAAACTAGATTCGC
CCAAGGAGTCTCTTTCTTCGCCCTAGCATACTGTTGCTTCGCCCTAGCATCCTCTTACTTCATCCTAATC
TTCAATTACGCCCAAGCAAATACATTTTACGGTGAAGCAAGTAGCTGTTTCGGCGTTTCAACAGCCTGTT
TGTGTCGGCGAGTTGTGCTCTTTTTTCGCCGAAGTTCCATTGACTCAATGATAAGTAGCCTCGCCCAACC
TGCCTCTAACTTCGCCCAACCTGTCTCTAACTTCGCCCAAGCTGTCTCTAACTTCGCCCAATCATTTTGG
GGGGCTTTCTGGTCTCTTGCATCGTGGTTTCTTCCTTCGCAAAGATTCAACCGGTTGATTCTTTCGTGAA
GCAAGTTCTTGCTTCACCATAAGGTCACGTGATTTCTAGGAACTTTTGGCGAGGAAGTTTAAGAAGCGCC
GTAGACGAAAATAGTGTCGCCGAATTGTTGAATGTAGTTTAAAAACCTCCCTCTTTTCTGGAAAAGCAAG
AAGCGACGCGACAAGAACCCTCTGTTACGTCTCCGTGAGAACTAGCCATAACATGCTGAGGACTCCCGCT
TTCTCCCCCATCCCATCAACTCTCATGAGCATCCAGAACTGTACTTGCAGCTTGAAGTAACTAGCTAGTT
TCTTCAAGCTTACAGGGTGGTATTTTATTGTAGGCCTATCTGTCTATAGATCTTGCTTGTGTGATAGTAA
GTTTTGATCAGCAGTAGTTTTCAGGGGAAAATAGTGGTTGTCTTTTACATCGAGCTCAGTAGAAGATACG
AGTGTTTAAAACATCACGAAATGTTATTCTACCTGTTCACAAAGTTGATTTTTAAAATTTCAAATAATAT
GTAAATGGCACTGCCATGAAATTCGCACAGCATTGTCACAGAAGTACTGCATTATCTTTAACTTTCCATG
CAGAAGGCCAATTTATATTTTTAAATGACTAAGCAAATGGGCATATAGATAGAATAGATAGATAAGATCA
GATCTATAGATAAGATTAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATA
GATAGATAGATTGCACTGACAGTTGTGTAATTCAAATCTGTGTTAAATTTGGATCTATAAATTTGGATCT
ATTATGTTAAATTGTTGATGTTACATAACTGAGATCAATAAAACAATAGCTATTCAGAGATGACTGTTTA
CTATTAGACTGTTACATAATTGTTGATAGTTAATGAAGATTATGTAACCTTAAGTGTTGTTGTGATTTCT
TACTTGGTGAGCCATTTAAGTCATTAACTTTTGTGTTGACTTTTAATTTTCAGTCACTTCTTATTACAAT
CAGGGGTATGGAAATAATTATTTCCAATGTTGATACACATGTTGATTTAAATCCCTGAAATATGTCATTG
GAGGTTGTTATACGACGAGACTGAAATTTTAAAGCTATTTTGGAAGATATTTGAGGTATTTTAATTATTT
ACCAAACTAAACTCTGACTAAACTACTAGCTCCACTTTTATGGCAACTACAGGCACTTCCTGTTTAGGCA
GTCACTGTAGACAGGAGCACTACACAGAATGAGCTGTCCTTGTGCTAAGTGAGCTTCGCTCATGGCTTCC
TTCGGCAGCCATGAGCTGCGCTCATATTATTTTCCTCACATAAAAAGCAAAAAAAATCTAAAATTTTAGG

AGATTTTGGTCACAAAAAGGAGACTTTTGCAAGAAAACTTTTTAGTCAAAAGGAGGTTCTGTCAGAAGGA
GATTTTGTCAAAAGATGATTCTGGGAAAGGCGATTTTTGAAAAAGGAGAGTTTGACTGTCCTCTTGAAAG
GAGTACGCTTTCGTTGTGTGGCTTGTTTTATTCAAAAGAAAGGGTAATATGTTTTTCAATATCTTTCATG
ACTGAGTTGTACACCTACATTGATCAGCACAAGGTCATTGAGACACTTGTTGTCGTAGATCCAGCCGAAG
GCCAACTTCAAAGTCAGTCTTTGCTTGTACATGCACAGACATAAAGGTATCTTTCTATCCTGATAATATT
GCAATGAAATTGATTTCATTTAACTATAGGCCTACGTGCCTGTTGAGAAAGATATTCTGATGAGTAGAAT
GCTTGGAGTCTGCTAGATGAGTTCTTGGAAGATCTTAATGGAATTACATTAGTGGGATCCAGAAGCAAGG
GCAGATAAGAAGAAAAAAGAGACGTGCTTGACAAGGAGGGGGGGGGGGTAATAACACCCCCTCTCTGTTC
ACCCCCCCCCCACCTTCATCCTCCTATCTTCTCTCNATCTTGATGTAAAGGCCTCTCAGCGACTCTCTCA
GGCTTCTGAGTAGAGCTGCAATCTGATTGGCTGTTGGCGCGTGAGCTGCGCTGTGATTGGCCAGTGCCTT
TGAGTAGCCACGCCNCCTGCCTTGCAGCCCCTCAAGAGCTTTCACACTGCTTGATCAGGCTTAATACCAC
GAATCCCACCAAGTGTAGAGCACCCAGCCAACTCCGTGCGATTGTGGACAGGTCGAGGGGGATTGGGCGG
ATGCTGTGAAGTGGGACTTGCAACAGCAACAAGGAAAATCACAACAACAGGACTTGAAACCCAACTTCTT
CTGGAAAACGAGCATCTCTCACATACTCCCAGGTTTAAAACAAGGAGCTCCAGGACAACAGTGTCTGAAT
CAGGTAGGTGATGTTGGATAGCATCCATCTCCAACATCAGCAACAGGCTTCATCAGTAAGCCACATCAGC
AAGCCACATCAGCAGCCTCCACGGCTGAATATCAGTTGGTCCATCAGCTTTTGAGCAGCCTCCTAGCTTG
TGAGAAAATTCTAGGACTTCCTGGCTGCTGTAGACAATCCTTACAACATCCTTCCTGTTCCTGTAGCTTC
CTGTTGAGATGTGAATCTTGCTGATGAATGTGCTGATGTTGTTGATGATGAAGAATTCCTGATGCTTGTG
ATAATTATCGATGTTGTTGTGTCGGTGCTGTTGTCGATATGTCTGTAATTTGTTGATTATATTTTTATCT
TCCGATGATGATGCACATATCCAGCTTCTTTCACCTCCCTGAGATAACATGCAGAATCTTCTCGAAACCT
TCTGAATCACCTTGAATATATACGGGTTTTTTTCTGTTTCATTTCTGAATCCAATGATTTCCTGATTCTT
GTGCTTTATATATTCTCAAATTTATCCGAGAACGCAACCATTTTTCACAGACTCTGCATGGGTGACAGAA
GACATGTTGGAAAGTTCGTTCCATGCACTGGCAGTTAAGCTATGTTCTTTTAGCTTAAAAAGTTGACCCA
TTTGCACAAACTGAGAAGGGTCTATAAAGTGAACATTTTAAGCAGAATTCTTGCAGGCAAAATATCCCTT
CCAAAAAGTACTAGGTAGTAAATATTTTAGGCGACATTATAAATCATACTAGCGGTAATCCAGAAATGTA
ACTTGATTTATGCTTGTAAAATTCTACCATGCAAGTTAGGCCTCTGGAACAGTCCATGATGTAAAAGCAG
GACAAGATTAGGATGTTTTGTACCTGTGATGCTTCAGGTTTCCTCAACTAGATTAGCCTTATAAGCCTTT
TGCAGGTGTTTTTGCTTTTGACATAGACCTAGACCTCCAAATGGAGCTTATATGCCAAACTCGTGAGCAG
TGGGAGGTGCCAGGTCTCTATCTGTCGTTTTGGAGCTTCTCGAATCTTCACAGCCTTGCACTCTTACAAG
TTTTCGAGCTGCTTAACACCACGAAACCAACAAATGCAAATCCACTGGCTCCCTAAGCTTTGAGAGCTCT
CTTATTTGATAGGTCTCTTATTTGAGCTCTTATTCGAGCTCTCCCATTTGCTCCTATTAGAGTTGCTCAC
AAGTCGCACAACTGGGCTTAGAGGAGCTTCAAGGGAACACCCATGACAGACAGTGTTTATAAGGCATCTC
ATCAGCCCCTTCCCTCCAAAAGATTCCCTATAAATATACTTACAATCGAATCAGCCTGAATCAGATTGGC
CTAGCAGCATTTGCCTGCAGCTTTGCCTGCACCTGCCTGCTACTGCTCACTGCAGGAGAGGAGTAAAAGT
TGCCCATCTTCCAACTGCATACATTCAGCATTTTGGCAACACCTACACCACATCTACTTGGATTTTTGTG
AACATATTTCCATTTTGAAATATTTCATCTGACTGAACTTGGACTTGGAAGTCTTGGACATATCAGGAGT
TAGAAATCTGCGCAGGCCAGCATTATGCAGTGAGAATTTGGACACCACTGACCCATCAGCCCAGCTTTGT
GTGCTTCTGGAAGACACTCACACTTGTCTGGCAAACCATGGATCTATTTCTTTTAAATTATCCAGTTCTG
GACTGAAATGTCACAAAGATGGTAAACTAGCTTTCTGACGAGGACTTTGAAAAACTTTTATACACCATTA
CACCTGCAGTCATTAGCTTCAGTTCTTAGTTTTTGACAATGGTGAGCCATTCAGTTTGGCTACAAACTTA
GGAGACTTGGATCACTATTTGATACTTCTGTGTACTGGAACAAGAATTGTATCCTTCTATTGTGATGTCC
AGTTTTGAATATTGGAAACTAACTTCATGTTACCATTTTCAATTTCTAATTCTCACTCGCGCTTGGGATT
TTCGTTTCTGGTATTCTCACATTGATCTTTGACAAGTCGTCTCATATTACCAACACTGGACTTCACCACG
AACCTTTGAGATAAATATTTCACAGAATACTCACTTTTTGAAAATTTTGGTAACCCGCTGAAATTTTGAG
CGGTGTGAGCATCATGTGGAATCAGCTACATCATATTTTTGTCGTCTCTTATATTTTTTTAATGTCTCAA
AATTGATGTTTAAACAATTCGAGACAAATCTATTCTAGTATGAGCCCAGTCATTCGGGCCTAAAGAGCTC
TGAAAATTGAGAAACTTTCAGAAAAACTGTTATTTTGTACGGAAAAACACACATTTGACATTTCTGAATC
GGTAATGCGCAGTTCCCAGAATGTTCCAGAATGTACGCCATTTTGAATTTTGTGACGGGCAGACATTTTG
TCGTTTTGGTAATGCTGCTGGAGTTGATTCTCGTACAGCGAGCAGGTTATCATTGAATGCCAAGGATTTT
TGATGGACTGAATTTTCCCCCTTAGTTTGGATTAAATAAAATAGGTTTTGGAAAAAA*ATG*CTAAACGTTC
GGATGACTGTGGAggcgcgcctt

5. GENERAL DISCUSSION

Some sections of this chapter are currently being modified, and may be published as part of a review on photoreceptor development in the future.

5.1. *glass* mutant photoreceptor precursors develop abnormally in the *Drosophila* retina

Metazoans have evolved a diversity of cell types – like myocytes, adipocytes, or lymphocytes – that differ from each other both from a morphological and a molecular point of view. These differences originate during development due to extrinsic (e.g. morphogens and cell–cell interactions) and intrinsic signals (e.g. transcription factors), which act in dissimilar combinations across the body (Arendt, 2008; Arendt et al., 2016; Basson, 2012; Christian, 2012; Soustelle and Giangrande, 2005). As a consequence, each cell type has a characteristic location and a unique set of functions. In the case of photoreceptor neurons (PRs), these cells typically appear in the eye, and they are specialised in detecting light. In fact, animals have evolved different PR subtypes for detecting specific light properties, such as colour, intensity, or polarisation (Fain et al., 2010; Purves et al., 2004b; Roberts et al., 2011). This allows animals to obtain information about their environment, which is useful for surviving. Here I have investigated how the ability of *Drosophila* PRs to sense light is controlled by the zinc finger transcription factor Glass.

Glass is enriched in the *Drosophila* visual system and it is expressed in all compound eye PRs (Ellis et al., 1993; Moses and Rubin, 1991), which is noteworthy, given that the fly retina contains eight different PR subtypes (called R1, R2, R3, R4, R5, R6, R7, and R8). These subtypes can be distinguished because of their position, the opsins that they possess, the targets of their axonal projections... (Morante and Desplan, 2004; Tomlinson and Ready, 1987; Treisman, 2013). Importantly, each PR subtype expresses a distinctive set of transcription factors that are responsible for establishing and maintaining its identity, and there are many examples in which misexpressing a transcription factor (e.g. Spalt major, Senseless, or Seven up) in the developing eye imaginal disc causes the transdifferentiation of one particular PR subtype into another (Domingos et al., 2004; Mollereau and Domingos, 2005; Treisman, 2013; Tsachaki and Sprecher, 2012). In the case of the *glass* mutant eye disc, the different PR precursors do not

express any correct set of transcription factors (Treisman and Rubin, 1996), indicating that Glass contributes to establishing PR subtype identity.

However, the analysis of the *glass* mutant phenotype was not previously extended to mature PRs: before I started my Ph.D., most research on Glass had been done by the group of G. Rubin in the 1990s, and, at that time, authors could not find PRs in adult *glass* mutant flies. It had been shown that there are no rhabdomeres in the *glass* mutant retina, and that it loses the expression of a PR marker (recognised by the mAb 302 antibody) during metamorphosis (Moses et al., 1989; Ready et al., 1986; Stark et al., 1984). As a consequence, it was believed that *glass* mutant PR precursors died during development. The starting point of my research was the discovery of a Gal4 driver that specifically labels a subset of *glass* mutant PR precursors, which allowed me to observe that these cells are still present in the adult retina. This finding was extremely meaningful because it indicated that, contrary to the previous belief, *glass* mutant PR precursors do not die during development: they are present in the *glass* mutant retina, but they do not possess a rhabdomere, nor express the protein that is targeted by mAb 302 (Bernardo-Garcia et al., 2016). Later, these results were confirmed by another lab, led by A. Zelhof, which followed a different methodology. A. Zelhof's group demonstrated that *glass* mutant PR precursors do not die during metamorphosis by staining with antibodies against Death caspase-1 (a marker for dying cells) and by using a TUNEL assay (Liang et al., 2016). Therefore, Glass is not required for PR survival, but it is important for correct PR development.

Most probably – not counting those mutations that cause lethality or eye loss – *glass* mutant alleles are the ones that cause the strongest developmental defects in the fly retina. This structure appears extremely disorganised in *glass* mutants, but it is still possible to identify specific cell types with the help of Gal4 drivers and antibody stainings, which have been useful for investigating the fate of *glass* mutant PR precursors (Bernardo-Garcia et al., 2016).

5.2. *glass* mutant photoreceptor precursors differentiate as neurons

By investigating a *glass* amorphic allele, I found that *glass* mutant PR precursors differentiate as neurons in the compound eye: they express the neuronal marker Embryonic lethal abnormal vision (Elav), possess the neurotransmitter histamine, and form axons (which contain presynaptic specialisations) (Bernardo-Garcia et al., 2016). Moreover, the proneural transcription factor Atonal, which is crucial for

specifying PR precursors as neurons, is not required for *glass* expression (Bernardo-Garcia et al., 2016), and Glass is also not necessary for *atonal* expression (Jarman et al., 1995). This shows that both transcription factors act in parallel, and that Glass is not involved in the specification of neuronal identity.

Interestingly, the axonal projections of *glass* mutant PR precursors are misrouted, indicating that these cells lack some (not yet identified) proteins that are required for axon guidance. In addition, the lamina of *glass* mutants appears very reduced in size (Bernardo-Garcia et al., 2016), which is most likely a non-cell-autonomous defect caused because *glass* mutant PR precursors fail to innervate their targets correctly. In wild-type, the developing PRs innervate the lamina and medulla neuropils, and secrete morphogens – Spitz and Hedgehog – that are necessary for the maturation of lamina neurons (Huang and Kunes, 1996; Huang et al., 1998; Ting and Lee, 2007; Yogev et al., 2010). Therefore, given that the axons of *glass* mutant PR precursors project abnormally, it is expected that lamina neurons present defects caused by the lack of signalling from their presynaptic partners, but we still know little about the non-cell-autonomous consequences of mutating *glass* (Selleck and Steller, 1991).

5.3. Glass instructs photoreceptor maturation

In *Drosophila*, to sense light, all mature PRs express a similar phototransduction machinery, independently of their subtype identity (Hardie, 2012; Montell, 2012). Remarkably, I found that the retina of *glass* mutants lacks virtually all the components that participate in the phototransduction cascade, and, in agreement with this, these animals are blind (Bernardo-Garcia et al., 2016; Bernardo-Garcia et al., 2017). The only PR-specific feature that *glass* mutant flies retain is the presence of capitate projections on some axons in the brain (Stark et al., 1989). A similar mutant phenotype – so strong and restricted to PRs – has never been shown for other genes and, therefore, my Ph.D. work is crucial for understanding how immature PR precursors differentiate into functional, light-sensing cells.

How does Glass regulate PR development? To some extent, it seems that Glass instructs PR terminal differentiation by activating the promoter of the homeodomain transcription factor *hazy*, which was previously shown to activate the expression of some phototransduction proteins (Mishra et al., 2016; Rister et al., 2015; Zelhof et al., 2003). My work demonstrates that Glass is both required and sufficient for *hazy* expression (*glass* mutant PRs lack Hazy, and misexpressing Glass can ectopically

induce Hazy) (Bernardo-Garcia et al., 2016). In addition, working in parallel, a competing lab and I found that artificially inducing Hazy in the *glass* mutant retina can restore the presence of some phototransduction proteins (Bernardo-Garcia et al., 2016), and it also rescues rhabdomere formation (Liang et al., 2016). Therefore, our data on this aspect are complementary. We also obtained similar results about the mechanism by which Glass activates *hazy*. Particularly, we found that Glass directly binds to the *hazy* promoter, mainly through two evolutionarily conserved Glass binding sites (shown by ChIP-qPCR), which are required for the expression of a *hazy(wt)-GFP* reporter (shown by mutating both sites, alone and in combination) (Bernardo-Garcia et al., 2016; Liang et al., 2016). Notably, Glass is important for activating *hazy* in all PRs in the three visual organs of *Drosophila*: the Bolwig organ, the ocelli, and the compound eye (Bernardo-Garcia et al., 2017).

My work also shows that misexpressing either Glass or Hazy alone is sufficient to ectopically induce a subset of phototransduction proteins. However, misexpressing both Glass and Hazy simultaneously causes the ectopic expression of most phototransduction components (Bernardo-Garcia et al., 2016). This indicates that Glass and Hazy act synergistically to activate the expression of PR genes through a coherent feed-forward loop. The coherent feed-forward loop is a common network motif, which is present in many transcriptional pathways (also in distantly related species, like bacteria and yeast). This motif consists of three genes: a regulator, called X (in this case, Glass), that activates Y (Hazy) and Z (a phototransduction protein), and Z is activated by both X and Y (Alon, 2007). Interestingly, in the course of these experiments, I observed that the different phototransduction proteins are ectopically induced in a context-dependent manner, which varies for each of them: some appear most often in the brain (e.g. Transient receptor potential-like) and others in the ventral nerve cord (e.g. Rhodopsin 2), indicating that Glass and Hazy probably interact with additional, endogenously expressed proteins. A similar effect has been described with other transcription factors: for example, Eyeless can ectopically induce its targets more efficiently in those tissues that express the morphogens Hedgehog and Decapentaplegic (Kango-Singh et al., 2003). It would be interesting to know what endogenous elements cooperate with Glass and Hazy for the ectopic induction of PR proteins, but this has not yet been investigated.

How important is Hazy for activating the expression of phototransduction proteins in PRs? Interestingly, a previous report showed that most phototransduction components are either absent, or have strongly reduced levels in the retina of a

hazy amorphic allele (Zelhof et al., 2003), but I have demonstrated that this is a time-dependent phenotype. Young *hazy* mutants have functional PRs, which correctly express most of the components that participate in the phototransduction cascade. However, as they age, the expression levels of most phototransduction components become strongly reduced in *hazy* mutant flies, and these animals appear to be blind 10 days after their eclosion. Therefore, it seems that Glass is the main responsible transcription factor for initiating the expression of PR genes in the retina, and that Hazy is required for maintaining these cells functional in adult flies.

5.4. Glass links eye development with photoreceptor differentiation

My work indicates that Glass is hierarchically at the top of a transcriptional pathway leading to PR formation. Therefore, Glass can be classified together with other transcription factors that are key regulators of specific cell identities, such as Glial cells missing (Gcm, which is required for the development of *Drosophila* glial cells) (Jones, 2005), or myoblast determination protein (MYOD, which participates in the development of muscle cells in vertebrates) (Tapscott, 2005). But, what is upstream of Glass? PRs are characteristically located in the eye, and eye development is regulated across metazoans by a group of evolutionarily conserved transcription factors called the 'retinal determination network' (RDN) (Chow et al., 1999; Halder et al., 1998; Halder et al., 1995; Hoge, 1915; Loosli et al., 1999; Pignoni et al., 1997; Quiring et al., 1994; Silver and Rebay, 2005; Zuber et al., 2003). In this thesis I show that one RDN component, called Sine oculis, directly binds to the *glass* promoter, and that Sine oculis is required for the expression of *glass* in the eye imaginal disc (i.e. Glass is not present in *sine oculis* mutant clones) (Bernardo-Garcia et al., 2016; Jusiak et al., 2014). As a consequence, Glass is a downstream target of the RDN.

RDN transcription factors direct eye formation both in *Drosophila* and in vertebrates (Silver and Rebay, 2005), but *Drosophila* PRs are not evolutionarily homologous to the main types of vertebrate PRs: rods and cones. *Drosophila* PRs are called 'rhabdomeric PRs', and they have a different phototransduction machinery from that of rods and cones, which are called 'ciliary PRs' (Fain et al., 2010). My work shows that vertebrates do not have a clear Glass homologue, rather, vertebrate ciliary PRs require the transcription factor cone-rod homeobox protein (CRX) for the expression of their phototransduction proteins (hence, CRX plays in these cells a similar role to that of Glass in the *Drosophila* compound eye,) (Brzezinski and Reh, 2015; Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997; Furukawa et

al., 1999; Peng and Chen, 2005). It is not yet understood how the *Crx* promoter is activated: the RDN member PAX6 (which is the mammalian homologue of Eyeless) represses *Crx* expression (Brzezinski and Reh, 2015; Oron-Karni et al., 2008). Therefore, my work describes for the first time a series of direct transcriptional links between the processes of eye development and PR formation (that is: the RDN component Sine oculis activates *glass*, Glass activates *hazy*, and both Glass and Hazy activate the expression of phototransduction proteins).

5.5. Outlook

Can the results of my Ph.D. help to point the direction of future research? Related to the topic of PR development, I expect that one of the most interesting questions that will be investigated in the future is the evolutionary conservation of this process. My work shows that vertebrates lack a clear Glass homologue, and, in addition, Glass is not expressed in *Platynereis* rhabdomeric PRs (which are presumably homologous to *Drosophila* PRs). Therefore, if we aim at understanding the general principles that underlie PR formation across metazoans, it will be crucial to obtain more information about how this transcriptional pathway works. At the moment – with the exception of the RDN transcription factors – we have little information that can be compared between different species. For example, the RDN acts through Glass in *Drosophila*, but independently of Glass in *Platynereis* rhabdomeric PRs, and, for this reason, it will be interesting to explore what other RDN targets contribute to regulating the expression of phototransduction proteins. There is high-throughput information available about which genes are induced upon ectopic expression of RDN components (including both Eyeless and Sine oculis), and also ChIP-seq data about the binding sites of Sine oculis (Jusiak et al., 2014; Michaut et al., 2003). Notably, the zinc finger transcription factor Lola, which is a target of Sine oculis, has already been investigated. *lola* RNAi knock-down causes a reduction of some PR markers in the *Drosophila* ocelli (including the transcription factor Hazy, and also the phototransduction components NorpA, Trpl, InaD and Gαq) (Mishra et al., 2016). This indicates that Lola functions in parallel with Glass during PR differentiation. Therefore, it will be interesting to test if Lola also contributes to the development of PRs in other species (e.g. in *Platynereis*), and to screen further RDN targets for a role in regulating PR maturation.

Also, Glass does not entirely depend on Hazy for initiating the expression of phototransduction proteins in *Drosophila*, given that the retina of young *hazy* mutant flies correctly expresses most phototransduction components (Bernardo-

Garcia et al., 2016; Bernardo-Garcia et al., 2017). It will be interesting to investigate deeper how Glass directs PR differentiation: it could be possible that Glass directly binds and activates the promoters of phototransduction proteins, or that it regulates the expression of additional intermediary transcription factors. To investigate this, Glass targets could be identified by using either ChIP-seq or the TaDa technique (Marshall et al., 2016; Ozsolak and Milos, 2011). Moreover, it would also be interesting to investigate if Glass activates the same targets in different cell types: apart of the visual system, Glass is also required for the development of the corpus cardiacum (an endocrine organ) (De Velasco et al., 2004; Park et al., 2011), and it is expressed in some neurons in the *Drosophila* brain, including a subset of adult DN1 cells (which are involved in circadian rhythmicity) (Ellis et al., 1993; Klarsfeld et al., 2004; Shafer et al., 2006). However, we still know very little about the function of Glass outside the PRs.

To conclude, the work that I have done during my Ph.D. places Glass in a key position for the terminal differentiation of *Drosophila* PRs, and I expect that these results will serve to inspire future research. Much about the development of vision remains an open field for scientific exploration.

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CURRICULUM VITAE

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Personal information

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Date of birth: 25th July, 1988

Education

Ph.D. in Natural Sciences (I am currently finishing it). University of Fribourg, Switzerland (February 2013–present).

M.Sc. in Neurosciences. University of Salamanca, Spain (July 2012).

B.Sc. in Biology. University of Salamanca, Spain (February 2012).

B.Sc. in Biochemistry. University of Salamanca, Spain (June 2011).

Publications

Bernardo-Garcia, F. J., Humberg, T.-H., Fritsch, C. and Sprecher, S. G. (2017). Successive requirement of Glass and Hazy for photoreceptor specification and maintenance in *Drosophila*. Fly 11, pp. 112–120.
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► One of my images for this paper got the cover of Fly.

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. (2016). The transcription factor Glass links eye field specification with photoreceptor differentiation in *Drosophila*. Development 143, pp. 1413–1423.
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Honours and awards

SNF Early Postdoc Mobility grant, to do a research project on neuronal homeostasis in G. Davis' lab, at the University of California, San Francisco $78,550 (November 2017).

Best talk prize (2nd position), received at the Swiss *Drosophila* Meeting (Bern, June 2017).

Travel grant, awarded by the LS^2 Society to attend the Neurofly Meeting in Crete, CHF1,200 (July 2016).

Best poster prize, it was obtained at the Swiss *Drosophila* Meeting (Lausanne, April 2015).

Relevant experience

Visiting researcher in the laboratory of A. Huber. I investigated the ability of several *Drosophila* stocks to detect light by performing electroretinograms. Particularly, I tested *glass* mutant alleles, which were generated with the CRISPR–Cas9 technique (University of Hohenheim, Germany, November 2017).

Visiting researcher in G. Jékely's lab. Here, I assessed whether the Glass protein plays an evolutionarily conserved function for photoreceptor development in *Platynereis* (Max Planck Institute for Developmental Biology, Germany, January–June 2016).

Erasmus Placement in the group of A. Prokop. I engaged in a research project on the role of microtubule acetylation in neurons, using *Drosophila* as model organism (The University of Manchester, United Kingdom, June–November 2012).

Collaborator student in the group of E. Saldaña. Before I started studying a master's degree, I undertook a research project on the neuronal pathways of the rat auditory brain (Neuroscience Institute of Castilla y León, Spain, August 2010–September 2011).

Internship in an Animal House for Genetically Modified Organisms. I worked in the cleaning and maintenance of transgenic SPF mice stocks, and was trained in the caretaking of rats, rabbits, and frogs (University of Salamanca, Spain, September–December 2008).

Techniques

- *Drosophila* genetics, microdissections, cryosections and calcium imaging.
- Neuronal cell culture from *Drosophila* embryos.
- Antibody staining and *in situ* hybridisation of *Drosophila* samples.
- Confocal imaging.
- Molecular biology: PCR, cloning...
- Stereotactic surgery for tracer injections in the rat brain. Also, I can do perfusion fixation and histological procedures for Nissl staining, and I have experience on identifying specific anatomical regions in the mammalian brain.
- *Platynereis* microinjections.
- *Drosophila* electroretinogram.

Supervision of students

I have supervised **Maryam Syed**, a student who joined our lab for an **internship** before starting her Ph.D. elsewhere. From **January to June 2015**, I was responsible for teaching her how to work with *Drosophila* as well as for guiding her in her project. She was successful in setting up a technique to perform *in situ* hybridisation on cryosections.

Teaching activities

I have presented two talks for students:

- 'The transcription factor Glass links eye field specification with photoreceptor differentiation in *Drosophila*', at the Systems Biology of the Brain course by the StarOmics doctoral programme (University of Fribourg, August 2015).
- 'Regulation of photoreceptor cell fate in *Drosophila*', at the A Bern–Fribourg Colloquium on Development course for master's students (University of Fribourg, April 2014).

In the case of the talk that I presented in 2014, master's students had to write a summary about my presentation, and I was responsible for correcting and evaluating their works.

Contributions to conferences

I have given two oral presentations:

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the Swiss *Drosophila* Meeting (Bern, June 2017).

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the French *Drosophila* Meeting (Grasse, September 2016).

I have presented the following posters:

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the European *Drosophila* Research Conference (London, September 2017).

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the French *Drosophila* Meeting (Grasse, September 2016).

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the German *Drosophila* Meeting (Cologne, September 2016).

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'The transcription factor Glass links eye field specification with photoreceptor differentiation'. In the Neurofly Meeting (Crete, September 2016).

Bernardo-Garcia, F. J., Fritsch, C. and Sprecher, S. G. 'Glass regulates photoreceptor identity in the *Drosophila* eye'. In the Swiss *Drosophila* Meeting (Lausanne, April 2015).

Bernardo-Garcia F. J., Sprecher S. G. 'Regulation of photoreceptor cell fate in *Drosophila*'. In the Swiss *Drosophila* Meeting (Fribourg, June 2014).

Bernardo-Garcia F. J., Sprecher S. G. 'Studying cell fate determination and differentiation in *Drosophila* photoreceptors'. In the Swiss *Drosophila* Meeting (Zurich, June 2013).

Bernardo-Garcia F. J., Saldaña E. 'Sources of projections to the auditory thalamus in the rat. II. Projections of the dorsal cortex of the inferior colliculus'. In the FENS Forum of Neuroscience (Barcelona, July 2012).

Memberships in scientific societies

Member of the Community Advisory Group of Flybase, since November 2017

Member of the Life Sciences Switzerland (LS^2) society, since November 2013

Language skills

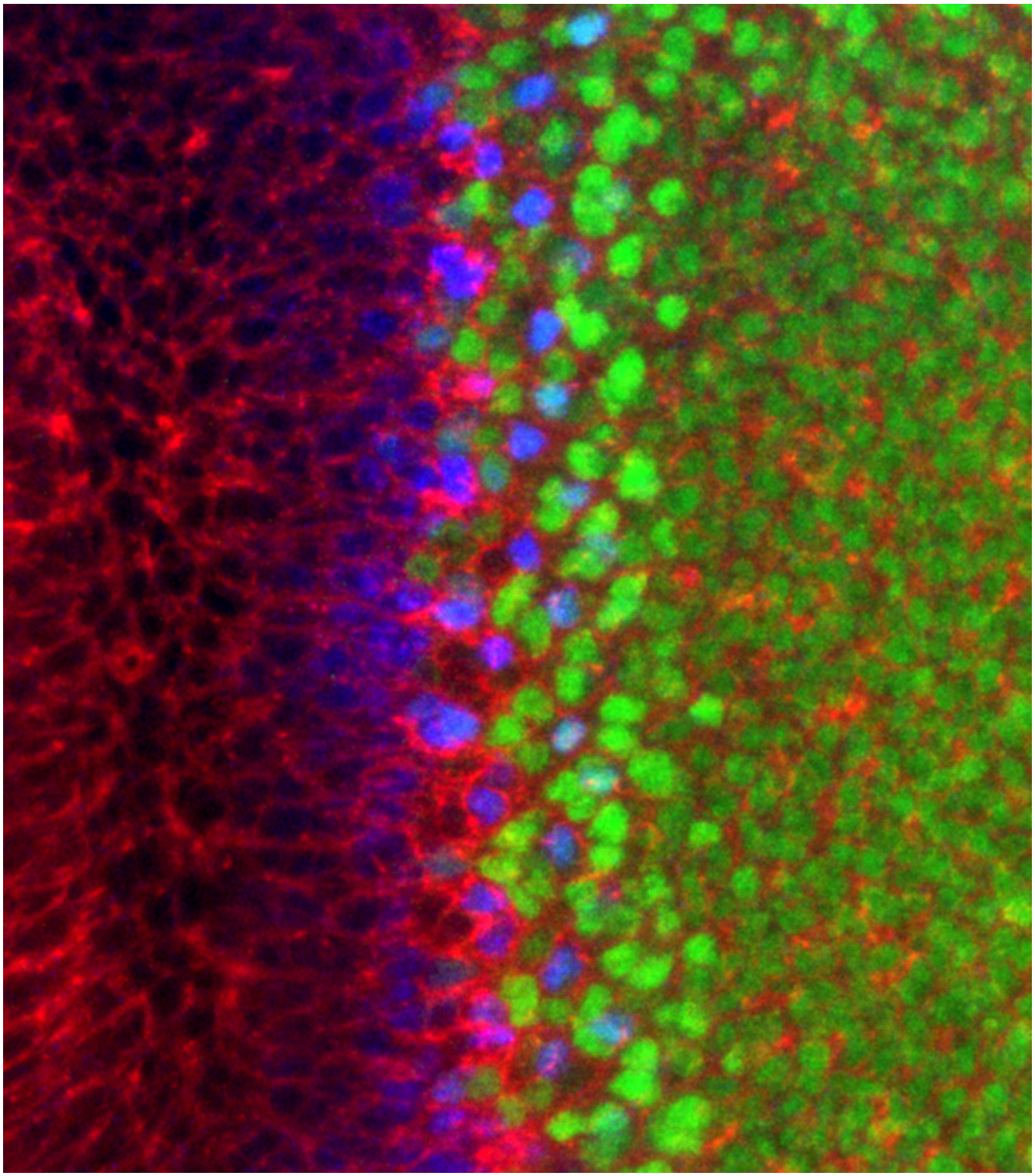
Spanish: native speaker

English: excellent command (total score of 7.0 in IELTS test, October 2012)

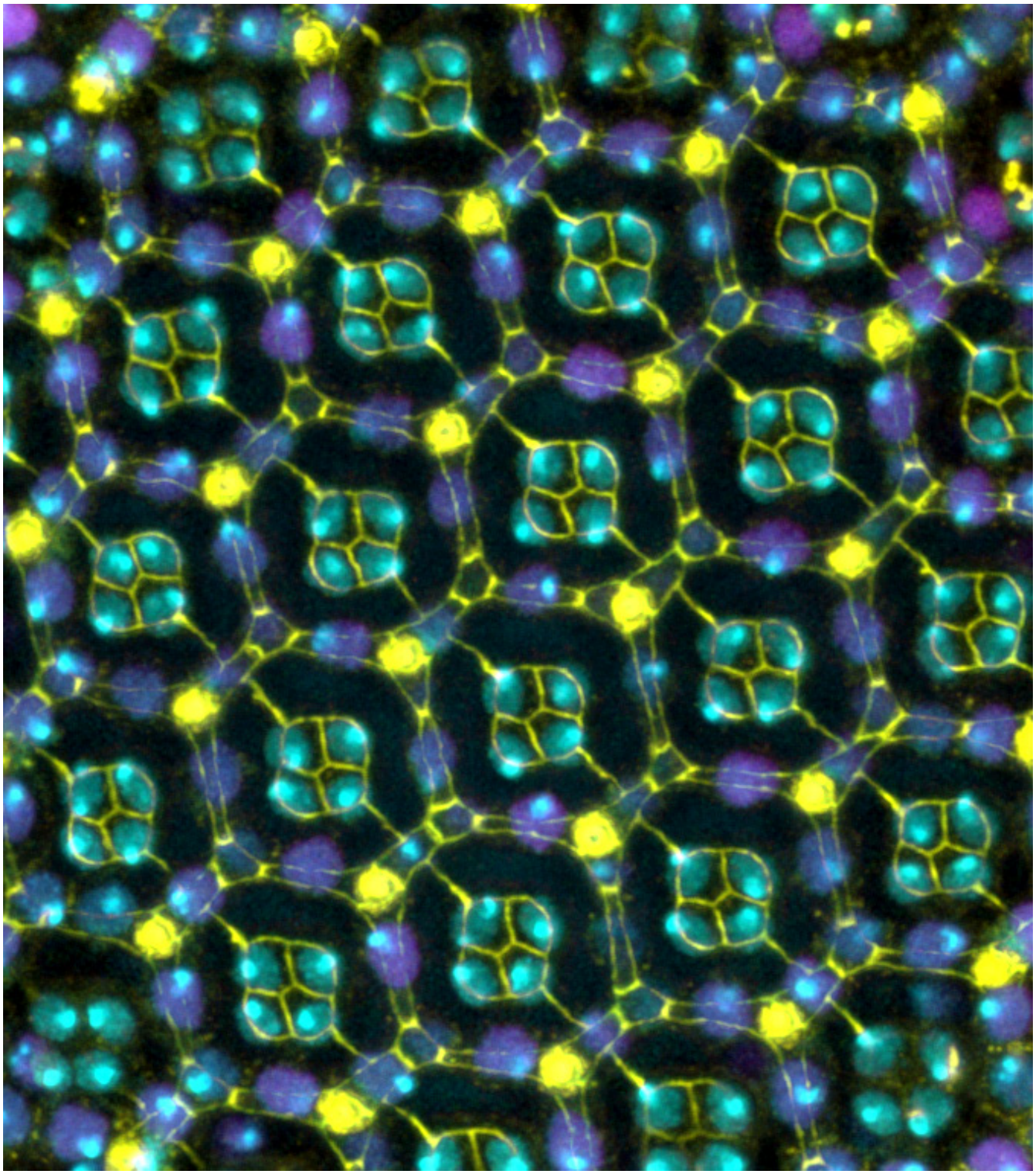
German: basic communication skills in German (B1 in BULATS test, taken on December 2016, currently studying the B2 level at the Migros Klubschule)

French: basic communication skills

***hazy>mCD8::GFP*, late *Drosophila* embryo**. I immunostained this specimen with antibodies against GFP (yellow), Fas2 (cyan) and Elav (magenta).



Third instar eye imaginal disc of a CantonS *Drosophila* larva. Stained for Glass (green) and Ato (blue). Actin was labelled by using phalloidin (red).



***Drosophila* retina belonging to a late *glass-DsRed* pupa**. I stained it by using Hoechst (cyan) and also antibodies against RFP (magenta) and E-cadherin (yellow).

***salm>H2B::YFP, Syt::eGFP, mCD8::Cherry* brain and retina of a late *Drosophila* pupa**. It was immunostained with antibodies against YFP/GFP (green), Cherry (red) and Elav (blue).

► **This image was selected as a cover in Fly (volume 11, issue 2, 2017)**. Nowadays it decorates Simon's lab, and also a meeting room of the SNF.

Simon's lab in October 2013, during our retreat at the Moulin de Bayerel. From left to right, on the front row: Boris, Abhishek, Simon, Sören, Michael, Yves, Yvonne and Lena. On the back: Silvia, I, Martin, Tim, Nanae, Christophe, Oriane, Pauline and Cornelia.

Lab members inside the lab, on December 2017. From left to right, on the front: Boris, Simon, Larisa, Cornelia and Clarisse. On the back: I, Tim, Jenifer, Jules and Yves.