Homothorax Controls a Binary Rhodopsin Switch in *Drosophila* Ocelli

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Running title: Hth regulates binary rhodopsin switch in *Drosophila* ocelli

Abstract

Visual perception of the environment is mediated by specialized photoreceptor (PR) neurons of the eye. Each PR expresses photosensitive opsins, which are activated by a particular wavelength of light. In most insects, the visual system comprises a pair of compound eyes that are mainly associated with motion detection, color or polarized light perception and a triplet of ocelli that are thought to be critical during flight to detect horizon and movements. It is widely believed that evolutionary diversification of compound eye and ocelli in insects occurred from an ancestral visual organ around 500 million years ago. Concurrently, opsin genes were also duplicated to provide distinct spectral sensitivities to different PRs of compound eye and ocelli. In the fruit fly *Drosophila melanogaster*, Rhodopsin1 (Rh1) and Rh2 are closely related opsins that are originated from the duplication of a single ancestral gene. However, in the visual organs, Rh2 is uniquely expressed in ocelli whereas Rh1 is uniquely expressed in outer PRs of the compound eye. It is currently unknown how this differential expression of Rh1 and Rh2 in the two visual organs is controlled to provide unique spectral sensitivities to ocelli and compound eyes. Here, we show that Homothorax (Hth) is expressed in ocelli and confers proper *rhodopsin* expression. We find that Hth controls a binary rhodopsin switch in ocelli to promote Rh2 expression and repress Rh1 expression. Genetic and molecular analysis of *rh1* and *rh2* supports that Hth acts through their promoters to regulate rhodopsin expression in the ocelli. Finally, we also show that when ectopically expressed in the retina, *hth* is sufficient to induce Rh2 expression only at the outer PRs in a cell autonomous manner. We therefore propose that the diversification of *rhodpsins* in the ocelli and retinal outer PRs occurred by duplication of an ancestral gene, which is under the control of Homothorax.

Introduction

The ability to perceive and discriminate a broad range of environmental stimuli in nature is essential for many aspects of life. Animals rely heavily on visual cues to perform complex tasks such as navigation to find food, mates and shelter as well as social interactions. Visual cues are perceived by visual organs that contain photoreceptors (PRs) as light-sensing structures. PRs are specialized cells that gather information from the surrounding world which is subsequently processed by the brain. Each PR expresses a unique photosensitive *opsin/rhodopsin* and they define the wavelength of light by which a PR will be activated.

It is believed that eyes have evolved separately due to fundamental differences between visual organs of different animals [1]. However, it is also known that eye development in different animal phyla share a common genetic network initiated by *Pax6* gene orthologs [2]. Finding similarities in the gene regulatory network that controls eye development further strengthened the idea that phylogenetically diverse eye types may share a conserved eye developmental program [3-5]. Insects are among the largest and most diverse animal groups. Decoding eye development in insects offers great opportunity to unravel developmental insights that lead to the emergence of evolutionary complexity.

In most insects, compound eyes represent the prominent visual organ that is responsible for providing major share of the visual information. In the fruit fly *Drosophila melanogaster*, each compound eye consists of approximately 850 ommatidia and each ommatidium houses eight PRs: six outer PRs and two inner PRs [6]. Additionally, winged insects (such as *Drosophila*) possess ocelli that are comparatively simple photosensory organs embedded in the dorsal head cuticle [7]. In *Drosophila*, a triplet of ocelli (one medial and two lateral) are arranged in a triangular shape between the two compound eyes and the dorsal vertex of the head [8]. It is

72 believed that insect compound eye- and ocellus-like precursor structures have
73 segregated from an ancestral eye over 500 million years ago [9, 10]. In order for these
74 visual organs to perform different functions that require distinct spectral sensitivities,
75 new opsin genes evolved by gene duplication during evolution [11]. In *Drosophila,*
76 phylogenetic analysis supports that Rhodopsin1 (Rh1), Rh2 and Rh6 have originated
77 from a common ancestral gene [12]. A first gene duplication may have separated Rh6
78 from Rh1/Rh2 and a second gene duplication may have separated the closely related
79 Rh1 and Rh2 [12]. The green sensitive Rh6 is expressed in the inner PRs of the
80 compound eye and is critically involved in color perception [13-15]. The blue-green
81 sensitive Rh1 is expressed in the outer PRs of the compound eye and is mainly
82 associated with motion detection [16-18]. Conversely, the UV-blue sensitive Rh2 is
83 expressed in PRs of the ocelli [19-21] and is proposed to be involved in horizon
84 sensing and flight stabilization [22, 23]. While it is known that *rh1, rh2* and *rh6* are
85 genetically linked [24], it is still unknown how they are differentially expressed in
86 different PRs in *Drosophila*.

87 Here we show that the homeodomain transcription factor Homothorax (Hth)
88 regulates Rh2 expression in the ocelli. We demonstrate that Hth is expressed in ocellar
89 PRs and controls a binary *rhodopsin* switch by promoting Rh2 expression and
90 repressing Rh1 expression in ocelli. We also demonstrate that misexpression of Hth
91 forces outer PR of the retina to induce Rh2 expression and clonal expression in the
92 retina suggest that this process is cell autonomous. Furthermore, genetic and
93 molecular analysis of *rh1* and *rh2* shows that the *rhodopsin* switch in ocelli is
94 transcriptionally controlled by Hth and that it may act directly through *rh1* and *rh2*
95 promoter sequences. Finally, we argue that while Hth maintains Rh3 fate in the DRA
96 [25], it initiates Rh2 fate in the ocelli. The results presented here greatly adds to our

97 understanding of how genetically linked opsins are spatiotemporally controlled to
98 provide distinct spectral sensitivity to different visual organs.
99
100

101 ## Results

102 ### Differential expression of *rhodopsins* in the compound eye and ocelli

103 It is believed that the hexapod ancestor of extant insects only had a single visual
104 organ (also referred as ancestral eye) and that other visual organs (such as compound
105 eye and ocelli) evolved as a result of a morphological bifurcation event over 500 million
106 years ago [10, 11] (Figure 1A). It is hypothesised that the different functions of
107 compound eye and ocelli in conjunction of gene duplication events of opsins led to the
108 emergence of an ocelli specific *rhodopsin* gene, with a distinct spectral sensitivity [11].
109 In most insects, three different opsin subfamilies are present that show distinct spectral
110 sensitivity to different wavelengths of light and are associated with different functions.
111 Ultraviolet-sensing (UV) opsins are sensitive to 300-400 nm range of wavelength
112 whereas blue-sensing (B) opsins are sensitive to a wavelength of 400-500 nm. Long
113 wavelength-sensing (LW) opsins are sensitive to a range between 500-600 nm [26-
114 28]. In the *Drosophila* compound eye, brightness detection is achieved by inputs from
115 six outer-PRs (R1-R6) that express the LW-opsin Rhodopsin 1 (Rh1). *Drosophila* uses
116 outer PRs mainly for motion detection and dim light vision [16-18]. Color vision
117 requires the two inner PRs (R7 and R8) that encode either one of the UV- (Rh3 or
118 Rh4) or the B- (Rh5) or LW-opsins (Rh6) [29, 30]. In most insects, ocelli express a
119 LW-opsin, which is different from the one present in the compound eye [11, 21, 31]. In
120 *Drosophila*, PRs in the ocelli express the LW-opsin Rh2 that is sensitive to a
121 wavelength range of 418-506 nm [19-21]. The different spectral sensitivity of ocelli is
122 also reflected by their involvement to perform different functions than the compound
123 eye and they are believed to detect horizon, control head orientation and stabilize flight
124 posture while flying [22]. To get an insight of *rhodopsin* gene duplications in
125 *Drosophila*, we compared the nucleotide sequences of *rh1* to *rh6* by generating a

126 phylogenetic tree (by using MUSCLE online tool; phylogeny.fr) [32]. The phylogenetic
127 tree made by maximum likelihood method showed two clades with branching support
128 value of 1 each (Figure 1B). Clade-I showed tandem gene duplication that separated
129 Rh5 from the closely related Rh3/Rh4. Clade-II represents LW-opsin gene
130 duplications in *Drosophila* where first a tandem gene duplication separated Rh6 from
131 Rh1/Rh2 (Figure 1B). Rh6 subsequently got expressed in the inner PRs of the
132 compound eye (Figure 1H). A further gene duplication led to the separation of the blue
133 shifted rhodopsins Rh1 and Rh2 (Figure 1B). While Rh1 subsequently got expressed
134 in the outer PRs of the compound eye (Figure 1F), Rh2 got exclusively expressed in
135 ocelli (Figure 1D).
136
137 **Homothorax regulates a binary Rhodopsin switch in ocelli**
138 In the compound eye, the homeodomain transcription factor Homothorax (Hth)
139 is expressed in the dorsal rim area (DRA) of inner PRs, where it has been shown to
140 be both necessary and sufficient to regulate Rh3 expression and thereby critically
141 contributes to the polarized-light sensing system [25, 33]. We found that Hth is also
142 expressed in all PRs of the ocelli (Figure 2A). Since ocellar PRs expresse Rh2, we
143 first analysed if Hth is involved in regulating Rh2 expression. We performed
144 knockdown of *hth* using the pan-photoreceptor driver *IGMR*-Gal4 and observed a
145 complete loss of Rh2 expression in PRs of the ocelli (Figure 2B). Additionally, we also
146 observed that in *hth* knockdown, the loss of Rh2 expression in the ocelli is
147 compensated by gain of Rh1 (Figure 2B), a Rhodopsin that is normally exclusively
148 expressed in the outer PRs of the compound eye. Thus, Hth regulates a binary switch
149 of Rhodopsin in the ocelli where it promotes Rh2 expression and represses Rh1
150 expression.

151 In the retina of the compound eye, a feedback mechanism has been described
152 that allows Rh6 to transcriptionally repress Rh5 in yellow R8 PRs [34]. Since in *hth*
153 knockdown, ectopic Rh1 expression in the ocelli replaces Rh2, we speculated that Hth
154 may repress Rh1 in order to allow Rh2 expression. Knockdown of *hth* in turn removed
155 this repression and as a result Rh1 gets activated and represses Rh2 in ocelli. To
156 check this hypothesis, we misexpressed Rh1 in ocelli by using *IGMR-Gal4* and found
157 that ectopic expression of Rh1 was not sufficient to repress Rh2 and in fact in this case
158 ocelli co-expressed both Rh1 and Rh2 (Supplemental figure 1A). Thus, Rh2 loss in
159 *hth* knockdown seems to be independent of Rh1 repression.

160 To further test whether switching Rhodopsin expression might also alter PR
161 identity based on other molecular markers of outer PRs in the retina, we monitored
162 Seven-up (svp) and BarH1 expression in the ocelli in *hth* knockdown. Svp is a steroid
163 hormone receptor and is expressed in R3/R4 and R1/R6 pairs whereas BarH1 is a
164 homeobox transcription factor expressed in the R1/R6 pair of the developing
165 compound eye [35, 36]. We found that neither Svp nor BarH1 are expressed in
166 wildtype nor in *hth* knockdown ocelli (Supplemental figure 2A, 2B, 2C and 2D).
167 Therefore, although ocellar PRs gained Rh1 and lost Rh2 expression when *hth* was
168 knocked down, they do not appear to undergo an identity change towards retinal outer
169 PRs.

170 We next investigated whether regulation of this Rhodopsin switch by Hth occurs
171 at the transcriptional level by using ocelli specific *rh2-lacZ* [20] and outer PR specific
172 *rh1-lacZ* [36] reporter lines. In wildtype control animals, *β*-gal expression was
173 specifically observed in ocelli in case of *rh2-lacZ* (Figure 2C) whereas no expression
174 was observed in ocelli in the case of *rh1-lacZ* (Figure 2E). In *hth* knockdown

175 background, we found that *β*-gal expression from *rh2-lacZ* is completely abolished

176 from ocelli (Figure 2D), whereas ectopic *β*-gal expression from *rh1-lacZ* is now seen

177 in ocellar PRs (Figure 2F). Therefore, Hth is indeed involved in regulating a binary
178 rhodopsin switch in the ocelli by promoting transcription of *rh2* and repressing
179 transcription of *rh1*.

180 We have previously shown that the homeodomain transcription factor Hazy
181 (Flybase: Pph13 for PvuII-PstI homology 13) controls expression of Rh2 in the ocelli
182 [37, 38]. We therefore next asked if Hazy and Hth act jointly to regulate the rhodopsin
183 switch in ocelli. However, we find that in *hazy*$^{-/-}$ null mutant flies, the absence of Rh2
184 expression is not accompanied with the ectopic expression of Rh1 in ocellar PRs
185 (Supplemental figure 1B). Moreover, we found that Hth is still expressed in the ocelli
186 of *hazy*$^{-/-}$ mutant flies (Supplemental figure 1C). Also, the expression of Hazy remains
187 unchanged in ocellar PRs when knocking down *hth* (Supplemental figure 1D). Thus,
188 the Hth-dependent Rhodopsin switch in ocelli does not depend on Hazy.

189

190 **Hth controls a binary rhodopsin switch in ocelli by acting through the promoters**
191 **of *rh1* and *rh2***

192 Since Hth encodes a homeodomain transcription factor we next investigated if
193 Hth may act by directly regulating the *rhodopsin* promotors. We identified three
194 potential Hth binding sites in the minimal promoter region of *rh2* (-293/+55) [20],
195 whereas the *rh1* minimal promoter region (-247/+73) [36] contains two potential Hth
196 binding sites. To test if the Hth binding sites in the *rh1* and *rh2* promoter regions may
197 be involved in the rhodopsin switch in ocelli, we created transgenic flies containing
198 transgenes with the minimal promoter regions of *rh1* or *rh2* driving GFP (*rh1-GFP* and

199 *rh2-GFP* [37]). Next, in order to abolish Hth binding, we introduced point mutations in
200 the Hth binding regions of the *rh1* and *rh2* promoters and created *rh1(hth mut)-GFP*
201 and *rh2(hth mut)-GFP* transgenic flies (Figure 3A and 3D) (See material and methods
202 for details). In support with the previous observations, we do observe *rh2-GFP*
203 expression in the ocelli (Figure 3B) whereas *rh1-GFP* is not expressed in ocellar PRs
204 (Figure 3E). However, by mutatingthe Hth binding sites in the promoter region of *rh2*
205 (*Rh2 (hth mut)-GFP*), we observed a loss of GFP expression in the ocelli (Figure 3C).
206 Conversely, we found that deleting the Hth binding sites in the promoter region of *rh1*
207 (*rh1 (hth mut)-GFP)* leads to ectopic GFP expression in ocelli (Figure 3F).
208

209 **Hth is sufficient to induce Rh2 expression in the outer PRs of retina**

210 In the dorsal rim area of the retina, Hth is required in inner PRs to promote Rh3
211 expression and ectopic expression of Hth under the control of *lGMR*-Gal4 was
212 sufficient to block the expression of inner PR rhodopsins (Rh4, Rh5 and Rh6) and to
213 induce Rh3 expression in all inner PRs. However, in outer PRs, expression of Rh1
214 was not affected suggesting that only inner PRs were responsive to Hth misexpression
215 [25]. We next investigated if misexpression of Hth in the retina was also sufficient to
216 induce Rh2 expression. In the wildtype retina, Rh1 was uniquely expressed in outer
217 PRs whereas Rh2 was absent (Figure 4A, 4A' and 4A''). When Hth was misexpressed
218 in the retina under the control of *lGMR*-Gal4, we observed that Rh2 was now
219 ectopically co-expressed in all outer PRs. However, we found that Rh1 expression
220 was unaffected and it was still expressed in all outer PRs (Figure 4B, 4B' and 4B'').
221 We also found that ectopic Rh2 expression was only limited to the outer PRs (and not
222 inner PRs) suggesting that only Rh1 expressing PRs were competent to induce Rh2
223 expression in the retina, while inner PRs were not.

224 To investigate if this process is cell autonomous, we generated outer PR versus
225 inner PR Hth gain-of-function clones in the retina using the “flip-out” technique [39]
226 (material and methods for details). Flip-out clones were marked by presence of GFP
227 expression. We found that when clones were generated in the inner PRs, Rh2
228 expression was not induced (Figure 5A, 5A’ and 5A’’). However, when clones were
229 generated in the outer PRs, Rh2 expression was ectopically induced in all GFP
230 expressing clones (Figure 5B, 5B’ and 5B’’). Thus, ectopic Rh2 expression in Rh1
231 expressing PRs of the retina is a cell-autonomous process. Expression of Hth in inner
232 PR clones did not have any effect on Rh1 expression in the neighbouring outer PRs
233 (Figure 5C, 5C’ and 5C’’) and Rh1 expression was maintained when clones were
234 generated in the outer PRs (Figure 5D, 5D’ and 5D’’).
235 Taken together, we showed that Hth regulates a binary rhodopsin switch in
236 ocelli by promoting Rh2 expression at the cost of Rh1. We found that regulation of
237 Rh1 and Rh2 is transcriptionally controlled by Hth. We also found that Hth regulates
238 the rhodopsin switch by acting through the promoters of *rh1* and *rh2*. We further
239 demonstrated that misexpression of Hth in the retina modifies outer PRs to acquire
240 Rh2 expression resulting in outer PRs co-expressing Rh1 and Rh2 and we show that
241 this process is cell-autonomous (Figure 6).
242
243
244

245 ## Discussion

246 **Homothorax expression provides new insights to understand Rhodopsin fate in**
247 ***Drosophila***

248 In insects, ocelli represent a fundamentally simpler visual organ whose spectral
249 sensitivity is different from the compound eye. The unique spectral sensitivity of ocellar
250 PRs in *Drosophila* is provided by the UV-blue sensing Rh2 [21]. The presence of this
251 particular Rhodopsin exclusively in ocelli and not in the retina may explain why ocelli
252 perform different functions than the compound eye. We have characterized the roles
253 of Hth during terminal differentiation of ocellar PRs and showed that Hth regulates a
254 binary rhodopsin switch in ocelli that promotes Rh2 expression and represses Rh1
255 expression. Hth is known to be expressed in the ocellar primordium of the early third
256 instar laval (L3) eye antennal imaginal disc but gets downregulated later at mid- to
257 late-L3 [40]. In our study, we show that Hth is expressed in all mature and terminally
258 differentiated PRs of ocelli.
259 Hth performs similar function in PRs of the ocelli and the DRA of the retina: in
260 ocelli, it induces Rh2 expression by repressing Rh1 whereas in the DRA of the retina
261 it induces Rh3 by repressing inner-PR Rhodopsins [25]. Loss of *hth* transforms the
262 DRA into odd-coupled ommatidia where Rh3 is expressed in R7 and Rh6 in R8
263 suggesting that the Rh3/Rh6 pair represents the default state of rhodopsins in inner
264 PRs of the retina [25]. In ocelli, we find ectopic expression of Rh1 by loss of *hth*
265 suggesting that Rh1 may be the default state in ocellar PRs. Loss of Hth leads to
266 ectopic expression of the R8 specific transcription factor Senseless in the DRA, which
267 is otherwise not expressed in the wildtype DRA suggesting a fate change of the DRA
268 to become odd-coupled ommatidia [33]. Interestingly, Hth loss in ocelli does not induce
269 expression of outer PR-specific transcription factors (such as Svp and BarH1)
270 suggesting that the ocellar PR fate may not have been changed.
271
272 **Genetic and molecular dissection of Hth in the ocelli during PR development**

273 The DNA binding property of Hth is required for proper DRA fate in the retina
274 [33]. Similarly, we observed that Hth regulates binary rhodopsin switch in ocelli by
275 transcriptionally controlling *rh1* and *rh2* expression through binding to their upstream
276 DNA sequences. The homeodomain transcription factor Hazy controls rhodopsin
277 expression in the retina and in ocelli [37, 38]. However, epistatic analysis showed that
278 both Hth and Hazy acted independently to control Rh2 expression in ocelli.
279 Misexpression of *hth* in all developing PRs of the retina is sufficient to induce a fate
280 switch where inner PRs are transformed into PRs of DRA [33]. Rh3 is expanded to all
281 inner PRs whereas specific loss of Rh4, Rh5 and Rh6 was seen. However, outer PR
282 fate was not changed suggesting that only those PRs, which were previously
283 committed to become inner PRs during development, were responsive to Hth [33]. We
284 additionally found that outer PRs, too were responsive to *hth* misexpression since they
285 induce Rh2 expression while maintaining its default fate by expressing Rh1. However,
286 the genetic program activated by Hth misexpression to induce Rh2 expression in outer
287 PRs is still unknown.
288
289 **Rhodopsin duplication and role of Hth during ocelli diversification.**
290 Photoreception is achieved by expression of different opsins in conjunction with an
291 array of proteins of the phototransduction cascade. Each PR contains different opsins,
292 which are light sensing structures and they define the particular wavelength of light to
293 which a given PR will respond. In most insect species, opsins were categorized into
294 three major clades: 1) short-wavelength (SW) opsins, which are mostly UV-sensitive,
295 2) middle-wavelength (MW) opsins, which are mainly blue-sensitive and 3) long-
296 wavelength (LW) opsins, which are ranging from blue-violet such as Rh2 in *Drosophila*
297 [41] to green-red in some butterflies [42]. Presence of all three opsins in most insect

species may imply that the ancient retina was trichromatic [43]. Further phylogenetic analyses support ancestral trichromacy in insects and show that SW, MW and LW sensitive opsins must have derived from a single ancestral opsin as a result of gene duplications [12]. In many insect species, spectral sensitivity of opsin gene clades is represented by more than one paralog such as the LW-opsin clade consisting of three paralogs in *Drosophila* (Rh1, Rh2 and Rh6), four in the mosquito *Anopheles gambiae* and two in the honeybee *Apis mellifera* [12]. It is believed that a first tandem gene duplication in *Drosophila* separated Rh6 from its sister paralog Rh1/Rh2 and while Rh6 is retained in the inner PRs of compound eye, Rh1/Rh2 was associated with the outer PRs of retina and PRs of ocelli. A second gene duplication event led to the diversification of Rh1 and Rh2 by accumulation of further amino acid changes in their sequence. While Rh1 expression was retained in the outer PRs of the compound eye, Rh2 became associated with the PRs of the ocelli. Our results also suggest that the ability of Hth to bind to both promoters may have been a prerequisite during evolution to differentially regulate the expression of the two paralogs and thus, provide spectral sensitivity of ocelli by promoting Rh2 expression and repressing Rh1 expression. However, it would be interesting to know if a change in the spectral sensitivity of ocelli upon Hth loss would in turn change their specific functions. Also, if gene duplications of opsins occurred during evolution to allow the diversification of different spectral sensitivities in different visual organs, it would be interesting to know what kind of spectral sensitivity was present in the ancestral visual organ. We conclude that Hth may act as an evolutionary factor required by ocelli to provide their unique spectral identity to ocellar PRs that are marked by expression of Rh2. To maintain this unique spectral identity, Hth controls a binary rhodopsin switch to repress outer PR fate in ocelli by repressing Rh1 expression.

323

324 **The opposing regulatory function of Hth on its direct targets.**

325 We show here that Hth functions both as a transcriptional activator of *rh2* and as a
326 transcriptional repressor of *rh1*. Usually, Hth in conjunction with its binding partner
327 Extradenticle acts as a transcriptional activator. However, it has also been shown to
328 function in gene repression, for example by recruiting the transcriptional repressor
329 Engrailed to the regulatory elements of target genes [44]. In this case the formation of
330 the repressor complex occurs directly at the regulatory regions of the repressed gene
331 and depends on the proximity of DNA-binding sites for different components of the
332 complex in the regulatory region. The same mechanism could also explain the
333 opposing regulatory effect that Hth has on *rh1* in comparison to *rh2* in the ocelli.
334 Analysis of transcription profiles of different PR types would provide a list of Hth
335 interactors expressed in the ocelli versus the outer PRs of the retina. Binding analysis
336 of these interactors to the *rh1* and rh2 minimal promoter sequences would help to
337 identify the potential repressor complex forming on the *rh1* promoter, helping to better
338 understand the regulatory functions of Hth.

339

340

341 ## Author contributions

342 AKM and SGS designed all experiments. AKM, CF and RV performed experiments
343 and generated reagents/materials. AKM, CF, RV, RSM and SGS wrote the
344 manuscript.

345

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354

355 Materials and methods

356 Fly stocks

357 Wildtype Canton S flies have been used in this study. Other fly strains used are: UAS-
358 *hth*^{RNAi} (BL27655 and BL34637; we do see rhodopsin switch phenotype in both RNAi
359 lines. However, all the experiments were done in BL27655), *IGMR*-Gal4
360 (Bloomington's stock center). UAS-*rh1* [34], UAS-*hth* [45], *rh1*(-252/+57)-*lacZ* [36], *rh2*
361 (-309/+32)-*lacZ* [20], *rh2* (-293/+55)-*GFP* [37], *hazy*$^{-/-}$ [46], *hsFLP*; UAS-*mCD8::GFP*;
362 *IGMR*<wt>Gal4 (a gift from Claude Desplan lab). The following transgenic lines were
363 made in this study: *rh2 (hth mut)-GFP*, *rh1-GFP* and *rh1 (hth mut)-GFP*. Flies were
364 reared on standard food medium and at 25°C. Knockdown experiments were
365 performed at 29°C temperature. For *hth* overexpression clones by ''flip-out'' technique,
366 flies were grown at 25°C and heat shock at 37°C was performed for half an hour at
367 the late pupal stage. Vials were placed back at 25°C and freshly hatched flies were
368 dissected.

369

370 Generation of *rh1* and *rh2* reporter transgenes

371 The *rh1* minimal promoter (-247 to +73) was PCR amplified from genomic DNA with
372 primers “rh1 enh Kpn fw” (gcggtacCTGGAGACTCAAGAATAATACTCGGCCAG) and
373 “rh1 enh Xba re” (gatctagAGGGTTCCTGGATTCTGAATATTTCACTG) and cloned
374 into pBluescript vector using the KpnI and XbaI sites added to the primers. For cloning
375 of the *rh2* minimal promoter see [37]. The same *rh1* and *rh2* promoter fragments were
376 *in vitro* synthesized (BioCat) altering the sequences of the potential Hth binding sites.
377 The first Hth site in the *rh1* promoter (TGACAT) was changed to TaAgcT creating a
378 HindIII restriction site. The second Hth site in the *rh1* promoter (CTGTCG) was
379 changed to CTaaaG. The first Hth site in the *rh2* promoter (GGACAG) was changed
380 to GtttAG, the second Hth site in the *rh2* promoter (GTGTCA) was changed into
381 agcTgA and the third Hth site (CTGTCC) was changed to CTaaaC. Both versions of
382 the two enhancers were cloned into a GFP reporter plasmid containing *eGFP,* a
383 *miniwhite* marker *and an attB* site kindly provided by Jens Rister. The plasmids were
384 injected into nos-φC31; attP40 flies for integration on the second choromosome using
385 the φC31 site-specific integration system [47].
386
387 **Immunohistochemistry**
388 Adult ocelli were dissected and stained by a protocol published in [37] whereas
389 dissection and immunohistochemistry of adult retinas were done according to [48].
390 After the immunostainings, tissue samples were mounted by using Vectashield H-
391 1000 (Vector laboratories). Primary antibodies and their dilutions were as follows:
392 Rabbit anti-Rh2 1:100 [37], Rabbit anti-Rh6 1:10,000 [49], Rabbit anti-Hth 1:500 [50],
393 Chicken anti-βGal 1:1000 (Abcam), Chicken anti-GFP 1:2000 (Life technologies),
394 Rabbit anti-Hazy 1:500 [46], Mouse anti-Svp 1:100 [51], Rat anti-BarH1 1:200 [52],

395 Mouse anti-Rh1 1:20, Rat anti-Elav 1:20 and Mouse anti-Chp 1:20 (Developmental
396 Studies Hybridoma bank). Phalloidin conjugated with Alexa-568 and Alexa-647
397 (Sigma-Aldrich, Life Technologies) marks F-actin and were used (1:1000) during
398 incubation with secondary antibodies. The following secondary antibodies were used:
399 Goat anti-rabbit, Goat anti-mouse, Goat anti-rat and Goat anti-chicken that are
400 conjugated with Alexa-488, Alexa-555 and Alexa-647 (Jackson Immunoresearch). All
401 secondary antibodies are used at 1:200 dilution.
402
403 **Confocal microscopy and Image analysis**
404 Tissue samples were imaged with a Leica TCS SP5 confocal microscope at a
405 resolution of 1024x1024 pixels and optical sections were taken in the range of 1-2 µm
406 depending on the sample size. Images were further processed and analysed in
407 Fiji/ImageJ and Adobe photoshop 2020 software.
408

409 ## Figure legends

410 **Figure 1: Model representing the evolutionary diversification of the insect**
411 **ancestral eye with rhodopsin duplication and its expression in the *Drosophila***
412 **adult visual system.** (A) A morphological bifurcation from an ancestral eye of a
413 hexapod ancestor to extent insects over 500 million years ago (mya) is believed to
414 form ocelli and compound eye. (B) Gene duplications of rhodopsin in *Drosophila* where
415 the blue dotted square box marks a clade consisting of Rh1, Rh2 and Rh6. A first
416 tandem gene duplication separated Rh6 from Rh1/Rh2 and a second gene duplication
417 separated the closely related Rh1 and Rh2. (C, F) Expression of Rh1 (green) in the
418 wildtype ocelli (C) and retina (F). Rh1 is exclusively expressed in the outer PRs of the
419 retina but not in ocelli. (D, G) Expression of Rh2 (green) in the wildtype ocelli (D) and

420 retina (G). Rh2 is uniquely expressed in all PRs of the ocelli but not in the retina. (E,
421 H) Expression of Rh6 (green) in ocelli (E) and retina (H). Rh6 is normally expressed
422 in the inner PRs in retina but not in ocelli. PRs of ocelli are marked by Elav (Red)
423 whereas rhabdomeres in the retina are marked by Phalloidin (Red).
424
425 **Figure 2: Hth expression and phenotype in ocelli.** (A) Antibody staining of anti-Hth
426 showed that it is expressed in all PRs of wildtype ocelli. Anti-Chp (green) and anti-Elav
427 (blue) mark the PRs of ocelli. (B) Antibody staining of anti-Rh2 (green) and anti-Rh1
428 (Red) in *hth* knockdown (*IGMR*>UAS-*hth*^{RNAi}) ocelli shows loss of Rh2 expression and
429 gain of Rh1 expression. (C, D) Antibody staining of anti-β-Galactosidase (β-Gal) in the
430 control (*rh2-lacZ; IGMR/+*) and in *hth* knockdown (*rh2-lacZ; IGMR>hth*^{RNAi}) ocelli. Anti-
431 βGal (green) expression is seen in control ocelli, also marked by anti-Rh2 (blue) (C).
432 However, anti-βGal (green) expression is absent in *hth* knockdown ocelli, marked by
433 anti-Rh1 (red) (D). (E, F) Antibody staining of βGal in the control (*rh1-lacZ; IGMR/+*)
434 and in *hth* knockdown (*rh1-lacZ; IGMR>hth*^{RNAi}) ocelli. In the control ocelli marked by
435 anti-Rh2 (blue), anti-βGal (green) expression is absent (E) whereas it is ectopically
436 expressed in case of *hth* knockdown ocelli marked by anti-Rh1 (red) (F).
437
438 **Figure 3: Expression of *rh1* and *rh2* reporter constructs in ocelli.** (A) Schematic
439 representation of the *rh2* minimal promoter used for making GFP reporter constructs
440 ranging from positions -293 to +55 of the transcription start (+1, arrow). The wildtype
441 promoter (*rh2-GFP*; in the top) contains three potential Hth binding sites (green) with
442 their corresponding sequences shown. In the *hth* mutant promoter (*rh2(hth mut)-GFP*;

443 in the bottom), the mutated sequences are shown with altered residues depicted in
444 red. (B, C) Expression of the wildtype *rh2-GFP* and mutant *rh2(hth mut)-GFP* reporter
445 lines (green) in ocelli stained with antibodies against GFP (green), Rh2 (red) and Rh1
446 (blue). Wildtype *rh2-GFP* (marked by anti-GFP) is expressed in PRs of ocelli (marked
447 by anti-Rh2) staining the entire rhabdomeres (B) whereas the mutant *rh2(hth-mut)-*
448 *GFP* (marked by anti-GFP) is not expressed in ocelli (C). (D) Schematic representation
449 of the *rh1* minimal promoter used for making the GFP reporter constructs ranging from
450 positions -247 to +73 of the transcription start (+1, arrow). The wildtype promoter (top)
451 contains two potential Hth binding sites (green; with its corresponding sequence
452 shown). In the *hth* mutant promoter (bottom), the mutated sequences are shown with
453 the altered residues depicted in red. (E, F) Expression of the wildtype *rh1-GFP* and
454 mutant *rh1(hth mut)-GFP* reporter lines in ocelli stained with antibodies against GFP
455 (green) Rh2 (red) and Rh1 (blue). (E) Like Rh1, the *rh1-GFP* reporter is not expressed
456 in the PRs of ocelli expressing only Rh2. (F) The mutant *rh1(hth-mut)-GFP* reporter
457 line is expressed in PRs of ocelli.
458
459 **Figure 4: Hth misexpression phenotype in the retina.** (A) Antibody staining to show
460 expression of Rh2 and Rh1 in the wildtype retina. Rh2 (green) is normally not
461 expressed in the retina (A') whereas Rh1 (blue) is expressed in the outer PRs of retina
462 (A''). PRs of the retina were stained with phalloidin (red). (B) Expression of Rh1 and
463 Rh2 in the *hth* overexpressing retina. Rh2 expression (green) can be seen now in the
464 outer PRs of retina (B') whereas Rh1 expression (blue) remained unaffected (B'').
465
466 **Figure 5: Hth overexpression clones in the retina.** (A, B, C, D) Antibody staining to
467 mark *hth* overexpression in clones (green) in the retina generated by the flip-out

468 technique. (A, A') Antibody staining against GFP to identify inner PR clones of the
469 retina, also co-stained by Rh2 (blue) and Phalloidin (red). Rh2 expression was not
470 induced (A'') when clones were generated in the inner PRs. (B, B') Antibody staining
471 against GFP to identify outer PR clones of the retina, also co-stained by Rh2 (blue)
472 and Phalloidin (red). When clones were generated in the outer PRs, Rh2 expression
473 was induced (B''). White arrows marked inner and outer PR clones (A, A', B, B') and
474 their corresponding Rh2 expression. (C, C', D, D') Antibody staining against GFP to
475 identify inner and outer PR clones of the retina, also co-stained by Rh1 (blue) and
476 Phalloidin (red). When clones were either induced in the inner PRs (C, C') or outer
477 PRs (D, D') of the retina, Rh1 expression is unaffected (C'', D'').
478
479 **Figure 6: Model of Rh2 cell fate in the ocelli.** Schematic representation showing
480 origin of ocelli and compound eye from an ancestral eye in insects around 500 million
481 years ago. We showed that Rh2 is normally expressed in ocelli and gets independently
482 regulated by Hazy and Hth. Hth regulates a binary rhodopsin switch to promote Rh2
483 expression in ocelli while repressing Rh1. Overexpression of *hth* is sufficient to
484 promote Rh2 expression but only at the outer-PRs of retina.
485
486 **Supplemental figure 1: Independent roles of Hazy and Hth to regulate Rh2**
487 **expression in ocelli.** (A) Overexpression of *rh1* in all PRs of ocelli by using pan-
488 photoreceptor *IGMR*-Gal4. Antibody staining against Rh2 (green) and Rh1 (red) were
489 performed to show that overexpression of *rh1* does not inhibit Rh2 expression in ocelli.
490 (B) Antibody staining to show expression of Rh2 (green) and Rh1 (red) in *hazy*$^{-/-}$ null
491 mutant ocelli. Rh2 expression is lost is absent in *hazy*$^{-/-}$ mutants but they don't show
492 ectopic Rh1 expression in ocelli. (C) Antibody staining to show Hth expression (blue)

493 in *hazy*$^{-/-}$ mutant ocelli, also marked by anti-Chp (green). Hth expression is not affected
494 in ocellar PRs in *hazy*$^{-/-}$ mutants. (D) Antibody staining to show Hazy expression (blue)
495 in *hth* knockdown ocelli. Ocelli in *hth* knockdown is marked by staining against Rh1
496 (red). Hazy is expressed during rhodopsin switch in *hth* knockdown ocelli.
497
498 **Supplemental figure 2: Knockdown of Hth does not alter ocellar PR cell fate.** (A,
499 B) Antibody staining against Svp (green) in the wildtype and in *hth* knockdown ocelli.
500 Svp is normally expressed in R3/R4 and R1/R6 pairs of the developing retinal PRs
501 [36] and not in wildtype ocelli (A). During rhodopsin switch by *hth* knockdown in ocelli,
502 Svp is still not expressed in ocellar PRs (B). (C, D) Antibody staining against BarH1
503 (green) in the wildtype and in *hth* knockdown ocelli. BarH1 is normally expressed in
504 R1/R6 pair of the developing retina [35] and not in wildtype ocelli (C). Upon rhodopsin
505 switch by *hth* knockdown, BarH1 is still not expressed in the ocellar PRs (D). Ocellar
506 PRs are marked by antibody staining against Elav (blue, in A and B) and Chp (red, in
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508

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690

Figure 1

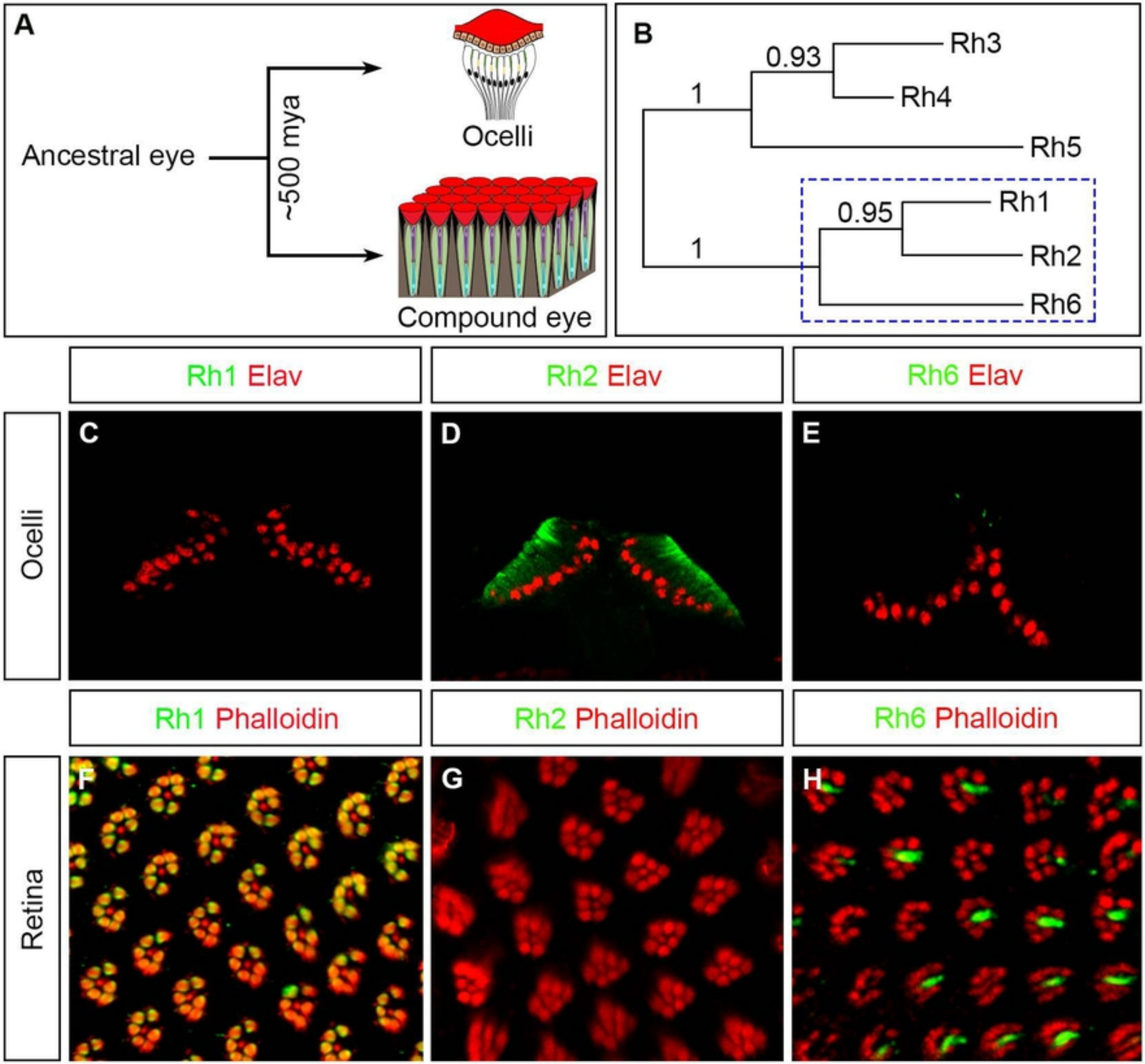

A
Ancestral eye
~500 mya
Ocelli
Compound eye
B
0.93
1
Rh3
Rh4
Rh5
0.95
1
Rh1
Rh2
Rh6
Rh1 Elav
Rh2 Elav
Rh6 Elav
Ocelli
C
D
E
Rh1 Phalloidin
Rh2 Phalloidin
Rh6 Phalloidin
Retina
F
G
H

Figure 2

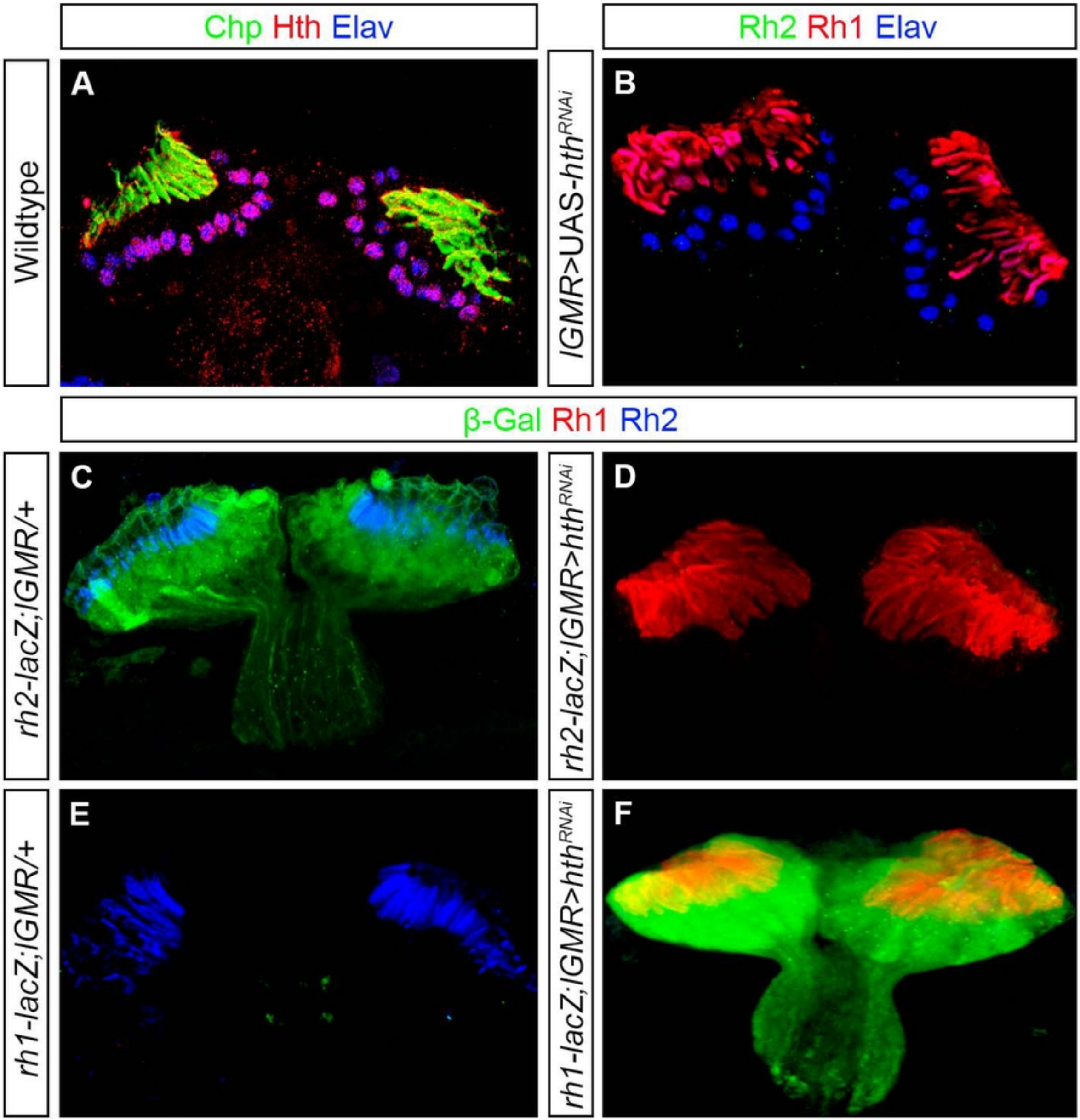

Chp Hth Elav
Rh2 Rh1 Elav
Wildtype
IGMR>UAS-hth^RNAi
A
B
β-Gal Rh1 Rh2
rh2-lacZ;IGMR/+
rh2-lacZ;IGMR>hth^RNAi
rh1-lacZ;IGMR/+
rh1-lacZ;IGMR>hth^RNAi
C
D
E
F

Figure 3

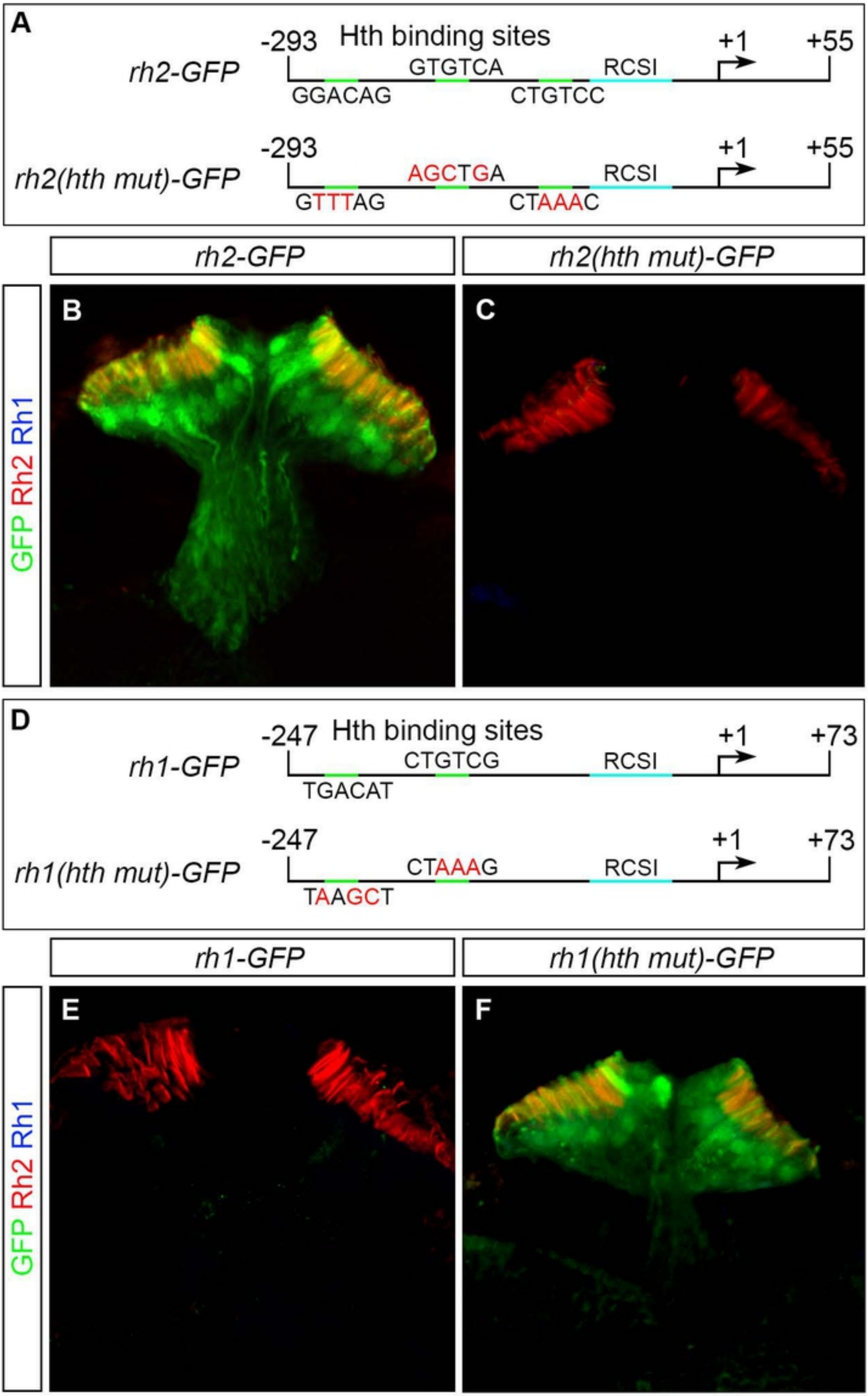

A
-293
Hth binding sites
+1
+55
rh2-GFP
GTGTCA
RCSI
GGACAG
CTGTCC
-293
+1
+55
rh2(hth mut)-GFP
AGCTGA
RCSI
GTTTAG
CTAAAC
rh2-GFP
rh2(hth mut)-GFP
GFP Rh2 Rh1
B
C
D
-247
Hth binding sites
+1
+73
rh1-GFP
CTGTCG
RCSI
TGACAT
-247
+1
+73
rh1(hth mut)-GFP
CTAAAG
RCSI
TAAGCT
rh1-GFP
rh1(hth mut)-GFP
GFP Rh2 Rh1
E
F

Figure 4

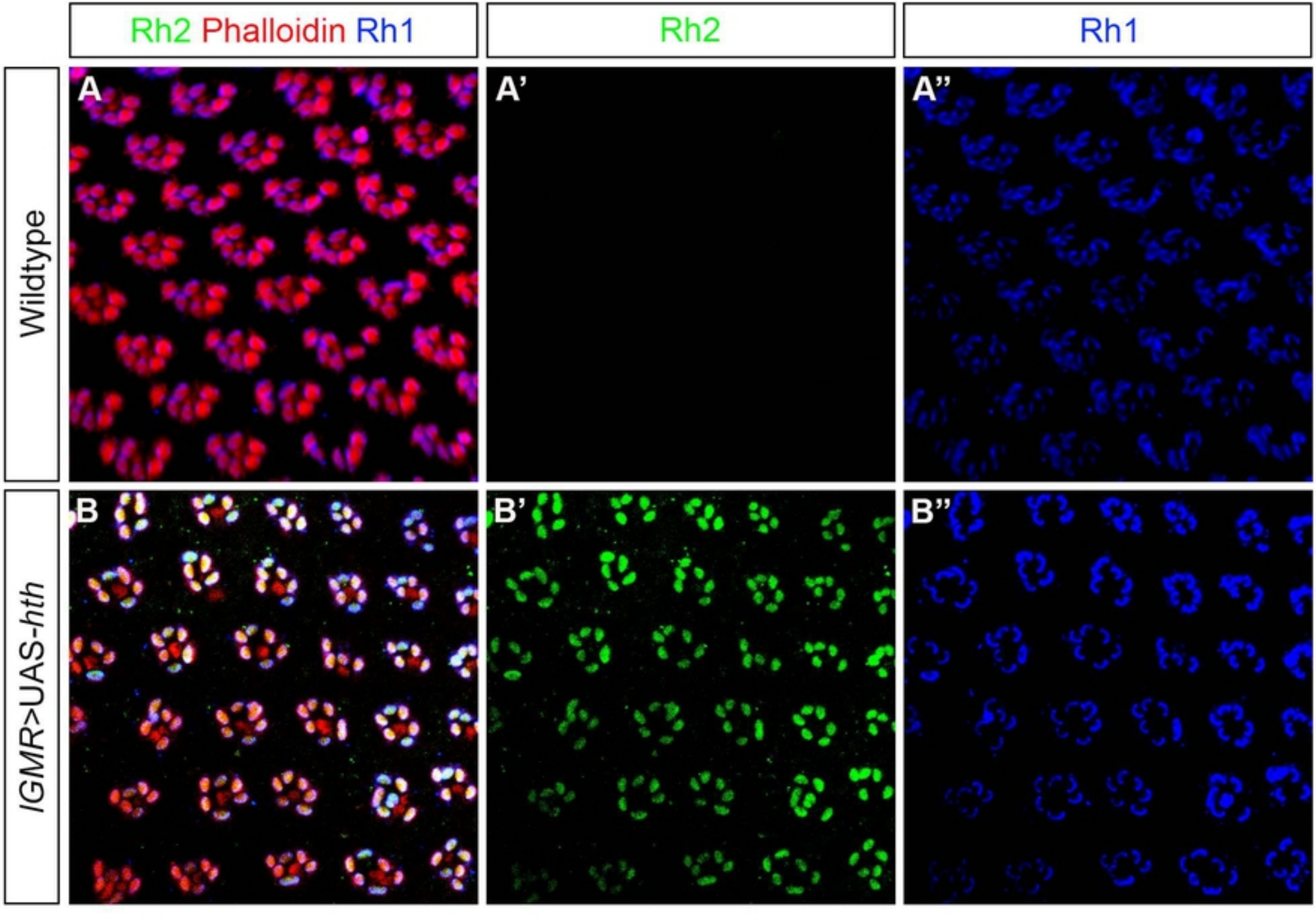


Figure 5

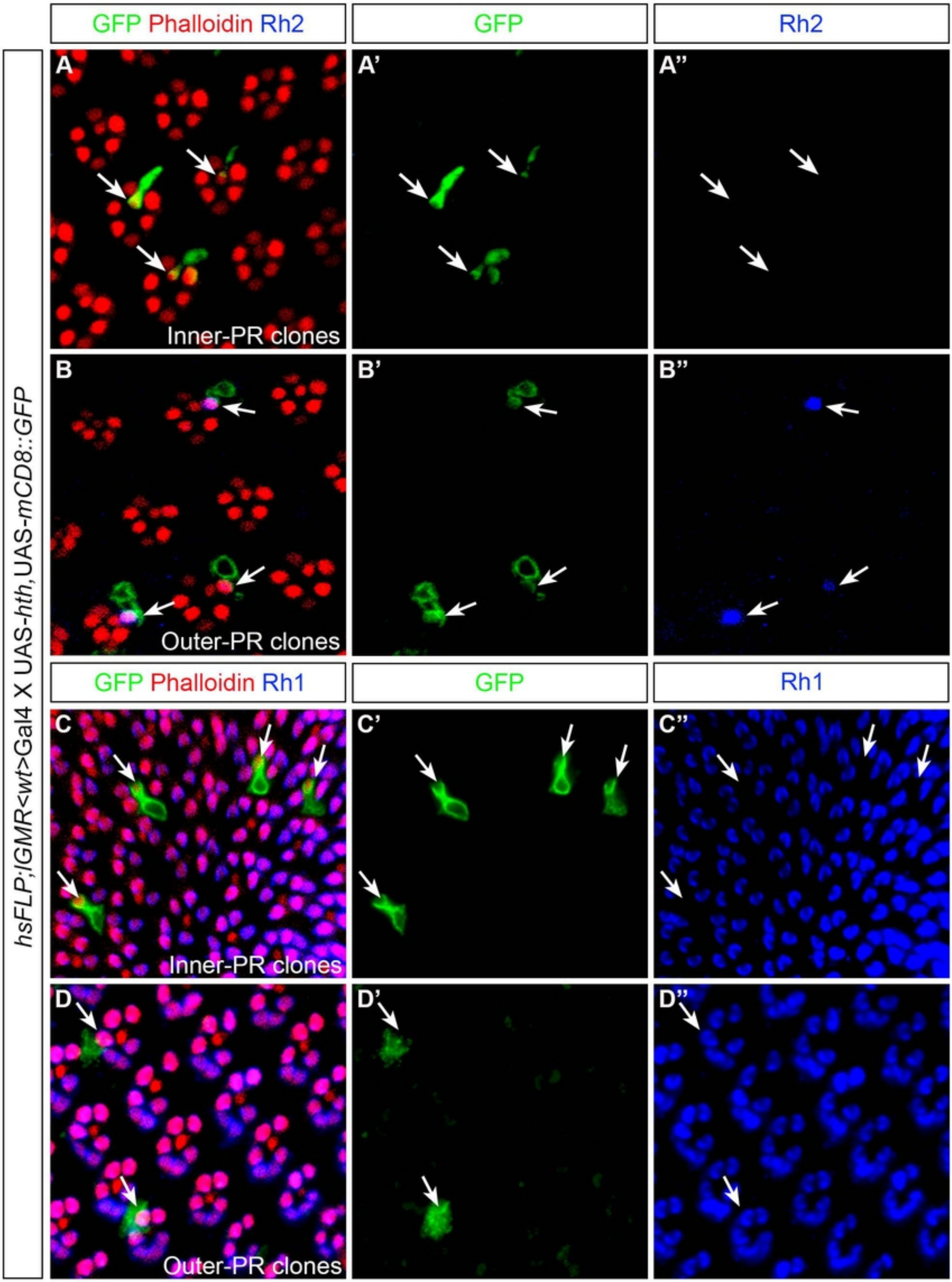
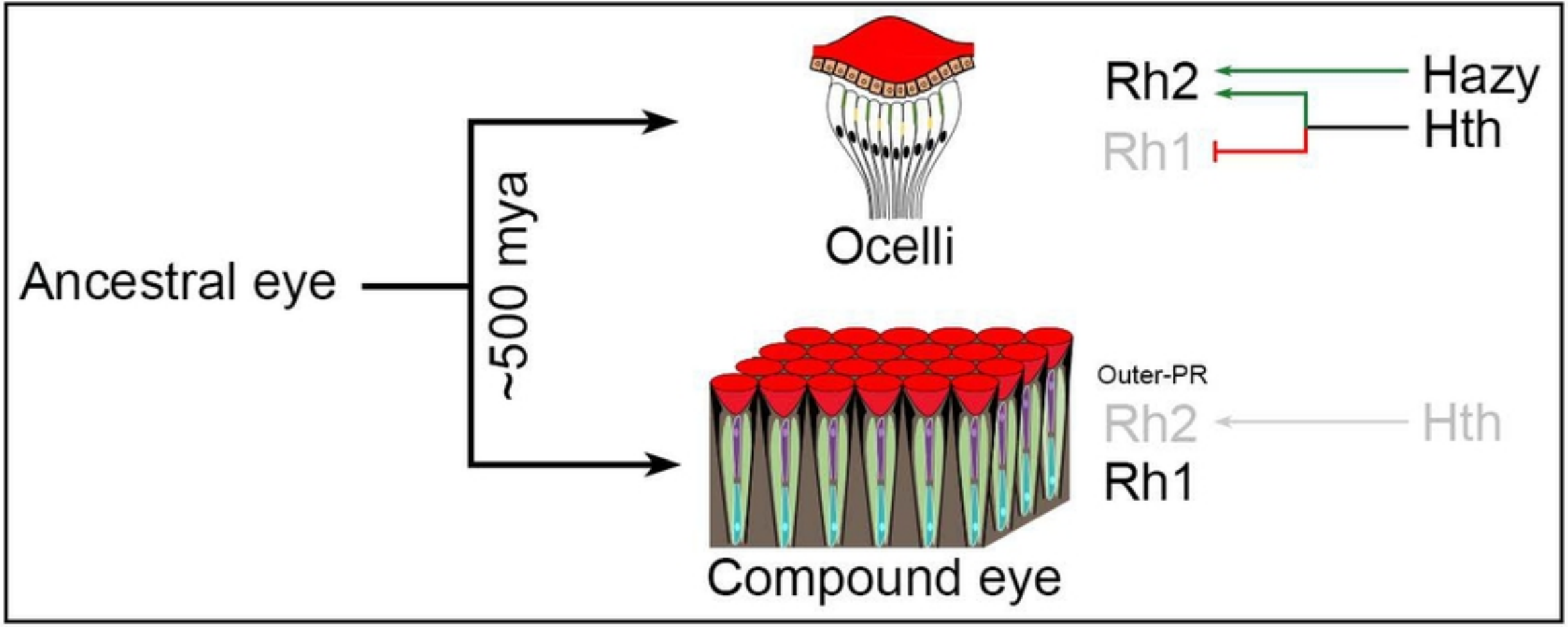
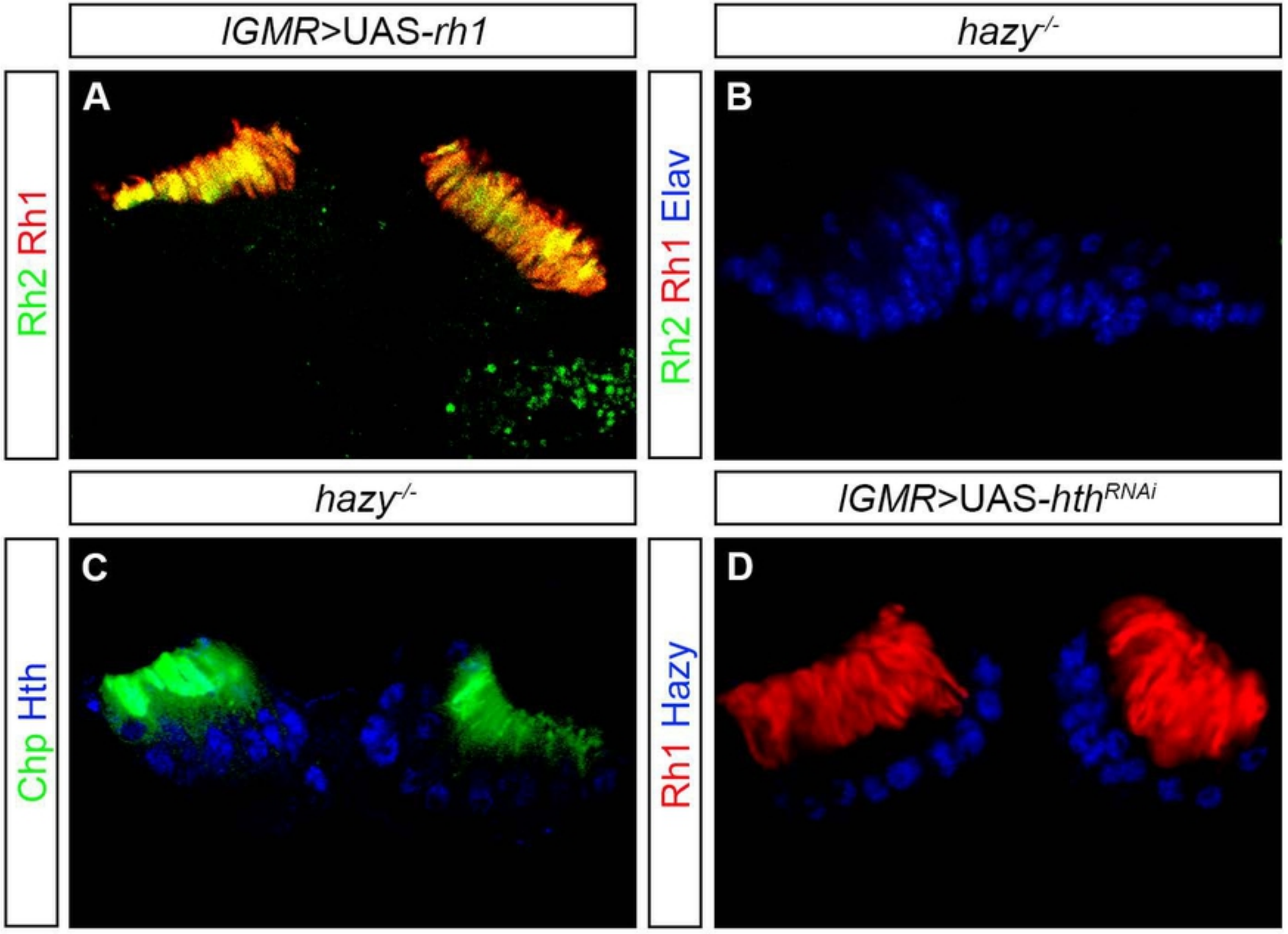
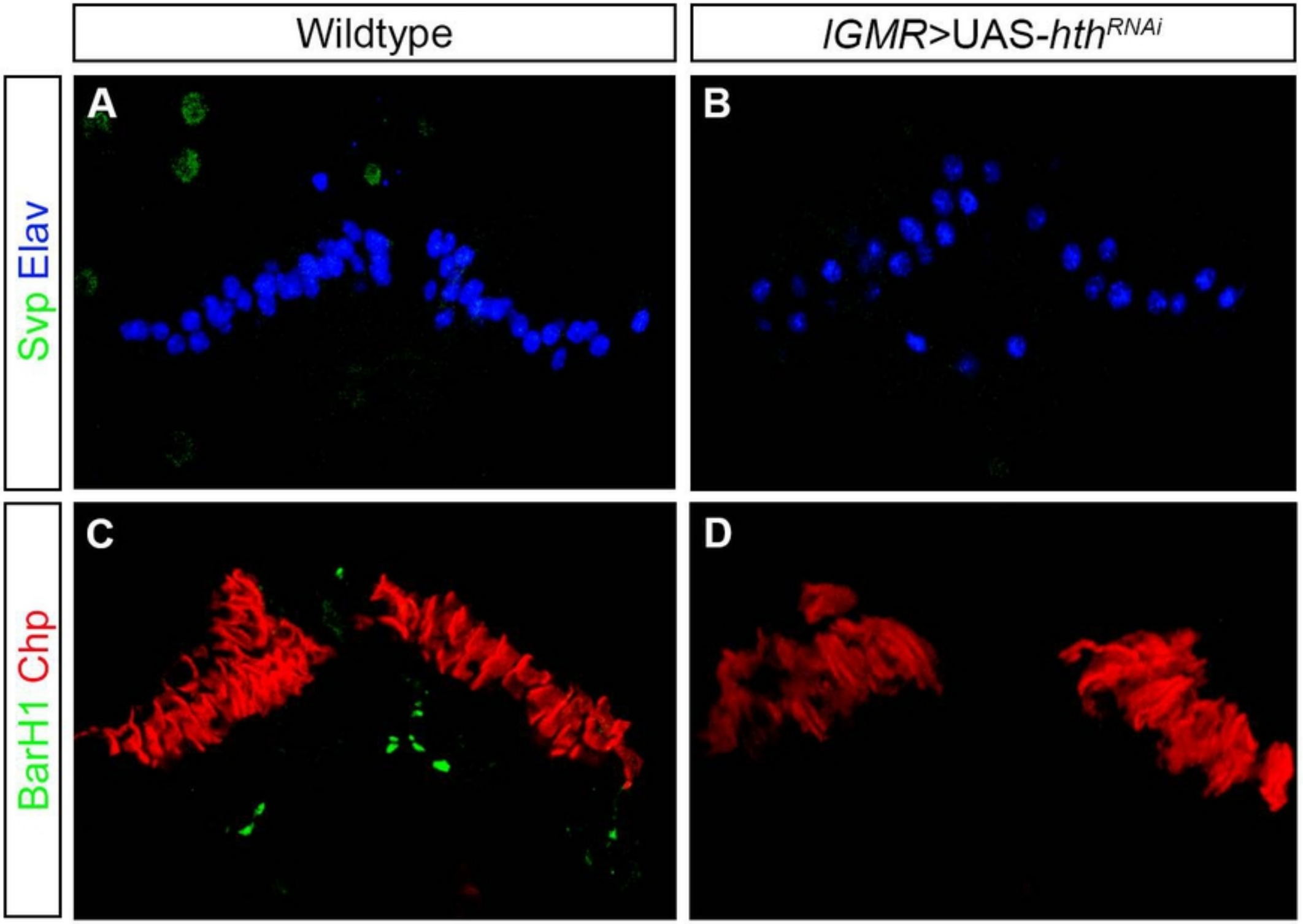

GFP Phalloidin Rh2
GFP
Rh2
A
A'
A''
Inner-PR clones
B
B'
B''
Outer-PR clones
GFP Phalloidin Rh1
GFP
Rh1
C
C'
C''
Inner-PR clones
D
D'
D''
Outer-PR clones
hsFLP;IGMR<wt>Gal4 X UAS-hth,UAS-mCD8::GFP

Figure 6


Ancestral eye
~500 mya
Ocelli
Rh2
Hazy
Hth
Rh1
Outer-PR
Rh2
Hth
Rh1
Compound eye

Supplemental figure 1



Supplemental figure 2