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 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 mediteranea* 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 Gαq. Gαq 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 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, by analysing recently published single-cell sequencing data of *Schmidtea* we found that *glass* is not expressed in rhabdomeric PRs in this species. Moreover, using *in situ* hybridisation we could not detect *glass* expression in 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

Glass sequence conservation analysis

We used BLAST [22] to retrieve NCBI-deposited sequences belonging to clear Glass homologues from several species, including *Amphimedon queenslandica*, *Aplysia californica*, *Caenorhabditis elegans*, *Strongylocentrotus purpuratus*, and *Branchiostoma belcheri*. Details about these sequences appear in the text and in Figure 2. Also, we found a Glass homologue in the genome of *Schmidtea mediterranea* [23] and in the transcriptome of *Platynereis dumerilii* [24]. For more information about the *Platynereis* Glass homologue, see Additional file 1: Supplementary methods and sequences.

We used either BLAST [22] or MUSCLE [25] to align protein sequences, and analysed them with 'DNA-binding site predictor for Cys_2His_2 Zinc Finger Proteins' [26, 27]. To investigate the DNA-binding specificity of each candidate, we copied its full amino acid sequence as input, and asked the software to search for Cys_2His_2 domains [26]. Then, we predicted the binding sites for those regions that best aligned with the 4th and the 5th zinc fingers of Glass, which are responsible for recognising its targets *in vivo* [28-31]. We used 'expanded linear SVM' as prediction model.

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) [32] 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) [14]. 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 [33]. The larvae that hatched from these eggs were kept at 18 °C.

111

112 **Immunohistochemistry and in *situ* hybridisation**

113

114 In the case of *Drosophila* antibody stainings, these were performed on cryosections of
115 *glass>mCD8::RFP* flies, as previously described [9, 34]. We dissected heads (removing the
116 proboscis to improve the penetration of our reagents) and fixed them for 20 minutes with 3.7%
117 formaldehyde in 0.01 M phosphate buffer (PB; pH 7.4). Then, we washed our samples with PBT
118 (Triton X-100 0.3% in PB) and incubated them with a cryoprotectant solution (sucrose 25% in
119 PB) overnight at 4 °C. The following day we embedded the fly heads in OCT, froze them with
120 liquid nitrogen, and cut 14 µm cryosections in the transverse plane. Once the samples were dry,
121 we proceeded to immunostain them. For this, we washed the slides with PBT (this buffer was
122 also used in subsequent washing steps) and incubated them in primary antibody (rabbit anti-
123 DsRed, 1:100, Clontech, no. 632496) at 4 °C overnight. Then, we washed the cryosections and
124 incubated them in secondary antibody (goat anti-rabbit conjugated to Alexa Fluor 568, 1:200,
125 Molecular Probes, no. A-11011) at 4 °C overnight, and washed again the next day. We mounted
126 our samples by using Vectashield that contained DAPI (Vector, H-1200) and took images with a
127 Leica SP5 confocal microscope.

128

129 To detect the *glass* transcript in *Drosophila*, we used the ViewRNA *in situ* hybridisation kit of
130 Affimetrix (no. QVT0012) – which is a proprietary method – and proceeded according to the
131 instructions of the company. Briefly, we took head cryosections (as described above for
132 antibody stainings) and ordered a mix of labelled RNA probes against *glass* from Affimetrix.
133 Then, we processed the samples by digesting them with protease QF, and washed with PB and
134 with various commercial solutions included in the kit. We incubated our cryosections with the
135 *glass* probes for 2 hours, at 40 °C. After this, we continued with a series of washing and signal
136 amplification steps, followed by a colour reaction (we used Fast Red as a fluorophore). We
137 finished by washing the samples with PB, and used Vectashield containing DAPI (Vector, H-
138 1200) to cover the slides. Imaging was done with a Leica SP5 confocal microscope.

139

140 To perform double *in situ* hybridisation in *Platynereis*, we followed – with few modifications – a
141 protocol that has been previously used for characterising the expression pattern of *r-opsin1* [16,
142 35]. For the present work, we also produced an RNA probe against the *glass* transcript (for
143 details on the *glass* probe, see supplementary Materials and Methods). We fixed 3–5 day old
144 larvae in 4% formaldehyde, and we subjected them to a mild proteinase K digestion to improve
145 the penetration of our reagents. These larvae were prehybridised at 65 °C by using a
146 hybridisation mix (Hyb-Mix), containing 50% formamide, 5x saline-sodium citrate buffer (SSC),
147 50 µg/ml heparin, 0.1% Tween 20, and 5 mg/ml torula RNA. Then, we dissolved the probes
148 against *r-opsin1* and *glass* (labelled with either fluorescein-UTP or digoxigenin-UTP) in Hyb-Mix,
149 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 series of solutions 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 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 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.

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. 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) [36]. The fragment that we cloned included a 5,789 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 [37, 38]. For details on the sequence that we cloned, see Additional file 1: Supplementary methods and sequences.

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 microcapillaries (Eppendorf). Larvae were kept at 18 °C, and we imaged them with a Leica SP8 confocal microscope to study the expression of the reporter.

183 **RESULTS**

184

185 **Glass homologues are present throughout metazoans**

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187 Glass plays a fundamental role for the differentiation of rhabdomeric PRs in the fruit fly [9, 11,
188 39, 40]. To investigate if it provides a similar function in other animal clades, we first decided to
189 search for Glass-like sequences outside *Drosophila* by using BLAST [22].

190

191 Intriguingly, we did not find any protein very similar to Glass in choanoflagellates (the sister
192 group of metazoans), nor in vertebrates. However, we were able to retrieve multiple NCBI-
193 deposited sequences belonging to clear Glass homologues. These were distributed across
194 distantly related genera, including *Amphimedon* (Porifera), *Aplysia* (Mollusca), *Caenorhabditis*
195 (Nematoda), *Strongylocentrotus* (Echinodermata) and *Branchiostoma* (Cephalochordata). Also,
196 we found one single Glass homologue in the planarian *Schmidtea mediterranea*
197 (Platyhelminthes) [23] and in the marine ragworm *Platynereis* (Annelida) [24] (Fig. 1, Fig. 2).
198 Therefore, based on sequence alignment, Glass is broadly distributed across metazoans.

199

200 **Neither vertebrates nor choanoflagellates have clear Glass homologues**

201

202 Since we identified Glass in multiple animal clades, we wondered why we could not find its
203 vertebrate homologue. Several species have fully sequenced, well annotated genomes, like
204 zebrafish, mice, or humans [41-45]. For this reason, we decided to further investigate the
205 evolutionary conservation of Glass by scrutinising its sequence.

206

207 Glass homologues share a distinctive cluster of five Cys_2His_2 zinc fingers in most species (one
208 exception is *Caenorhabditis*, in which it only has four zinc fingers because the first one is
209 missing). Particularly, the 4th and the 5th zinc fingers are especially important because they are
210 responsible for guiding Glass towards its targets, given that they recognise its DNA-binding
211 motif *in vivo*, GAARCC [28-31]. Therefore, we modified our bait by using the consensus
212 sequence of either the full cluster of five zinc fingers, or only the 4th and 5th zinc fingers, and
213 we repeated our BLAST search against vertebrates and choanoflagellates. By doing this we
214 obtained results like, for example, ZSCAN22, ZNF253, or KOX 26 in humans, which still showed
215 less similarity to Glass than any of those homologues that we identified in other species (Fig. 2).
216 We also considered the human candidates that appeared annotated as putative Glass
217 orthologues in Flybase via DIOPT [46, 47], including ZNF764, ZNF683, or ZNF500, but,
218 likewise, they aligned poorly with the consensus sequence of the Glass zinc fingers (Fig. 2).
219 Next, we analysed if any of these proteins would be able to functionally substitute Glass by
220 recognising its DNA-binding motif, the GAARCC sequence [28, 29, 31]. For this, we used the
221 online tool 'DNA-binding site predictor for Cys_2His_2 Zinc Finger Proteins', which predicts the

222 DNA-binding behaviour of zinc finger proteins [26, 27]. This software indicates that those Glass-
223 like proteins that exist in vertebrates and choanoflagellates cannot recognise the GAARCC
224 motif, in contrast to the clear Glass homologues that we found in other animals (i.e. in
225 *Amphimedon*, *Schmidtea*, *Platynereis*, *Aplysia*, *Caenorhabditis*, *Drosophila*, *Strongylocentrotus*
226 and *Branchiostoma*) (Fig. 2). Consequently, it remains unclear what happened to the *glass*
227 gene during the evolution of vertebrates: it could be that they lost Glass, or that it severely
228 changed its amino acid sequence and its DNA-binding motif. Intriguingly, similar to *Drosophila*,
229 some cells in the vertebrate retina also use the rhabdomeric phototransduction cascade – the
230 ipRGCs, which detect irradiance [8] – and, based on our data, it seems highly probable that
231 these cells develop through different mechanisms in *Drosophila* and in vertebrates.
232
233 **_glass_ is not expressed in _Schmidtea_ rhabdomeric PRs**
234
235 Given that Glass is an essential transcription factor for activating the expression of
236 phototransduction proteins in all *Drosophila* PRs [9, 10], we investigated whether Glass has a
237 similar function in other organisms. For this, we tested if it is expressed in PRs in the eye of the
238 planarian *Schmidtea mediterranea*. Planarians typically possess one pair of eyes, located in the
239 head, that mediate light avoidance [5, 17, 48]. Importantly, their eyes contain rhabdomeric PRs,
240 which are evolutionarily homologous to *Drosophila* PRs [1, 17].
241
242 Recently, a single-cell transcriptome atlas has been published for *Schmidtea*, and it is available
243 online [18, 19, 49]. Using this database, rhabdomeric PRs can be identified because they form a
244 cluster of non-ciliated neurons that express phototransduction proteins, including the *opsin* gene
245 (Fig. 3A) [19]. Surprisingly, these cells do not co-express Glass (Fig. 3B), suggesting that, in
246 contrast to *Drosophila*, Glass is not important for the function of rhabdomeric PRs in *Schmidtea*.
247
248 **_glass_ is not expressed in _Platynereis_ rhabdomeric PRs**
249
250 We next tested whether Glass is expressed in rhabdomeric PRs in the marine ragworm
251 *Platynereis dumerilii*. The visual system of *Platynereis* has been well studied, both from a
252 molecular and a functional point of view. *Platynereis* possesses two types of bilateral eyes
253 containing rhabdomeric PRs, called the dorsal and ventral eyes (also known as adult and larval
254 eyes, respectively). These two eye types are able to detect the direction of light, thus mediating
255 phototaxis [3, 13-16].
256
257 In *Drosophila*, *glass* is expressed in all rhabdomeric PRs [12, 50]. We could detect *glass*
258 expression in the compound eye of adult flies both with *in situ* hybridisation and with a *glass-*
259 *Gal4* line crossed to *UAS-mCD8::RFP* (Figs. 4A–B′), which confirms previous data [12, 50]. By
260 contrast, in the case of *Platynereis*, *in situ* hybridisations performed in 3–5 day old larvae did not

261 show co-expression of the *glass* transcript with *rhabdomeric opsin 1* (*r-opsin1*), which is a
262 marker for rhabdomeric PRs in both the dorsal and the ventral eyes [14, 16], indicating that
263 *glass* is not present in these cells (Figs. 4C–C′′′′). In addition, we also generated a *Platynereis*
264 *glass* reporter by cloning 5.7 kb of its upstream sequence into a plasmid, where the *glass* start
265 codon was in frame with *Tomato* (a red fluorescent protein). We used this plasmid for transient
266 transgenesis by injecting it in 1-cell embryos containing a stable *r-opsin1-GFP* insertion [14]. *r-*
267 *opsin1-GFP* animals consistently showed strong GFP signal in their dorsal eye PRs, and this
268 signal was weaker in the ventral eye PRs. In the case of the dorsal eyes, all PRs project their
269 rhabdomeres into a pigment cup, and their axons form four nerves that innervate the optic
270 neuropil in the brain [3, 14, 16]. After microinjections, we tested 3–8 day old larvae (slightly
271 older than those that we used for *in situ*, to guarantee that positive cells had enough
272 fluorescence to distinguish them) but we did not observe co-expression of GFP and Tomato.
273 *glass-Tomato*-expressing neurons were consistently located in the head of *Platynereis*, distant
274 from the ventral eyes. Some of these Tomato-positive cells appeared close to the dorsal eyes,
275 but they did not project any rhabdomere-like extension into the pigment cup, and their axons did
276 not innervate the optic neuropil (Figs. 4D–E′′), indicating that they were not part of the eye
277 rhabdomeric PRs. We conclude that, while Glass is expressed in all types of rhabdomeric PRs
278 in *Drosophila*, it is not present in known rhabdomeric PRs in *Platynereis*.
279
280 **Glass is expressed in *Platynereis* sensory neurons**
281
282 Since *glass* is predominantly expressed in PRs in *Drosophila*, we wondered what type of cells
283 express *glass* in *Platynereis*. Most of the neurons that we saw labelled with the *glass-Tomato*
284 reporter innervated the neurosecretory neuropil, a region located posterior to the optic neuropil
285 (also, some Tomato-positive axons innervated a slightly more antero-ventral region, forming a
286 ring) (Figs. 4D–D′′). Interestingly, an ongoing electron microscopy (EM) reconstruction of the
287 *Platynereis* connectome shows that most neurons in the head of *Platynereis* that project to the
288 neurosecretory neuropil are sensory neurons, suggesting that *glass* is likely expressed in
289 sensory neurons [51]. To test this hypothesis, we examined the morphology of the cells that
290 were labelled with the *glass-Tomato* reporter. In EM reconstructions, sensory neurons can be
291 identified because they possess distinctive membranous specialisations that project towards the
292 surface, called sensory dendrites. We observed this type of structures on some *glass-Tomato-*
293 expressing neurons, further supporting that these cells were indeed sensory neurons (Fig. 5).
294 Notably, the *Caenorhabditis* homologue of Glass (called CHE-1) is expressed in ASE
295 chemosensory neurons, and it regulates their development [28, 52]. Therefore, it could be
296 possible that Glass plays a similar function in *Platynereis*.
297

298 ## CONCLUSIONS

299

300 Remarkably, the earliest steps of eye development are controlled by a group of transcription
301 factors, called the 'retinal determination network' (RDN), which is both required and sufficient for
302 eye formation in distantly related species [20, 53-59]. RDN members, such as Eyeless, Sine
303 oculis, or Eyes absent are important for inducing eye field specification. To achieve this, they
304 establish complex epistatic interactions with each other. These interactions occur similarly
305 across model organisms, suggesting that this is an evolutionarily conserved process [20, 60]. In
306 contrast to the early steps of eye field specification, subsequent mechanisms that specify the
307 cell fate of PRs are not well understood. Here we provide evidence that, during the late stages
308 of eye development, rhabdomeric PRs mature through different mechanisms in different
309 species.

310

311 In *Drosophila*, we have recently shown that Sine oculis (a core component of the RDN) directly
312 activates the expression of the transcription factor *glass*, which is crucial for activating the
313 expression of virtually all the phototransduction proteins in all types of *Drosophila* PRs [9, 10,
314 61]. Based on their similar light-sensing machinery, *Drosophila* PRs are considered homologous
315 to the ipRGCs of vertebrates, and also to the rhabdomeric PRs that exist in *Schmidtea* and
316 *Platynereis* [1, 6, 7, 15, 17, 19]. Intriguingly, while we did identify Glass homologues in most
317 metazoans, we could not find a clear Glass homologue in vertebrates. Moreover, our data
318 indicate that *glass* is not expressed in the rhabdomeric PRs of *Schmidtea* or *Platynereis*. This
319 suggests that metazoans have evolved alternative transcriptional pathways to direct the
320 formation of rhabdomeric PRs. One of these pathways requires Glass (e.g. in *Drosophila*), while
321 others do not (e.g. in vertebrates, *Schmidtea*, or *Platynereis*).

322

323 It could be possible that Glass started being expressed in rhabdomeric PRs at some point
324 during the evolution of ecdysozoans and that it became specialised in regulating the
325 differentiation of these cells. Therefore, comparing the differentiation of Glass-expressing and
326 non-Glass-expressing PRs provides a valuable entry point to dissect shared and dissimilar
327 aspects of the developmental programme. The absence of Glass in rhabdomeric PRs in some
328 species argues for other transcription factors being capable of activating the expression of
329 phototransduction proteins, however the underlying mechanism remains unknown. Our data
330 support a rather complex scenario for the evolution of rhabdomeric PRs, but future works on the
331 targets of the RDN may help to better understand how rhabdomeric PR identity is regulated.

332

333 ## ABBREVIATIONS

334

335 ac-Tub: acetylated Tubulin, EM: electron microscopy, PB: phosphate buffer, PBT: phosphate
336 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.

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors do not declare competing or financial interests.

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Authors' 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., G.J., and S.G.S. wrote the manuscript.

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ADDITIONAL FILE

Additional file 1: Supplementary methods and sequences. Contains information on plasmids and DNA/RNA sequences.

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FIGURES

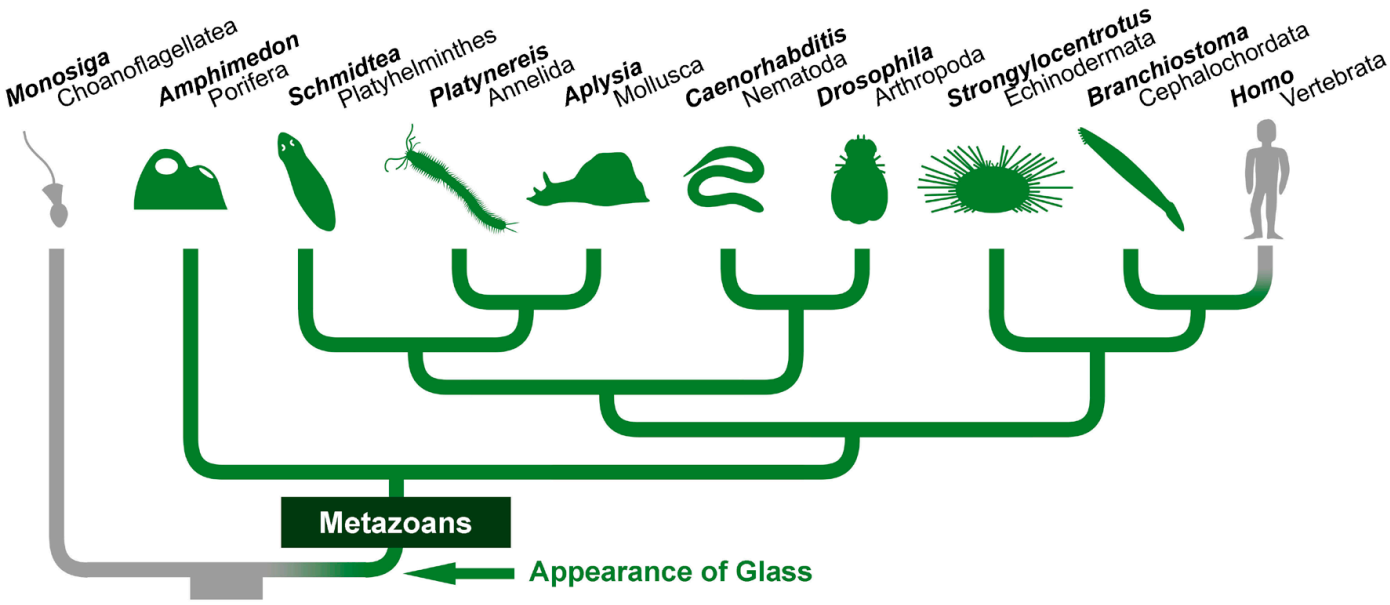

Fig. 1: Glass homologues exist in most animal groups. Based on sequence comparison (see Fig. 2), we infer that *glass* appeared in the common ancestor of all metazoans, and that it has been transmitted to most present-day animals (shown in green on the phylogenetic tree [62]). However, we were not able to identify *glass* in vertebrates.

Glass homologues	
>*Amphimedon*, https://blast.ncbi.nlm.nih.gov/ XP_019864430.1	CDYCGKCFADTSTLTKHKRIHSGEKPYRCKICNLGFSQSGNIHRHMKTH
>*Schmidtea*, http://smedgdkc03.stowers.org/ EGI57499.1	CHHCSKAFSDSSTLTKHTRVHSGEKPYKCRICLLKFSQSGNINRHMRVH
>*Platynereis*, http://jekely-lab.tuebingen.mpg.de/blast/ comp416373_c0_seq8	CRMCKKAFSDSSTLTKHLRIHSGEKPYQCKLCLLRFSQSGNINRHMRVH
>*Aplysia*, https://blast.ncbi.nlm.nih.gov/ XP_005091155.1	CRMCKKAFSDSSTLTKHLRIHSGEKPYQCKLCLLRFSQSGNINRHMRVH
>*Caenorhabditis*, https://blast.ncbi.nlm.nih.gov/ NP_001076597.1, CHE-1	CAQCEKAFTDSSTLTKHLRTHTGHKPYVCSICMMKFTQSGNIHRHMKTH
>*Drosophila*, http://flybase.org/ Glass	CSSCKKSFSDSSTLTKHLRIHSGEKPYQCKLCLLRFSQSGNINRHMRVH
>*Strongylocentrotus*, https://blast.ncbi.nlm.nih.gov/ XP_790671.3	CAYCRKAFADSSTLTKHLRIHSGEKPYQCKICLLRFSQSGNITRHMKVH
>*Branchiostoma*, https://blast.ncbi.nlm.nih.gov/ XP_019644085.1	CTVCPRAFADASTLTKHVRTHTNEKPYQCSECGQSFTQSGNIKRHLKVH

Glass-like proteins (vertebrates and choanoflagellates)	
>Human, https://blast.ncbi.nlm.nih.gov/ XP_011525220.1, ZSCAN22	CDACGRAFSDCSALIRHLRIHSGEKPYQCKVCPKAFAQSSSIIEHQRIH
>Human, https://blast.ncbi.nlm.nih.gov/ EAW84863.1, ZNF253	CEECGKAFNRSTDLTTHKIVHTGEKPYKCEECGKAFNQPSTLSKFESLY
>Human, https://blast.ncbi.nlm.nih.gov/ CAA36583.1, KOX 26	CKECGEAFSQSSTLTKHIKVYTGEKPYTCKDCRKAFSQSSSITQHQRVH
>Human, http://www.uniprot.org/ ZNF764	CPDCGRCFRQSSEMAAHRRTHSGEKPYPCPQCGRRFGQKSAVAKHQWVH
>Human, http://www.uniprot.org/ ZNF683	REVCHKRFSSSSNLKTHLRLHSGARPFQCSVCRSRFTQHIHTKLHHRLH
>Human, http://www.uniprot.org/ ZNF500	CTQCGKRFNNSSHFSAHRRTHTGEKPYTCPACGRGFRRGTDTHKHQRTH
>*Salpingoeca*, https://blast.ncbi.nlm.nih.gov/ XP_004993762.1	CSICGKSFITSSHVRMHERTHDDARHFSCPLCSKPFKTKPGTKQHLKTG
>*Salpingoeca*, https://blast.ncbi.nlm.nih.gov/ XP_004989424.1	CDQCSFKTAYKHLTTHKRIHTGEKPYKCDQCEYKTAYKRNMFRHKRIH

Fig. 2: 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 zinc fingers, which are responsible for recognising the DNA Glass-binding motif in PRs *in vivo* [28-31]. In the table, those aminoacids 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 Cys_2His_2 domain has been resolved [63], and it is expected that four aminoacids per zinc finger directly recognise three base pairs. These aminoacids 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 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 [64]. Similarly, we aligned Glass-like proteins from vertebrates (e.g. human) and choanoflagellates (e.g. *Salpingoeca*) with BLAST [22] and MUSCLE [25], but they did not entirely match the Glass consensus sequence (shown on the second column) and, therefore, they were not clear Glass homologues. Furthermore, a 'DNA-binding site predictor for Cys_2His_2 Zinc Finger Proteins' has been developed and is available online [26, 27]. This software predicts that, based on their aminoacid sequence, all Glass homologues (in the first column) can bind to the same DNA motif: GAAGCC, which was expected from experimental works in *Drosophila* and *Caenorhabditis* [28, 29]. By contrast, it appears that the Glass-like proteins of vertebrates and choanoflagellates (on the second column) would not be able to recognise this motif.

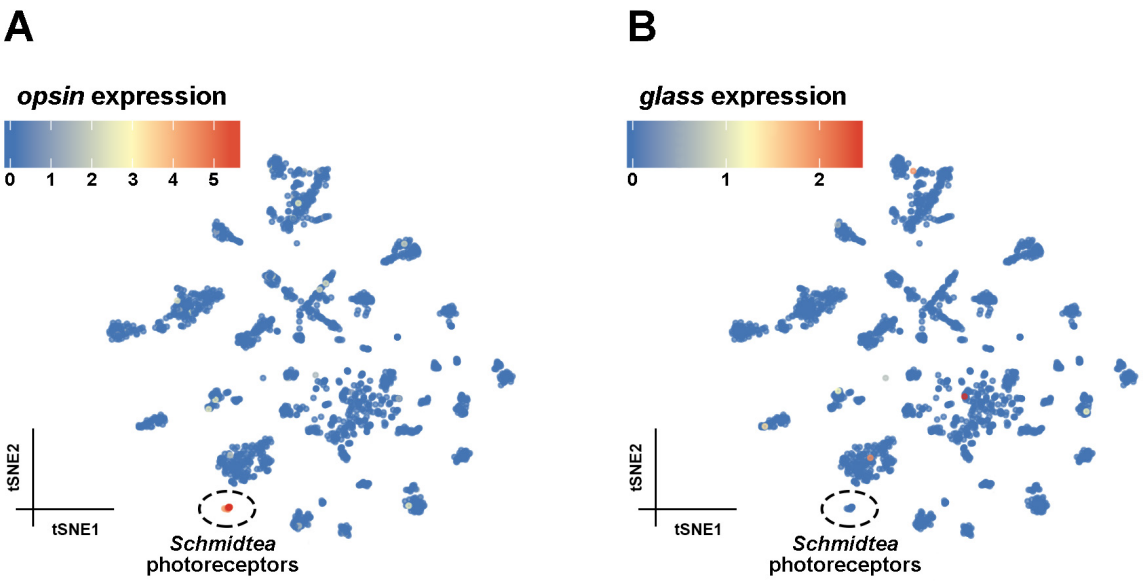

Fig. 3: *glass* is not expressed in rhabdomeric PRs in *Schmidtea*. These graphs were obtained from the Planarian Digiworm atlas, a single-cell transcriptome database for *Schmidtea mediterranea* [19, 23]. Each point corresponds to one single cell, and they are clustered according to the similarity of 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 *opsin* 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, 49], B).

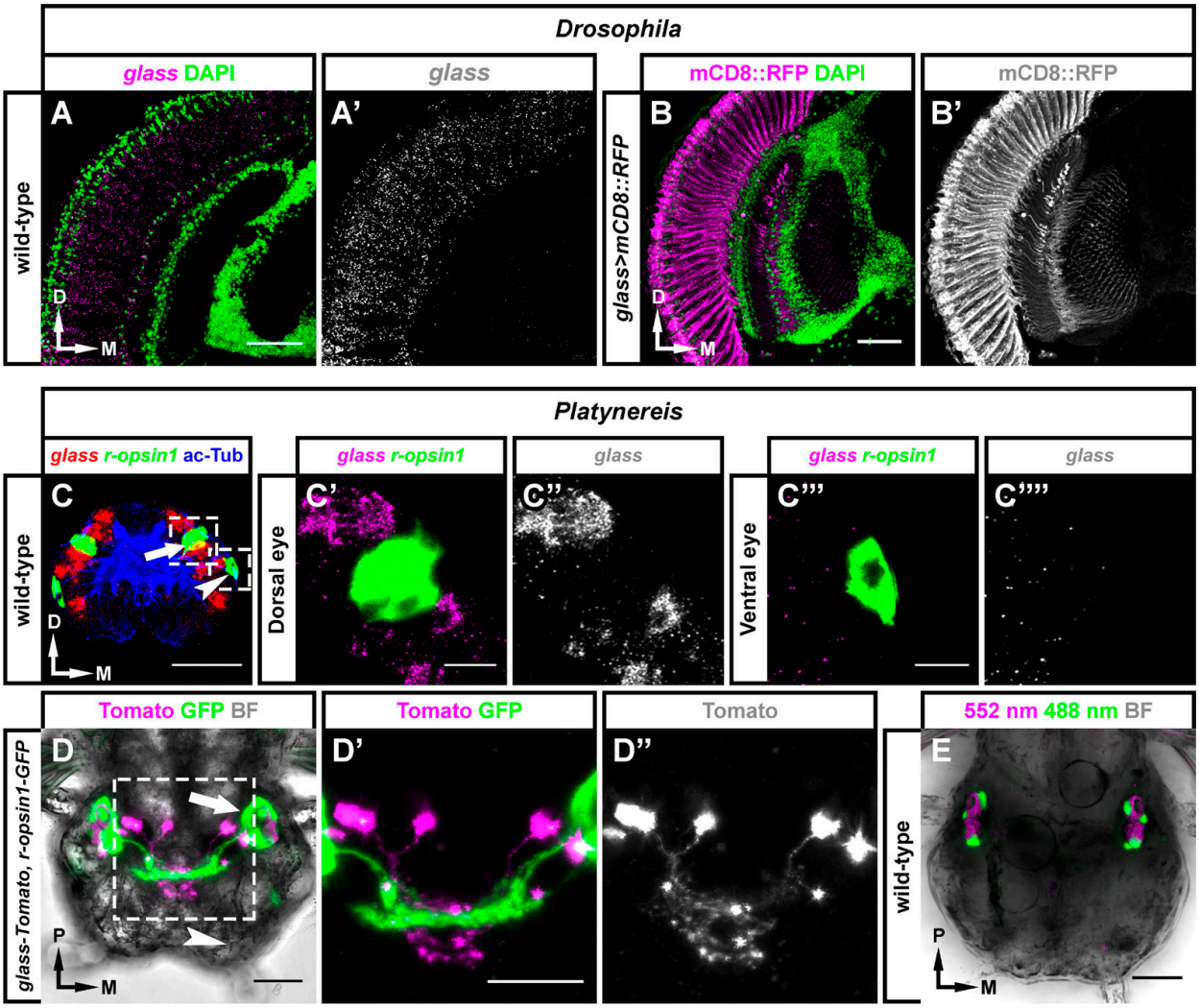

Fig. 4: *glass* is not expressed in rhabdomeric PRs in *Platynereis*. (A, B) *glass* is present in all *Drosophila* rhabdomeric PRs, including those in the compound eye [12, 50]. This can be observed in head cryosections, either by using *in situ* hybridisation (magenta in A, greyscale in A′) or with *glass>mCD8::RFP* flies (magenta in B, greyscale in B′). In both cases samples were counterstained with DAPI (green). (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″). 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.

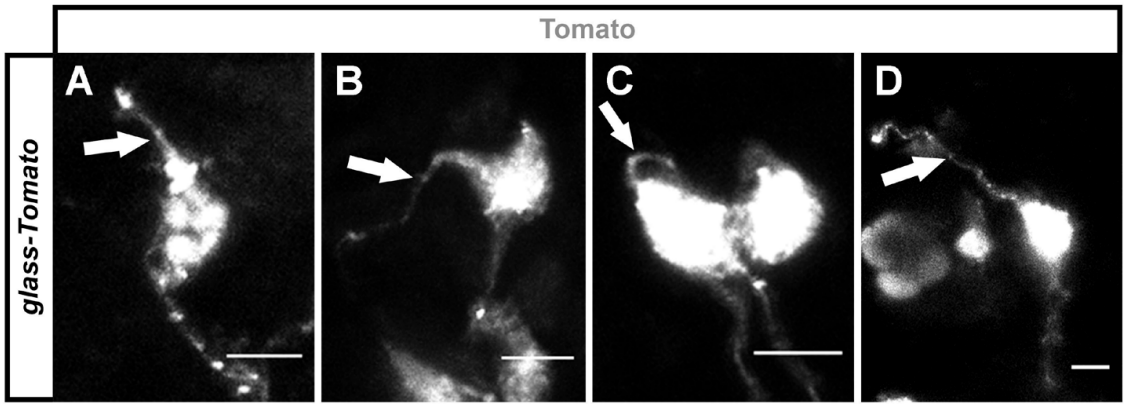

Fig. 5: Glass-expressing cells in *Platynereis* include sensory neurons. When we injected our *glass-Tomato* reporter, we observed that many of the neurons that appeared labelled in the *Platynereis* head were close to the surface, and they often possessed membranous specialisations resembling sensory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

535 **ADDITIONAL FILE 1: SUPPLEMENTARY METHODS AND SEQUENCES**

536

537 To find a Glass homologue in *Platynereis*, we searched a transcriptome database (http://jekely-
538 lab.tuebingen.mpg.de/blast/) (see Table 1 in the main text). We also had available an
539 unpublished reconstruction of the *Platynereis* genome. Both sources indicated that *Platynereis*
540 possesses one single Glass homologue, and that two different Glass isoforms can be produced
541 by alternative splicing. These are:

542

543 >Glass isoform 1, *Platynereis dumerilii*
544 MLNVRMTVDVPLCAKNTSYQKPQARMESCYLSAGGHSHGGHHGHGSHGGGGPGGHCGGGGGGSPGPSYYT
545 SSAAAAVAAAGELWRSSPLKPGSPASASEVCGPPRSSVDLSVNTFAMPPLDIDPLSNFFSFSSPAYKELS
546 VFKEKGPQDIADALLSLKHAVVHPGMNGQLSPLSPGLPPHLPQSISSAMAPSSSLSSYPMSHQHSQLQSY
547 GMSSQYGSPPPPPPPAPQYGETGSGQASPCHPQAPQHHSMFPAMSVNVSMNMNVAMGNQYNNSMDNWGHH
548 PTHQPSAQYSPAAAAAAAQMTSQYPSYGHHHHHHHHHHQNHSYSASYAFSPELRSSSSVSVRDMMYPHPH
549 KPSSASDSYKDVSSKLYSLSAMRRSPRGSCSPVGLGPPPGLRSHHPSGHLGSASSVDSKVNLCRICGKTY
550 ARPSTLKTHMRTHSGEKPYRCQTCSKSFSQAANLTAHLRTHSGEKPFRCPMCDRRFSQSSSVTTHMRTHS
551 GERPYRCRMCKKAFSDSSTLTKHLRIHSGEKPYQCKLCLLRFSQSGNLNRHMRVHANNA
552

553 >Glass isoform 2, *Platynereis dumerilii*
554 MESCYLSAGGHSHGGHHGHGSHGGGGPGGHCGGGGGGSPGPSYYTSSAAAAVAAAGELWRSSPLKPGSPA
555 SASEVCGPPRSSVDLSVNTFAMPPLDIDPLSNFFSFSSPAYKELSVFKEKGPQDIADALLSLKHAVVHPG
556 MNGQLSPLSPGLPPHLPQSISSAMAPSSSLSSYPMSHQHSQLQSYGMSSQYGSPPPPPPPAPQYGETGSG
557 QASPCHPQAPQHHSMFPAMSVNVSMNMNVAMGNQYNNSMDNWGHHPTHQPSAQYSPAAAAAAAQMTSQYP
558 SYGHHHHHHHHHHQNHSYSASYAFSPELRSSSSVSVRDMMYPHPHKPSSASDSYKDVSSKLYSLSAMRRS
559 PRGSCSPVGLGPPPGLRSHHPSGHLGSASSVDSKVNLCRICGKTYARPSTLKTHMRTHSGEKPYRCQTCS
560 KSFSQAANLTAHLRTHSGEKPFRCPMCDRRFSQSSSVTTHMRTHSGERPYRCRMCKKAFSDSSTLTKHLR
561 IHSGEKPYQCKLCLLRFSQSGNLNRHMRVHANNA
562

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

567

568 >DNA template for *glass* RNA probe
569 CTTGCTCCAAATCTTTCTCTCAAGCTGCTAATCTCACTGCACACTTGCGCACCCACTCTGGCGAGAAACC
570 TTTCAGATGTCCGATGTGTGACCGCCGTTTTTCCCAGTCGTCTTCGGTGACCACTCACATGAGAACACAT
571 AGCGGAGAACGGCCTTACAGATGTCGGATGTGCAAGAAGGCCTTCTCCGACAGCTCAACCCTCACCAAGC
572 ACCTAAGAATCCACAGTGGCGAAAAACCTTACCAGTGCAAACTCTGTTTATTAAGATTTTCTCAGTCTGG
573 AAATCTAAATAGGCACATGCGAGTACATGCTAACAATGCCTGATTATATTCTGAGCTATTCTGCTACTTC
574 ATTCATTCTGTGGACTGTGAACAATACCATAATGATCTGACAAGTTGTGACAGTTGTGAATTTACTGCTG
575 TAGTCTGTGGAATACAGCAATGTGAACTATGGACAGTTTCTTTTTAAATGTTGACAATGGACAATGTCTA
576 TTATGACTGTAACATTCGTGACATGAATGTGTTGTTTAATGATCAAAACATGTCATAGTTAATATATTTC
577 TTCATGACAACAAAATCTTGGTAATACACTTTCTTCTTTCCGCCTTGAAATTATTGTAGATTCAATAGTT
578 ATACCAATCCAATTGTTTTCATAATGATTTTTTCCCTTGCCTGTTGTTTTTTCAAATAAATTCCTTGATC
579 AATTCCTTCTATTTCTTTTTTACTTCCTTCACTTCTTTTTTTTCTCCATTTCGCTTTCCTTCCATAGGCC
580 TAATCCTTCTTTCCTTGCTTCCTTCATTGCCTGATTCACATTCCTTCTGTCTTCCTTCTTGTTGCATCCT
581 CTCCTTGCTTCTTTGTCTATCACTCATTCCTTTCTTCCTCGCTTCTTTCTATTTTTCCTTCCTTCCTTCC
582 TTTGCTTTCTCGTAACAATTGCTTGCTTGCTTCATTACTTTCTTCCTTCACCACTTGCTTCCTTCCTTGC
583 TCACTTTCTTGCCTCCTTCCTTTCTTGTTTCCTTCTCTTCTTCCTTCTGTGCTTCCTATTCTGCTTCATT
584 CCTTGCTTCCTACCTTGTTTGATTCCATAATCTTTTTCCTTCCTTGCTTTCTTGATAGTTTCTTCCTTGC
585 TTGCTTTCTTGAAAGTGTCTTGCTTCCTTGATTCATAAAAACTATAAAACAAATTTATACATACACACAA
586 CTTCAGATTCTTTTTGCTACACATGATTTGAATGTGAAACCATTTGTGCATAATATGTTGATATACATTT

GTTCTACTGGTGCTATTGACTATGATGTTTTATTCTGTCTTTACACTTAATTTCTTTACATCTCTTCTTT
ATTGATATGTAAAATTGTTCATTGCTGTTATTTTTGTTTTGTATATATAATAAACATTTTGTCTTGACTG
TGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAA

To generate a *glass-Tomato* reporter, we amplified the following sequence of the *glass* promoter by using *Platynereis* genomic DNA as a template (ApaI and SgsI were used as restriction enzymes, cut sites 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
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