Glass confers rhabdomeric photoreceptor identity in *Drosophila*, but not across all metazoans

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**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 possess an evolutionarily conserved phototransduction cascade. While the development of rhabdomeric PRs has been thoroughly studied in the fruit fly *Drosophila melanogaster*, we still know very little about how they form in other species. To investigate this question, we tested whether the transcription factor Glass, which is crucial for instructing rhabdomeric PR formation in *Drosophila*, may play a similar role in other metazoans. Glass homologues exist throughout the animal kingdom, indicating that this protein evolved prior to the metazoan radiation. Interestingly, our work indicates that *glass* is not expressed in rhabdomeric photoreceptors in the planarian *Schmidtea mediterranea* nor in the annelid *Platynereis dumerilii*. Combined with a comparative analysis of the Glass DNA-binding domain, our data suggest that the fate of rhabdomeric PRs is controlled by Glass-dependent and Glass-independent mechanisms in different animal clades.

**Keywords:** Glass, Transcription factor, *Drosophila*, *Schmidtea*, *Platynereis*, Photoreceptor development, Rhabdomeric photoreceptors, Eye development, Evolutionary conservation

**Introduction**

Most animals can detect visual cues, which provide them with detailed information about their environment. This information may include the shape of nearby objects, colours, movements, or 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 \(1, 2\), which play different roles in different animal species. For instance, rhabdomeric PRs are critical for image-forming vision (e.g. in *Drosophila* compound eye PRs) and for identifying the direction of a light source (e.g. in the planarian *Schmidtea mediterranea* and in the annelid *Platynereis dumerilii*) \(3–5\). Nevertheless, in the case of most metazoan clades, very little is known about how rhabdomeric PRs develop.

Interestingly, all known rhabdomeric PRs appear to use a similar assortment of phototransduction proteins. These PRs possess rhabdomeric-type opsins that can modify their spatial conformation upon light stimulation, which allows them to activate G\(\alpha\). G\(\alpha\) signals through phospholipase C (PLC) causing the opening of cation channels on the cytoplasmic membrane of PRs and thus leading to the formation of action potentials. This light-sensing machinery is present in distantly related animal phyla \(1, 6, 7\), including vertebrates (due to the ‘intrinsically photosensitive retinal ganglion cells’, ipRGCs \(8\)), 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? Using the fruit fly *Drosophila melanogaster* as a model system, we have recently shown that the zinc finger transcription factor Glass confers rhabdomeric PRs in *Drosophila*. However, we still know very little about how they form in other species. To investigate this question, we tested whether the transcription factor Glass, which is crucial for instructing rhabdomeric PR formation in *Drosophila*, may play a similar role in other metazoans. Glass homologues exist throughout the animal kingdom, indicating that this protein evolved prior to the metazoan radiation. Interestingly, our work indicates that *glass* is not expressed in rhabdomeric photoreceptors in the planarian *Schmidtea mediterranea* nor in the annelid *Platynereis dumerilii*. Combined with a comparative analysis of the Glass DNA-binding domain, our data suggest that the fate of rhabdomeric PRs is controlled by Glass-dependent and Glass-independent mechanisms in different animal clades.

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factor Glass acts as critical cell fate selector by directing the maturation of PR precursors into adult, light-sensing PRs. In *Drosophila*, Glass is required for the expression of virtually all the phototransduction proteins [9], and it regulates the development of all types of rhabdomeric PRs (including those in the Bolwig organ, the ocelli, and the compound eye) [10–12]. Therefore, we investigated whether Glass may also be involved in rhabdomeric PR differentiation in other animal species.

The planarian *Schmidtea* and the annelid *Platynereis* are emerging model organisms whose visual systems have been well characterised [3, 5, 13–19]. Interestingly, are emerging model organisms whose visual systems in situ hybridisation we could not detect glass expression in rhabdomeric PRs in this species. Moreover, using *Schmidtea* data of *Drosophila* identity in Glass is critical for the specification of rhabdomeric PRs in *Platynereis*. Thus, while Glass is critical for the specification of rhabdomeric PR identity in *Drosophila*, the absence of Glass in rhabdomeric PRs in *Schmidtea* and *Platynereis* supports that different genetic programmes are required for controlling rhabdomeric PR cell fate in different animal clades. Therefore, while the initial specification of the eye field appears to be controlled by an evolutionarily conserved group of transcription factors (called the retinal determination network, RDN) [17, 20, 21], the subsequent steps that diversify distinct cell types, including rhabdomeric PRs, are likely controlled by diverse developmental programmes.

Methods

Phylogenetic analysis

We searched for protein sequences similar to *Drosophila* Glass [22] and *Platynereis* Glass [23] (see sequences in Additional files 1 and 2) by using NCBI BLAST [24] and the *Schmidtea mediterranea* Genome Database [25]. Redundant sequences were removed from the collection using CD-HIT with an identity cut-off of 90% [26]. To obtain cluster maps based on all-against-all pairwise similarity, we used CLANS2 with the BLOSUM62 matrix and a p value cut-off of 1e−60 [27]. For phylogenetic tree construction, we selected a non-derived set of sequences from the glass cluster and aligned them with MUSCLE [28]. Sequences shorter than 300 amino acids were removed prior to the alignment. We trimmed the alignments with TriAlign in ‘Automated 1’ mode [29]. We identified the JTT + I + G4 model as the best by IQ-TREE [30]. Maximum likelihood trees and bootstrap analysis were performed with IQ-TREE. Trees were visualised with FigTree [31] (for the data corresponding to this analysis, see Additional file 3).

Glass-binding site analysis

We examined a subset of Glass-like protein sequences by aligning them with either BLAST [24] or MUSCLE [28], and analysed them with ‘DNA-binding site predictor for Cys2His2 Zinc Finger Proteins’ [32, 33] (for details on the sequences that we used, see Fig. 3 and Additional file 4). To investigate the DNA-binding specificity of each of these candidates, we copied its full amino acid sequence as input and asked the software to search for Cys2His2 domains [32]. Then, we predicted the binding sites for those regions that best aligned with the fourth and the fifth zinc fingers of Glass, which are responsible for recognising its targets in vivo [34–37]. We used ‘expanded linear SVM’ as prediction model.

Animal caretaking

*Drosophila melanogaster* stocks were cultured at 25 °C in a 12:12 h 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) [38] and *IAS-mCD8::RFP* (Bloomington Stock Center, no. 32219).

Our wild-type *P. dumerili* 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) [14]. These animals were kept in sea water at 22 °C, in a 16:8 h 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 [39]. The larvae that hatched from these eggs were kept at 18 °C.

Immunohistochemistry and in situ hybridisation

In the case of *Drosophila* antibody staining, these were performed on cryosections of *glass>mCD8::RFP* flies, as previously described [9, 40]. We dissected heads (removing the proboscis to improve the penetration of our reagents) and fixed them for 20 min 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 mounted our samples by using Vectashield that contained DAPI (Vector, H-1200) and took images with a Leica SP5 confocal microscope.

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 above for antibody staining) and ordered a mix of labelled RNA probes against glass from Affimetrix. Then, we processed the samples by digesting them with protease QF, and washed with PB and with various commercial solutions included in the kit. We incubated our cryosections with the glass probes for 2 h, 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. Imaging was done with a Leica SP5 confocal microscope.

To perform double in situ hybridisation in Platynereis, we followed—with few modifications—a protocol that has been previously used for characterising the expression pattern of r-opsin1 [16, 41]. For the present work, we also produced an RNA probe against the glass transcript (for details on the glass probe, see Additional file 1). 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. These larvae were prehybridised at 65 °C by using a hybridisation mix (Hyb-Mix), containing 50% formamide, 5× 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 glass (labelled with either fluorescein-UTP or digoxigenin-UTP) in Hyb-Mix, denatured them at 80 °C for 10 min, 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 series of solutions that initially contained 50% formamide and 2× SSCT (obtained from a stock solution with Tween 20 0.1% in 4× 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 immunostain them. We detected the two probes sequentially, by using peroxidase-conjugated primary antibodies against fluorescein (1:250, Roche) and digoxigenin (1:50, Roche). To detect the first probe, we incubated our samples overnight at 4 °C 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 by using a fluorescence microscope, and, when it was ready, we washed in TNT and stopped the peroxidase activity with H2O2. 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 (example confocal stacks can be found within the Additional file 5).

Microinjection of glass-Tomato

We used an unpublished assembly of the Platynereis genome (courtesy of D. Arendt, EMBL Heidelberg) for making a glass-Tomato reporter (see Additional files 1 and 2 for details). We PCR-amplified a fragment of the Platynereis glass promoter and cloned it into a plasmid in frame with the tandem dimer version of Tomato (courtesy of L. A. Bezares-Calderón) by using sticky end ligation with Apal and SgI [42]. The fragment that we cloned included a 5789 bp long upstream sequence and also the beginning of the Glass coding sequence: the first ATG codon was predicted both by aligning the Platynereis version of Glass to the Glass homologues of other species and by using the ATGpr software [43, 44]. For details on the plasmid that we injected, see its annotated sequence in Additional file 6.

For microinjections, we collected freshly fertilised Platynereis eggs and proceeded as previously described [14]. 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 micropipettes (Eppendorf). Larvae were kept at 18 °C, and we imaged them with a Leica SP8 confocal microscope to study the expression of the reporter (representative confocal stacks are available in the Additional file 5). The expression of this reporter showed some degree of mosaicism, given that it was not integrated into the genome, which allowed us to investigate the morphology of the individual neurons that expressed it. We investigated over 100 surviving, Tomato-positive Platynereis larvae.
Results

Glass homologues are present throughout metazoans

Glass plays a fundamental role for the differentiation of rhabdomeric PRs in the fruit fly [9, 11, 45, 46]. To investigate whether it provides a similar function across metazoans, we first decided to search for Glass homologues in other species.

To do this, we obtained Glass-like sequences by using NCBI BLAST [24] and the Schmidtea mediterranea Genome Database [25]. We analysed these sequences with the CLANS2 software (using the BLOSUM62 matrix and a p value cut-off of 1e−60) to produce a cluster map (Fig. 1a) [27]. In this type of graph, closely related sequences (represented as points) are clustered together and connected by dark lines. Based on their similarities, we were able to identify multiple Glass homologues across distantly related species. Some more derived sequences (e.g. from Strongylocentrotus and Saccoglossus) were also clearly supported as Glass homologues in our analysis. Using these data, we constructed a maximum likelihood phylogenetic tree for Glass, which was visualised with FigTree (Fig. 1b) [31] (for more details on our analysis, see the “Methods” section and the Additional file 3). Importantly, our data reveal that Glass homologues are widely present throughout the animal kingdom.

Neither vertebrates nor choanoflagellates have clear Glass homologues

Based on the distribution of Glass homologues, it seems this protein was present in the common ancestor of all metazoans, but not in choanoflagellates (the sister group of metazoans). Intriguingly, we could also not find any Glass homologue in vertebrates (Fig. 2). Since we identified Glass across multiple animal clades, we wondered why we could not find its vertebrate homologue. Several species have fully sequenced, well-annotated genomes, such as zebrafish, mice, or humans [47–51]. For this reason, we decided to further investigate the evolutionary conservation of Glass by scrutinising its sequence. Intriguingly, some cells in the vertebrate retina also use the rhabdomeric phototransduction cascade—the ipRGCs, which detect irradiance [8]—and, based on our data, it seems highly probable that these cells develop through different mechanisms in Drosophila and in vertebrates.

Glass is not expressed in rhabdomeric PRs in the Schmidtea eye

Given that Glass is an essential transcription factor for activating the expression of phototransduction proteins in all Drosophila PRs [9, 10], we investigated whether Glass has a similar function in other organisms. For this, we tested whether it is expressed in PRs in the eye of the planarian Schmidtea mediterranea. Planarians typically possess one pair of eyes, located in the head, that mediate light avoidance [5, 17, 53]. Importantly, their eyes contain...
Fig. 2. Glass homologues exist in most animal groups. Based on sequence comparison (Additional file 4, also see Fig. 3), we infer that glass appeared in the common ancestor of all metazoans, and that it has been transmitted to most present-day animals (shown in green on the phylogenetic tree [74]). However, we were not able to identify glass in vertebrates.

Fig. 3. Analysis of the Glass zinc fingers. Generally, Glass homologues possess a cluster of five Cys2His2 zinc fingers, each of them containing the following motif: Cys-X2,4-Cys-X12-His-X3,4,5-His. Of these, we compared the sequences of the fourth and the fifth zinc fingers, which are responsible for recognising the DNA Glass-binding motif in PRs in vivo [34–37], from the following species: Amphimedon (Porifera), Schmidtea (Platyhelminthes), Platynereis (Annelida), Aplysia (Mollusca), Caenorhabditis (Nematoda), Drosophila (Arthropoda), Strongylocentrotus (Echinodermata) and Branchiostoma (Cephalochordata). In the table, those amino acids that match the Glass consensus sequence (deduced by aligning the homologues of different species, in the first column) appear on black background. The 3D structure of the DNA-bound Cys2His2 domain has been resolved [75], and it is expected that four amino acids per zinc finger directly recognise three base pairs. These amino acids are well evolutionarily conserved across different Glass homologues and, in the sequences that we show, they are no. 10 (D), 12 (S), 13 (T), and 16 (K) in the fourth zinc finger, and no. 38 (Q), 40 (G), 41 (N), and 44 (R) in the fifth zinc finger. Other residues and neighbouring zinc fingers are also expected to contribute to the DNA-binding specificity of Glass [76]. Similarly, we aligned Glass-like proteins from vertebrates (e.g. human) and choanoflagellates (e.g. Salpingoeca) with BLAST [24] and MUSCLE [28], but they showed little similarity to the Glass consensus sequence (shown in the second column). Furthermore, a ‘DNA-binding site predictor for Cys2His2 Zinc Finger Proteins’ has been developed and is available online [32, 33]. This software predicts that, based on their amino acid sequence, all Glass homologues (in the first column) can bind to the same DNA motif: GAA GCC, which was expected from experimental works in Drosophila and Caenorhabditis [34, 35]. By contrast, it appears that the Glass-like proteins of vertebrates and choanoflagellates (in the second column) would not be able to recognise this motif. All sequences are available in the Additional file 4.
rhabdomeric PRs, which are evolutionarily homologous to Drosophila PRs [1, 17].

Recently, a single-cell transcriptome atlas has been published for Schmidtea, and it is available online [18, 19, 54]. Using this database, rhabdomeric PRs can be identified because they form a cluster of non-ciliated neurons that express phototransduction proteins, including the opsin gene (Fig. 4a) [19]. Surprisingly, these cells do not co-express Glass (Fig. 4b), suggesting that, in contrast to Drosophila, Glass is not important for the function of rhabdomeric PRs in the Schmidtea eye.

glass is not expressed in rhabdomeric PRs in the Platynereis eye

We next tested whether Glass is expressed in rhabdomeric PRs in the marine ragworm P. dumerilii. The visual system of Platynereis 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 [3, 13–16].

In Drosophila, glass is expressed in all rhabdomeric PRs [12, 55]. We could 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 (Fig. 5a–b′), which confirms previous data [12, 55]. 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 [14, 16], indicating that glass is not present in these cells (Fig. 5c–c‴‴, also see confocal stacks in Additional file 5). In addition, we also generated a Platynereis glass reporter by cloning 5.7 kb of its upstream sequence into a plasmid, where the glass start codon was in frame with Tomato (a red fluorescent protein). We used this plasmid for transient transgenesis by injecting it in 1-cell embryos containing a stable r-opsin1-GFP insertion [14]. r-opsin1-GFP animals consistently showed strong GFP signal in their dorsal eye PRs, and this signal was weaker in the ventral eye PRs. 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 [3, 14, 16]. 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. The expression of glass-Tomato showed some degree of mosaicism due to this reporter not being integrated into the genome, which allowed us to observe the morphology of individually labelled cells in detail. 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 (Fig. 5d–d‴, confocal stacks are available in Additional file 5), 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.

Glass is expressed in Platynereis sensory neurons

Since glass is predominantly expressed in PRs in Drosophila, we wondered what types of cells express glass in Platynereis. We observed that most of the neurons that were labelled with the glass-Tomato reporter innervated the neurosecretory neuropil (which is ventral to the optic neuropil, Fig. 5d–d‴) [56], and, interestingly, many of them were bipolar neurons (Fig. 6). These two features are relevant because an ongoing electron microscopy (EM) connectome reconstruction shows that, in Platynereis larvae, most neurons are either unipolar or pseudounipolar [3, 56–59]. Based on their position and on their morphology, all bipolar neurons in this EM reconstruction are considered sensory neurons because they possess distinctive membranous specialisations (called sensory dendrites) that project towards the surface [3, 56–59]. Therefore, it is very likely that a subset of glass-expressing cells in Platynereis are sensory neurons.

The neurosecretory neuropile of Platynereis contains multiple sensory neurons, and it has been characterised both from an anatomical and a molecular point of view [56]. However, it is still unknown whether this region is homologous to any structure of the Drosophila brain. Given that glass is also required for the development of the corpora cardica in Drosophila [60], it could be possible that Glass has an evolutionarily conserved function in neurosecretory cells. In addition, it could also be that Glass regulates the formation of other sensory neurons. Notably, the Caenorhabditis homologue of Glass (called CHE-1) is expressed in ASE chemosensory neurons, and it regulates their development [34, 61].
Conclusions

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 [20, 62–68]. RDN members, such as Eyeless, Sine oculis, or Eyes absent are important for inducing eye field specification. To achieve this, they establish complex epistatic interactions with each other. These interactions occur similarly across model organisms, suggesting that this is an evolutionarily conserved process [20, 69]. In contrast to the early steps of eye field specification, subsequent mechanisms that specify the cell fate of PRs are not well understood. Here, we provide evidence that, during the late stages of eye development, rhabdomeric PRs mature through different mechanisms in different species.

In *Drosophila*, we have recently shown that Sine oculis (a core component of the RDN) 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 [9, 10, 70]. Based on similarities in their light-sensing machinery, *Drosophila* PRs are considered homologous to the iPRGCs of vertebrates, and also to the rhabdomeric PRs that exist in *Schmidtea* and *Platynereis* [1, 6, 7, 15, 17, 19]. Intriguingly, while we did identify Glass homologues in most metazoans, we could not find a clear Glass homologue in vertebrates. Moreover, our data indicate that *glass* is not expressed in the rhabdomeric PRs of *Schmidtea* or *Platynereis*. This suggests that metazoans 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, *Schmidtea*, or *Platynereis* larvae).

It could be possible that Glass started being expressed in rhabdomeric PRs at some point during the evolution of ecdysozoans and that it became specialised in regulating the differentiation of these cells. Therefore, comparing the differentiation of Glass-expressing and non-Glass-expressing PRs provides a valuable entry point to dissect shared and dissimilar aspects of the developmental programme. Additionally, it would also be interesting to know the identity of Glass-expressing cells for understanding the ancestral function of Glass. The *glass* transcript is rare and lowly expressed in the *Schmidtea* single-cell transcriptome data that we have currently available [18, 19], and it was also lowly expressed in the single-cell transcriptome datasets of *Platynereis*, to the point of being removed from the analyses of the two papers in which the sequencing was published [71, 72], which makes it impossible to compare the function of *glass*-expressing cells between different species at this moment. It could be possible that this is because only a few cells in the brain express Glass, and these may not have been included in the samples that were sequenced. Therefore, we expect that, in the near future, increasing both the number and the quality of such single-cell transcriptomes for these and other species will be useful to address several questions about the evolution of specific

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**Fig. 4** *glass* is not expressed in rhabdomeric PRs in *Schmidtea*. These graphs were obtained from the Planarian Digiworm atlas, a single-cell transcriptome database for *Schmidtea mediterranea* [19, 25]. Each point corresponds to one single cell, and they are clustered according to the similarity of their transcriptome. One of the clusters shown, corresponding to non-ciliated neurons, is formed by 14 rhabdomeric PRs which can be identified because of the expression of the opsins gene (dd_Smed_v4_15036_0_1, a). However, these PRs do not appear to express the *Schmidtea* glass homologue (annotated as dd_Smed_v4_75162_0_1 in this website [19, 54], b).
cell fates. For example, some opsins may have other functions apart from light sensing [73], and it would be relevant to know whether glass regulates the expression of any such opsin outside the Platynereis eye (for example), at any stage.

The absence of Glass in rhabdomeric PRs in the eye of some species argues for other transcription factors being capable of activating the expression of phototransduction proteins; however, the underlying mechanism remains unknown. Our data support a rather complex

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**Fig. 5** glass is not expressed in rhabdomeric PRs in Platynereis. a, b glass is present in all Drosophila rhabdomeric PRs, including those in the compound eye [12, 55]. This can be observed in head cryosections, either by using in situ hybridisation (magenta in a and greyscale in a') or with glass>mCD8::RFP flies (magenta in b and greyscale in b'). In both cases, samples were counterstained with DAPI (green). c–e In contrast to Drosophila, double in situ hybridisation against the glass (red) and r-opsin1 (green) transcripts shows that glass is not present in Platynereis rhabdomeric PRs. Samples were counterstained with antibodies against acetylated Tubulin (ac-Tub, blue), which is a neuropil marker. c Transversal view of a whole-mounted, 5-day old larva. To the right, close-ups of the dorsal (arrow in c, c', c'') and ventral eyes (arrowhead in c, c'', c''') show that glass (in magenta/greyscale) is not expressed in either of these visual organs. Similarly, we found that a microinjected glass-Tomato reporter (magenta/greyscale) was not co-expressed with a stable r-opsin1-GFP insertion (green). Brightfield (BF, greyscale) was imaged as a reference (d–d'', dorsal view of a whole-mounted, 8-day-old larva). 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'', d'''; orthogonal views taken along the Z segment are shown below). As a control, we also imaged an 8-day-old, wild-type, uninjected larva to test its autofluorescence (using two excitation laser wavelengths: 552 nm, same as for Tomato; and 488 nm, same as 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; V, ventral.
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.

Additional files

Additional file 1. Supplementary methods.
Additional file 2. Platynereis glass supplementary nucleotide sequences (both genomic and transcriptomic).
Additional file 3. Glass phylogenetic tree and sequences data.
Additional file 4. Subset of glass-like sequences for which the DNA-binding affinity was investigated.
Additional file 5. Example confocal stacks.
Additional file 6. Annotated sequence of the glass–tomato plasmid that was used for Platynereis microinjections.

Abbreviations
ac-Tub: acetylated Tubulin; EM: electron microscopy; PB: phosphate buffer; PBT: phosphate buffer with Triton X-100; PR: photoreceptor neuron; RDN: retinal determination network; r-opsin1: rhabdomeric opsin 1; SSC: saline-sodium citrate buffer; SSCT: saline-sodium citrate buffer with Tween 20.

Authors’ contributions
FJB-G, GJ, and SGS conceived the study. FJB-G, GJ, and MS performed the experiments. FJB-G, GJ, and SGS wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors do not declare competing or financial interests.

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All data presented in this article are accessible online, as indicated in either the main text or in the Additional file section.

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