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## **Bilateral visual projections exist in non-teleost bony fish and predate the emergence of tetrapods**

Robin J Vigouroux, Karine Durore, Juliette Vouigny, Shahad Albadri, Peter Kozulin, Eloisa Herrera, Kim Nguyen-Ba-Charvet, Ingo Braasch, Rodrigo Suárez, Filippo del Bene, et al.

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2 **Bilateral visual projections exist in non-teleost bony fish and predate the**  
3 **emergence of tetrapods**  
4

5 One sentence summary: Bilateral vision preceded terrestrial life

6  
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30 **Abstract:**

31 **In most vertebrates, camera-style eyes contain retinal ganglion cell neurons projecting to**  
32 **visual centers on both sides of the brain. However, in fish, ganglion cells are thought to**  
33 **only innervate the contralateral side. This suggested that bilateral visual projections**  
34 **appeared in tetrapods. Here, we show that bilateral visual projections exist in non-teleost**  
35 **fishes and that the appearance of ipsilateral projections does not correlate with terrestrial**  
36 **transition or predatory behavior. We also report that the developmental program**  
37 **specifying visual system laterality differs between fishes and mammals as the *Zic2***  
38 **transcription factor which specifies ipsilateral retinal ganglion cells in tetrapods appears**  
39 **absent from fish ganglion cells. However, overexpressing human *ZIC2* induces ipsilateral**  
40 **visual projections in zebrafish. Therefore, the existence of bilateral visual projections**  
41 **likely preceded the emergence of binocular vision in tetrapods.**

42

43

44

45 Eye position on the head is highly variable between species, but frontal eyes have long been  
46 considered critical for depth perception (stereopsis) by increasing the overlap of the right and  
47 left eye visual fields (*1*). In vertebrates, ganglion cell axons from each eye cross through each  
48 other at the optic chiasm and enter the brain on the contralateral side. In mammals, visual axons  
49 from each eye meet and interweave at the chiasm. However, optic nerve crossing modalities  
50 are more diverse in fish and in most species the two optic nerves remain fully separated and  
51 only overlap at the chiasm (*2, 3*).

52 Classic neuroanatomical studies showed that in mammals, eye projections are bilateral with a  
53 variable fraction of retinal ganglion cell (referred to as ganglion cell thereafter) axons  
54 continuing in the ipsilateral optic tract after crossing the chiasm. The proportion of ipsilateral

55 projections is low (2-3%) in rodents but reaches around 40% in primates (4, 5). The comparative  
56 analysis of many vertebrate species conducted over several decades suggests that ipsilateral  
57 visual axons exist in all mammals, anuran amphibians, some reptiles, and that they are  
58 essentially absent or were secondarily lost in birds (5–8). Accordingly, developmental  
59 transcriptional programs specifying ipsilateral ganglion cells described in mammals are  
60 conserved in *Xenopus* but not in chick and zebrafish (5, 9). This textbook view implies that  
61 visual axon bilaterality emerged in early tetrapods and might have provided a visual advantage,  
62 in particular for nocturnal and predatory terrestrial species (10). However, a review of the  
63 extensive literature on fish visual systems gives a more complex image with reports, sometimes  
64 contradictory, of ipsilateral ganglion cell projections in some fish species (6, 11). Most of these  
65 pioneering studies relied on imprecise histological staining methods such as the Nauta-Gygax  
66 staining method or autoradiography (12). Here, we assessed the laterality of visual projections  
67 in bony fishes (**Fig. 1A**) with the B fragment of the cholera toxin (12) coupled to fluorescent  
68 dyes. Dye-coupled Cholera toxins have not been previously used in fish although they proved  
69 to be highly reliable tracers for visual projections in rodents due to their efficient endocytosis  
70 by neurons, slow elimination, high photostability and brightness (13). They are also compatible  
71 with whole-brain clearing and thereby allow mapping visual pathways in intact brain using 3D  
72 light sheet fluorescence microscopy (14, 15).

73 With more than 30,000 species, fishes account for at least half of the extant vertebrate species  
74 (16). We initially focused on ray-finned fishes (Actinopterygians; **Fig. 1A** and **fig. S1**) which  
75 separated from lobe-finned fish (Sarcopterygians, including tetrapods) around 450 Million  
76 years ago (Ma)(17). Within ray-finned fishes, we initially selected 6 species among the  
77 clupeocephalan lineage (**fig. S1**), the largest of the three lineages of teleost fishes which account  
78 for most (about 96%) of extant teleosts (18). Within clupeocephalans, three represent  
79 ostariophysians (Mexican tetra *Astyanax mexicanus*, redeye piranha *Serrasalmus rhombeus*,



80 and zebrafish *Danio rerio*) and three are percomorphs (green-spotted pufferfish *Tetraodon*  
81 *nigroviridis*, Atlantic mudskipper *Periophthalmus barbarus*, and four-eyed fish *Anableps*  
82 *anableps*). With 9,000 and 16,000 species respectively, ostariophysians and percomorphs are  
83 the two largest clades of teleosts. They have diverse eye positions, feeding behaviors, and  
84 habitats and some were previously reported to have ipsilateral visual projections (11).

85

### 86 **Totally crossed visual projections in teleosts**

87 The visual system of the zebrafish has been extensively studied using lipophilic dye tracing or  
88 genetic methods and shown to be exclusively contralateral (19). Accordingly, we found that  
89 fluorescent (Cholera toxin labelled) axons were only present on the contralateral side of adult  
90 zebrafish brains (**Fig. 1B** and **movie S1**; n=6). Previously identified retino-recipient visual  
91 nuclei (20) could be detected with cholera toxin, thereby validating the use of this tracing  
92 method in fish (**Fig. 1C**). Light-sheet microscopy imaging of cholera toxin injected fish, showed  
93 that visual projections were only contralateral in Mexican tetra surface fish (**Fig. 1, D and E**  
94 and **movie S1**; n=7), contradicting a previous study (21). Likewise, eyes in the redeye piranha  
95 only projected contralaterally (**Fig. 1, F and G** and **movie S1**; n= 3), in disagreement with earlier  
96 work in other piranha species (22, 23). This suggests that ostariophysians only have crossed  
97 visual projections.

98 In the fresh water green-spotted pufferfish (24), a percomorph, the 2 optic nerves stay separated  
99 at the chiasm and visual projections were exclusively contralateral (**Fig. 1, H and I** and **fig. S2**  
100 and **movie S1**; n= 4) as previously described in another pufferfish (25). We next studied the  
101 four-eyed fish, a percomorph surface dweller fish whose large protruding eyes with duplicated  
102 corneas and pupils allows seeing under and above the water (26). Again, one optic nerve passed  
103 over the other at the chiasm (**fig. S2**) and cholera toxin tracing showed that in this species, visual  
104 projections were also completely crossed (**Fig. 1, J and K**; n= 3). Similar results were obtained

105 in the mudskipper, a percomorph with amphibious lifestyle (**Fig. 1, L and M** and **fig. S2** and  
106 **movie S1**; n= 3). Together, these results show that in percomorph eyes, ganglion cells also  
107 likely only project to visual nuclei on the opposite side of the brain (**fig. S1**).

108 Osteoglossomorphs (bonytongues), a teleost sister groups of clupeocephalans (**Fig. 1A** and  
109 **fig. S1**), are considered a group of basal (i.e the group which gave rise to later forms) teleosts  
110 constituted of about 200 living species (16). We chose to trace visual projections in the African  
111 butterflyfish (*Pantodon buchholzi*), a predator living close to the surface of freshwater system,  
112 and found a small contingent of retinal axons (**Fig. 2, A and B** and **movie S1**,  $2.33\pm 0.23\%$  %  
113 ipsilateral projections in the optic tectum; n=3) project to the ipsilateral side, corroborating an  
114 earlier report (27). The main portion of ipsilateral axons targeted the tectum and some others  
115 targeted pretectal nuclei. Ipsilateral visual axons have also been described in a mormyrid  
116 electric fish (*Gnathonemus petersii*) (28), another osteoglossomorph with a more nocturnal  
117 predatory behavior that can orient by active electrolocation (29). These results show that  
118 bilateral visual projections exist in osteoglossomorph teleosts regardless of their predatory  
119 strategy and lifestyle history. Therefore, within teleosts, ipsilateral projections could have been  
120 secondarily lost in clupeocephalans or independently acquired in osteoglossomorphs. In  
121 mammals, binocular inputs to visual targets/areas are either segregated (thalamus, colliculus)  
122 or intermingled (suprachiasmatic nucleus). As both eyes were injected with 2 distinct Alexa-  
123 conjugated-cholera toxins, we also studied the relative distribution of ipsilateral and  
124 contralateral ganglion cell axons in butterflyfish brain areas innervated by both eyes. This  
125 showed that in African butterflyfish (**Fig. 2C**) retinal inputs from both sides segregated, as in  
126 the thalamus and superior colliculus of mammals.

127

128 **Bilateral visual projections exist in basal ray-finned fishes**

129 These results on teleosts led us to study retinal projections in non-teleost ray-finned fish  
130 lineages (Holosteans, Acipenseriforms, and Polypteriforms, **Fig. 1A**) which split from teleosts  
131 before the teleost whole genome duplication event (TGD, **Fig. 1A**) that occurred around 320  
132 Ma in the ancestor of extant teleosts (reviewed in (30)). Holosteans and Acipenseriforms are  
133 considered to have evolved slowly since they branched from other vertebrates 350 Ma (31).  
134 Bilateral visual projections (**movie S2**, n=5/5) were observed in the spotted gar (*Lepisosteus*  
135 *oculatus*), one of the seven extant species of garfish, and a representative of the holosteans. The  
136 spotted gar is a unique vertebrate model system as its genome is thought to provide a “bridge”  
137 between tetrapods and teleosts (32). Bilateral cholera toxin injections revealed an ipsilateral  
138 projection in the rostral optic tectum of the spotted gar, with visual axons targeting several  
139 pretectal nuclei. There was no overlap of the contralateral and ipsilateral axons (**Fig. 2, D to F**  
140 and **movie S2**, n=5/5) which represented  $4.78 \pm 0.46$  % of visual inputs in the tectum, a ratio  
141 comparable to rodents. Next, we traced visual projections in the acipenseriform sterlet sturgeon  
142 (*Acipenser ruthenus*, n=2/2). Cholera toxin tracing demonstrated the existence of a binocular  
143 domain in the tectum of the sterlet sturgeon as well as in several pretectal nuclei (**Fig. 2, G to**  
144 **J** and **movie S2**;  $9.77 \pm 1.28$  % ipsilateral projections in the tectum, n=2). No re-crossing of  
145 visual inputs after entering the brain were detected contrary to previous observations in the  
146 Russian sturgeon *Acipenser guldensädtii* (33). We then studied the armored bichir (*Polypterus*  
147 *delhezi*; n=2/2), a carnivorous nocturnal fish representing the most basally diverging lineage of  
148 extant ray-finned fishes, the Polypteriforms. In the bichir, the two optic nerves meet at the  
149 chiasm and ganglion cells axons interweave during decussation (**fig. S2**). Ipsilateral axons  
150 projected to numerous pretectal nuclei such as the nucleus opticus dorsolateralis anterior  
151 thalami, the area optica ventrolateralis thalami, and the nucleus commissurae posterior par  
152 magnocellularis (**Fig. 2, K to N** and **movie S2**). This corroborates previous studies in gray bichir  
153 *Polypterus senegalus* (34). These results, together with similar observations in the holosteans

154 longnose gar (*Lepisosteus osseus*) and the bowfin (*Amia calva*) (35, 36) indicate that bilateral  
155 visual projections likely are ancestral among actinopterygians and arose before their  
156 diversification and that the ipsilateral component likely was subsequently lost in  
157 clupeocephalans. To further test this hypothesis, we analyzed visual projections in the  
158 Australian lungfish (*Neoceratodus forsteri*) a basal member of the lobe-finned fishes  
159 (Sarcopterygians) the monophyletic group that includes tetrapods. Lobe-finned fishes diverged  
160 from ray-finned fishes about 450 Ma and lungfish are now considered the closest living fish  
161 relative of tetrapods (17). In all injected animals (n=6/6) a small ipsilateral projection was found  
162 innervating the optic tectum (**Fig. 2, O to S** and **movie S3**, n=6). In contrast to other fish species  
163 analyzed here, ipsilateral projections intermingled with contralateral ones (**Fig. 2, R** and **S**;  
164 n=6). This was consistent with an earlier analysis of a single specimen of Australian lungfish  
165 (37) and supports the existence of bilateral visual projections in the bony vertebrate ancestor of  
166 actinopterygians and sarcopterygians (**fig.S1**). This intermingling of ipsilateral and contralateral  
167 axons could have some functional implications as it suggests that some tectal neurons might  
168 receive and integrate inputs from both eyes. Alternatively, it could represent an immature stage  
169 of visual system development that could be resolved in adult animals in an activity-dependent  
170 manner as it is the case in mammals. Together, these results indicate that the bilateral  
171 organization of the visual system likely did not appear in amniotes but that it is an ancestral  
172 vertebrate feature that emerged much earlier in evolution, before water-to-land transition and  
173 aerial vision adaptation in tetrapods.

174

### 175 **Zic2 expression in the ipsilateral human embryonic retina**

176 Our results raised questions about the evolution and conservation of the genetic mechanisms  
177 underlying visual system binocularity. Are they conserved in ray-finned fish with bilateral  
178 visual projections? The zinc-finger transcription factor Zic2 specifies the ipsilateral identity of

179 ganglion cells in developing mice, ferrets and *Xenopus* (5). In *Xenopus*, *Zic2* is absent from  
180 the neural retina of pre-metamorphic tadpoles that have only crossed visual projections, but  
181 *Zic2* is expressed in ipsilaterally projecting ganglion cells after metamorphosis (5). To further  
182 evaluate and support the implication of *Zic2* in the control of mammalian ganglion cell  
183 laterality, we analyzed the expression of *ZIC2* in the human eye and compared it to mice by  
184 performing immunohistochemistry in whole-mount retinas. In human embryos, ganglion cell  
185 axons reach the brain by the 7th post-conception week (pcw7) and the optic nerve is well formed  
186 at pcw10 (38). Using the EyeDISCO clearing protocol (15) in mice, we observed that at  
187 embryonic day 16 (E16), the peak of *Zic2* expression in mice (5), the retinal domain positive  
188 for *Zic2* represented about  $5.34 \pm 0.36$  % of the total retinal surface (**Fig. 3, A to C** and **fig. S3A**;  
189  $n=6$  eyes). Post-mitotic ganglion cells, the first neurons generated in the retina(39), migrate to  
190 the basal side to accumulate at the inner surface of the retina and express the transcription  
191 factors *Islet1* (40) and RNA-binding protein with multiple splicing (*RBPMS*)(41) (**Fig. 3**). At  
192 pcw9, *RBPMS*<sup>+</sup> and *ISLET1*<sup>+</sup> ganglion cells were present all over the retina (**Fig. 3, D to I**).  
193 Flat-mounted and sections of human retinas from pcw9 embryos, an age equivalent to E16 in  
194 mice (40), showed that *ZIC2*<sup>+</sup> cells were restricted to the temporal quadrant of the retina  
195 (representing about  $18.95 \pm 0.98$  % of the retina surface), which contains ipsilaterally projecting  
196 ganglion cells in primates (**Fig. 3, D to J** and **movie S4**;  $n=3$  eyes). *ZIC2*<sup>+</sup> were ganglion cells  
197 as they co-expressed *ISLET1* and *RBPMS* (**Fig. 3, F to H**). In the temporal retina, the density  
198 of *ZIC2*<sup>+</sup>/*ISLET1*<sup>+</sup> ganglion cells was higher close to the ciliary marginal zone, at the edge of  
199 the retina (**Fig. 3G**), than in more medial regions where it was absent from the most superficial  
200 *ISLET1*<sup>+</sup> (**Fig. 3H** and **fig. S3B**) and *RBPMS*<sup>+</sup> ganglion cells (**Fig. 3F** and **fig. S3**). *ZIC2* was  
201 not detectable in the nasal retina (**Fig. 3, D, E** and **I**). Unlike in the mouse (42), *ZIC2* was not  
202 present in the neuroblastic layer which contains *SOX2* (sex determining region Y-box 2) +  
203 progenitors (**Fig. 3, J to L** and **fig. S3C**). At pcw14, at the end of ganglion cell neurogenesis

204 (39), ZIC2 was still only present in the temporal retina (**Fig.3, K and L**). Although we could  
205 not access later stages of development, the absence of ZIC2 from the most superficial ganglion  
206 cells in the inner retina suggest that human ZIC2 is expressed in recently differentiated  
207 ipsilateral ganglion cells in the temporal retina and might be down-regulated as they mature, as  
208 is the case in mice.

209

### 210 **Zic2 is not expressed in differentiating ganglion cells in fish with bilateral visual** 211 **projections**

212 The pivotal role for Zic2 in the specification of an ipsilateral axonal growth program in  
213 mammals was also well correlated with the absence of transcripts of the two *zic2* co-orthologs  
214 (*zic2a* and *zic2b*, generated in the TGD) in zebrafish ganglion cells (43) (**fig. S4, A to C and E**  
215 **to G**). Double fluorescent *in situ* hybridization for *zic2b* and *atoh7* (a committed precursor  
216 marker) from 24 to 48 hours post fertilization confirmed that *zic2b* did not colocalize with  
217 differentiating *atoh7*+ ganglion cells. By contrast, and as reported for *zic2* in the mouse (44),  
218 *zic2b* was detected in the ciliary marginal zone, (**fig. S4M**, n=3) which contains dividing  
219 progenitors and stem cells producing all retinal cell types, even in adult teleosts (45, 46) (**fig.**  
220 **S4, H to L**; n=5).

221 The presence of an ipsilateral visual projection in the spotted gar, as extensive as that of mice,  
222 together with its well characterized genome and the accessibility of gar embryos (32, 47), led  
223 us to evaluate the expression of *zic2* in the developing gar retina. We first analyzed the  
224 development of the gar visual system using whole-mount immunolabelling, iDISCO+ clearing  
225 and light-sheet microscopy (**Table S1**) (15). In the spotted gar, a few Islet1- immunoreactive  
226 ganglion cells were detected at 2-3 days post fertilization (dpf) (**Fig. 4, A and B and fig.S5C**  
227 **and movie S5**; n=5). At 6-7dpf, optic nerves could be observed and had reached the optic  
228 chiasm (**Fig. 4, C and D and movie S5**; n=5). Development is slower in gar than in zebrafish

229 and it is temperature-dependent (48). The next 10 days of development are only characterized  
230 by changes in fin opercular and gill formation but not in eye morphogenesis (48). By 17-18dpf  
231 the retina contained many ganglion cells and the optic tract was well developed (**Fig. 4, E to G**  
232 and **movie S5**; n=5). The highly proliferative ciliary marginal zone could be identified by the  
233 presence of cells expressing the S-phase marker, proliferating cell nuclear antigen marker and  
234 overlapping with *zic2* expression, which was absent from neighboring post-mitotic Islet1+  
235 ganglion cells (**Fig. 4, H to L**). At 2-3dpf and 6-7 dpf, *zic2* mRNA was detected in proliferating  
236 cells of the developing neuroretina and progressively became restricted to the ciliary marginal  
237 zone (**fig. S5, A to D**). Its paralogs, *zic1* and *zic5*, also enriched in ipsilateral ganglion cell in  
238 mice (49), were absent from the embryonic gar retina (**fig. S5, E to L**). These results show that  
239 neither *Zic2*, *Zic1* nor *Zic5*, specify ipsilateral ganglion cells in the spotted gar, suggesting that  
240 *zic* genes might be dispensable for gar ipsilateral projections. By contrast, the presence of *Zic2*  
241 in the ciliary marginal zone of fish and mammals suggests that *Zic2* might have a function in  
242 retinal precursors that is evolutionarily conserved.

243 In mammals, *Zic2* acts in part by activating the expression of the receptor tyrosine kinase  
244 EphB1 in ipsilateral axons (8, 50), whose ligand ephrinB2, localized at the chiasm, prevents  
245 crossing (7). According to the lack of *Zic2* in the gar ganglion cells, we did not detect *ephB1*  
246 mRNA in the developing retina and *ephrinB2* was absent from the chiasm (**fig. S5, M to W**).

247

#### 248 ***Zic2* overexpression induces ipsilateral projections in zebrafish**

249 In mice, *Zic2* overexpression in the retina, outside the ipsilateral domain, increases the  
250 proportion of ipsilaterally projecting ganglion cell axons (50). Therefore, we tested the  
251 hypothesis that despite its absence in fish ganglion cells, the forced expression of *Zic2* in  
252 zebrafish ganglion cells could affect their axonal targeting. We used a human *ZIC2-T2A-GFP*  
253 overexpression construct to express *ZIC2* and GFP in the zebrafish eye under the control of the

254 *atoh7* promoter. Vertebrate *Zic2* proteins are highly conserved (51), with 81.1% identity  
255 between Human ZIC2 and zebrafish *Zic2a* and more than 93% similarity in the zinc finger  
256 domain (**fig. S6**). To visualize the projections coming from ZIC2 overexpressing ganglion cells  
257 derived from a single eye, we removed one eye at 2 dpf. As previously reported, under normal  
258 conditions retinal fibers from the remaining eye projected exclusively to the contralateral  
259 tectum (**Fig. 4, M and N**; n=10) (52, 53). In contrast, ZIC2-expressing ganglion cells generated  
260 ipsilateral retinotectal afferent fibers representing a mean of  $19.6 \pm 8.5\%$  of the GFP+ axons  
261 (**Fig. 4, O and P**; n=10/13). Expression of ZIC2 did not seem to bias the targeting of ganglion  
262 cell axons to their topographic position as previously reported in mouse (54). These results  
263 show that *zic2*, although not normally expressed in zebrafish ganglion cells, can still specify an  
264 ipsilateral program. In mice, the receptor tyrosine kinase EphB1 is expressed by ipsilateral  
265 ganglion cell axons and the ectopic expression of *Zic2* in the contralateral retina induces EphB1  
266 and reduce midline crossing at the chiasm (8, 50). However, we could not detect EphB1 protein  
267 or mRNA (**fig. S7**) in zebrafish ganglion cells, neither in controls injected with GFP (n=13) nor  
268 in ZIC2-overexpressing fish (n=11) suggesting that these may be guided by alternative cues.

269

## 270 **Discussion**

271 It has been proposed that the evolution of terrestrial vertebrates followed an increase of eye size  
272 in aquatic vertebrates able to see through air, in a process that has occurred also in modern  
273 crocodiles or fish species similar to the four-eye fish and mudskippers (55). The existence of  
274 ipsilateral projections in the most basally branching groups of both actinopterygians and  
275 sarcopterygians indicates that ipsilateral connections were likely already present in the common  
276 ancestor of bony vertebrates, a bony fish, thus preceding aerial vision adaptation of tetrapods.  
277 This example highlights how the comparative study of a variety of species outside the list of  
278 the classical model species allows drawing evolutionary conclusions that may otherwise remain



279 obscured (56). Moreover, all the teleosts species analyzed in our study can be described as  
280 diurnal predators that heavily rely on visual cues to detect and consume their preys. These preys  
281 may vary in size from large vertebrates (redeye piranha) to small invertebrates (Mexican tetra,  
282 zebrafish, green-spotted puffer, Atlantic mudskipper and the four-eyed fish). In all cases our  
283 data show that ipsilateral projections in teleosts are not required for a visually mediated  
284 predatory behavior as it is usually assumed in mammals. Recent studies have confirmed this  
285 also in larval zebrafish where possible alternative neuronal circuits have been described (53).  
286 On the other hand, lungfish and some basally branching actinopterygians, where ipsilateral  
287 projections are present, show reduced visual system development, as they are bottom dwellers  
288 that show nocturnal predatory behavior (lungfish and bichir) or feed on benthic organisms  
289 (sturgeon). Overall, our data show that the presence of ipsilateral projections in the visual  
290 system of fishes appears to correlate with phylogeny and not with life style or predatory  
291 behavior. Along these lines, it is therefore unlikely that ipsilateral retinal projections serve a  
292 function similar to what is commonly considered in mammals. On the contrary, visual system  
293 bilaterality might have been used as the neural substrate to compute stereopsis following the  
294 acquisition in diurnal mammals of visual-based predatory abilities after the Cretaceous–  
295 Paleogene (K–Pg) extinction event. Supporting this view, the number of ipsilateral projections  
296 in reptiles (chelonians and squamates) correlates neither with eye position nor with the degree  
297 of binocular field (11, 57). It has been hypothesized that ipsilateral ganglion cells facilitate  
298 motor coordination by providing a direct visual feedback to the limb steering brain centers (6).  
299 However, the function of ipsilateral ganglion cells in fish remains elusive and behavioral studies  
300 in non-canonical model species such as the gar will be required to address this question.  
301 The conservation of the main families of axon guidance cues and receptors in Bilateria  
302 suggested that the mechanisms underlying the development of neuronal connectivity are  
303 evolutionarily conserved (58, 59). However, the loss of the gene encoding Deleted in Colorectal

304 Cancer receptor in some bird species (60) and the uniqueness of the Roundabout3 receptor in  
305 mammals (61) have challenged this view. Here we show that the guidance program specifying  
306 visual axon ipsilaterality does not appear to be entirely conserved as we failed to detect  
307 expression of a *zic2* and other *zic* genes or *ephB1* in spotted gar ganglion cells. Hence, the  
308 textbook model of *Zic2* and *EphB1* in orchestrating retinal ganglion cell laterality does not  
309 simply translate to fish. In rodents, the contralateral identity of ganglion cells is specified by  
310 *Islet2* (62) and *SoxC* (63) transcription factors but whether they influence the development of  
311 visual axons in fish is unknown. Therefore, further experiments are needed to address the  
312 molecular mechanisms underlying visual bilaterality in bony fish species. Are there other  
313 pathways, apart from *Zic2*, that could direct ipsilateral projections or which could block the  
314 contralateral fate that occurs normally? A recent study (64) shows that in mice, contralateral  
315 ganglion cells activate a non-canonical Wnt signaling pathway to cross the midline. In  
316 ipsilateral ganglion cells, *Zic2* prevents midline crossing by inducing a genetic module that  
317 changes the expression of a set of genes to jointly inhibit this non-canonical Wnt pathway.

318 In fact, the ectopic expression of human *ZIC2* in the developing zebrafish retina still induces  
319 the formation of ipsilateral visual axons without causing other targeting defects. This finding  
320 suggests a possible conservation in fishes of downstream components of the genetic program  
321 specifying ipsilateral axons in mammals although the factor initiating its expression and its  
322 relationship to *Zic2* remains unclear in the gar and in other non-teleost fish. By parsimony  
323 principle, given our data, we propose that the presence of ipsilaterality in the bony vertebrate  
324 ancestor is the most likely explanation. In particular, the Australian lungfish data show that  
325 ipsilateral projections were likely present in the sarcopterygian fish ancestor of tetrapods.  
326 Lungfish also functions as outgroup to the actinopterygians and thereby making an independent  
327 origin of ipsilateral visual axons within actinopterygians a less likely hypothesis. An alternative,

328 yet less parsimonious explanation, could be that ipsilaterality has evolved independently  
329 multiple times among bony vertebrates, using different genetic mechanisms.

330

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501

## 502 **Figure legends**

503

### 504 **Fig.1. Visual projections are only contralateral in clupecocephalan teleosts.**

505 (A) Simplified phylogenetic tree of the major groups of vertebrates. Divergence of each major  
506 group is displayed in million years. Asterisks indicate whole genome duplication events in the  
507 teleost (TGD) and sturgeon (AGD) ancestors. (B) Whole brain visualization of a juvenile  
508 zebrafish injected with an AlexaFluor-555-conjugated CTb (left eye) and AlexaFluor-647-  
509 conjugated CTb (right eye) highlighting complete contralateral projections. (C) High  
510 magnification showing pre-tectal nuclei. (D to M) 3D rendering of visual projections labelled  
511 by injecting AlexaFluor-555-conjugated CTb (left eye) and AlexaFluor-647-conjugated CTb  
512 (right eye) followed by iDISCO whole-brain clearing and 3D imaging using light-sheet

513 fluorescence microscopy. (D and E) Mexican tetra. (F and G) Redeye piranha. (H and I) Green-  
514 spotted pufferfish. (J and K) Four-eyed fish. (L and M) Atlantic mudskipper. In all species,  
515 visual axons only project to the brain on the contralateral side. Abbreviations: A, anterior; Cb,  
516 Cerebellum; Ctb, Cholera toxin B; D, dorsal; OB, Olfactory bulb; ON, Optic nerve; P, posterior;  
517 V, ventral; Sil, Silurian. Scale bars are 500  $\mu\text{m}$  in (B) and (D) to (M) and 200  $\mu\text{m}$  in (C).

518

519 **Fig. 2. Bilateral visual projections in basal ray-finned fishes and lobe-finned fishes**

520 (A to S) 3D light-sheet fluorescence microscopy images of iDISCO+ cleared brains from fish  
521 injected into the eyes with 2 cholera toxins. The left panels show only one channel. (C, F, J, N  
522 and S) are optical sections through the brain region receiving bilateral visual inputs. (I and M  
523 and R) high magnification from whole-mount brains. (A to C) Butterflyfish. (D to F) Spotted  
524 gar. (G to J) Sterlet sturgeon. (K to N) Armored bichir. (O to S) Australian lungfish. (C, F, J  
525 and N) In all fishes, contralateral and ipsilateral projections segregate in two different optic  
526 tectum (OT) layers except in the lungfish (S) where they are intermingled in the OT.  
527 Arrowheads indicate ipsilateral projections. Abbreviations: Cb, Cerebellum; OB, Olfactory  
528 bulb; OE, Olfactory epithelium; ON, Optic nerve; OC, Optic chiasm; pT, pretectal nuclei; T,  
529 Optic tract. Scale bars are 500  $\mu\text{m}$  in (A, B, D, E, G, H, K, L, P and Q) and 80  $\mu\text{m}$  in (C, G, K,  
530 N and S) and 100  $\mu\text{m}$  in (M, O) and 150  $\mu\text{m}$  in (I, R).

531

532 **Fig. 3. Zic2 is expressed in the temporal retina of mammal embryos**

533 (A to C) Whole-mount immunohistochemistry of an E16 mouse eye labeled with the pan  
534 ganglion cell marker RBPMS and the ipsilateral ganglion cell marker Zic2. (A) frontal view  
535 and (B) top view. (C) Optical section at the level indicated by the dashed line in (C). (D to F),  
536 3D light-sheet fluorescence microscopy of pcw9 human embryonic eye cleared using  
537 EyeDISCO and labeled for RBPMS and ZIC2. (D) frontal view and (E) top view. (F) Optical

538 section at the level indicated by the dashed line in (C). G, H, I indicate the approximate positions  
539 of images in panels G-I. (G to I) retinal cryosections of a pcw9 human embryo eye labeled with  
540 ZIC2 and ISLET1 at 3 different levels: temporal and close to retinal outer limit (G), temporo-  
541 medial (H) and nasal (I). ZIC2 cells are in the ganglion cell layer (GCL) and co-express  
542 ISLET1. They are absent from the neuroblastic layer (NBL). (J) retinal cryosection of a pcw9  
543 human embryo eye labeled with ZIC2 and SOX2. ZIC2 is absent from the NBL which contains  
544 SOX2+ progenitors. (K and L) retinal cryosections of a pcw14 human embryo eye labeled with  
545 ZIC2, SOX2 and ISLET1. ZIC2 is only present in ISLET1+ ganglion cells in the temporal  
546 retina and absent from SOX2+ progenitors (K). Abbreviations: D, dorsal ; V, ventral ; N, nasal ;  
547 T, temporal ; ON, optic nerve. Scale bars are 70  $\mu$ m in (A and B) and 20  $\mu$ m in (C) and 300  $\mu$ m  
548 in (D and E) and 50  $\mu$ m in (F to L).

549

550 **Fig. 4. Zic2 is not expressed by ganglion cells in spotted gar and zebrafish**

551 (A to F) Development of the visual system in the spotted gar. All images are 3D light-sheet  
552 fluorescence microscopy images of EyeDISCO-cleared spotted gar embryos labeled with Islet1  
553 and acetylated Tubulin. (A and C and E) Top (dorsal) views of spotted gars at 2-3dpf (A), 6-  
554 7dpf (C), and 17-18dpf (E). (B and D and F) frontal views of whole spotted gar eyes at 2-3dpf  
555 (B), 6-7dpf (D), and 17-18dpf (F). The optic nerve (ON and asterisk) starts to form by 6-7dpf  
556 and is well developed by 17-18dpf. The optic chiasm (OC) is formed by 6-7dpf. (G) Coronal  
557 cryosection of spotted gar embryos at 17-18dpf labeled for  $\beta$ III-tubulin and Islet1. (H to K)  
558 cryosection from 17-18dpf spotted gar eyes hybridized with *zic2* riboprobe (H and J) and  
559 labeled for proliferating cell nuclear antigen (PCNA) and Islet1 (I, K and L). (J to L) higher  
560 magnification of the ciliary margin zone (area framed in I). (M to P) 3D rendering of whole-  
561 brain viewed from the top of zebrafish injected with *Tg(atoh7:Gal4,14UASubc:T2A-eGFP-pA)*  
562 (M and N) or *Tg(atoh7:Gal4,14UASubc:ZIC2-T2A-eGFP-pA)* (O and P). N and P show



563 segmented ganglion cell projections. (P) A large ipsilateral projection (arrowhead) is seen in  
564 the *Tg(atox7:Gal4,14UASubc:ZIC2-T2A-eGFP-pA)*-injected fish. Abbreviations: ON, Optic  
565 nerve; OC, Optic chiasm; GCL, Ganglion cell layer; INL, Inner nuclear layer; ONL, Outer  
566 nuclear layer. Scale bars are 50  $\mu\text{m}$  in (A, D, F, J, K, L, M to P) and 15  $\mu\text{m}$  in (B) and 80  $\mu\text{m}$   
567 in (C, H and I) and 150  $\mu\text{m}$  in (E) and 200 $\mu\text{m}$  in (G).

568

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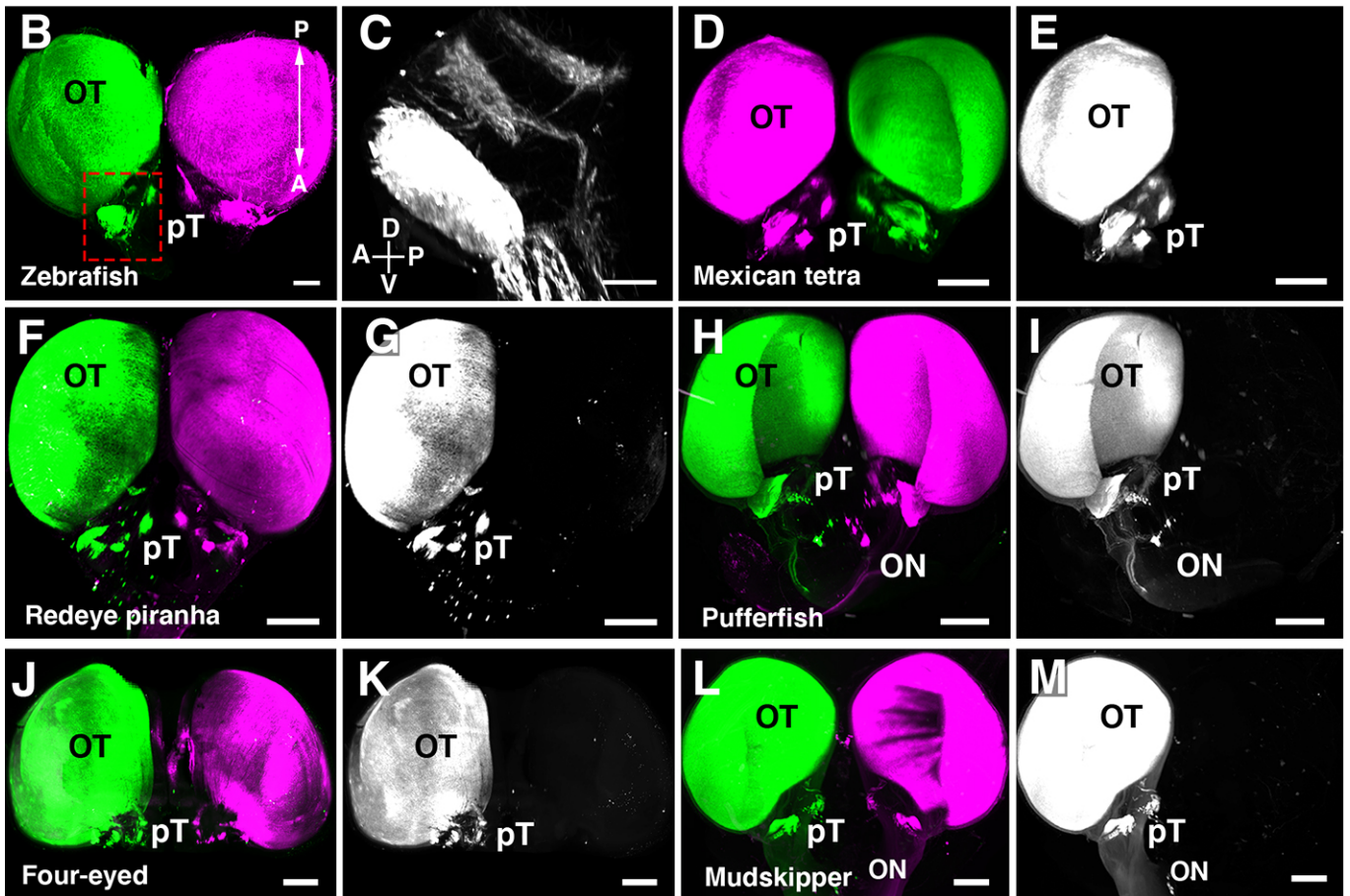
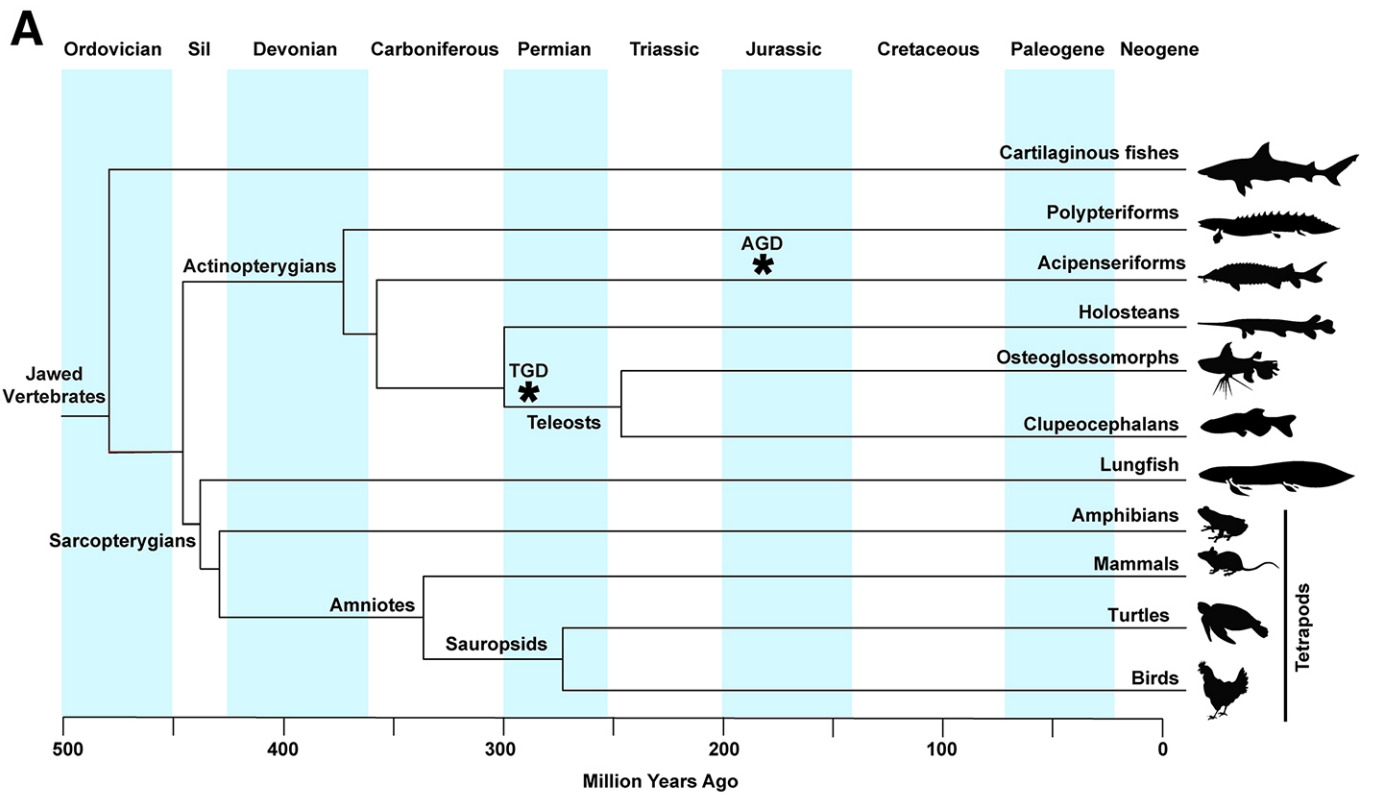
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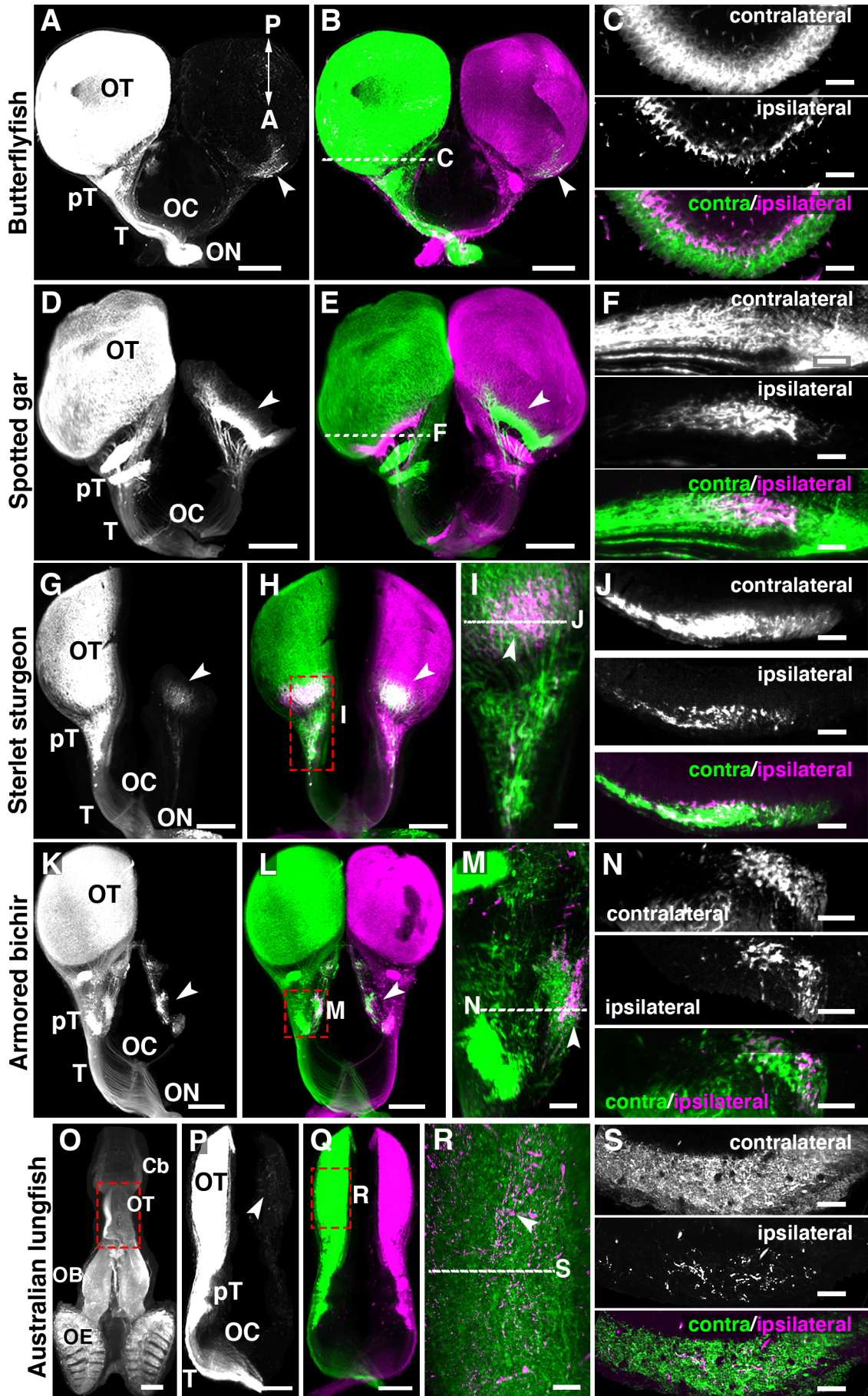
578 **Authors contribution:** AC, FDB and RV designed the experiments. FDB, KD, KNBC, JV,  
579 SA, RV, PK, RS conducted the experiments. AC, KNBC and FDB obtained funding for the  
580 project. IB provided *Lepisosteus* samples and EH the human *ZIC2* cDNA. AC, FDB and RV  
581 designed the figures and wrote the manuscript. All authors discussed the results and commented  
582 on the manuscript. **Competing interests:** The authors declare no competing interests. **Data and**  
583 **materials availability:** All data are available in the manuscript or the supplementary materials.

584

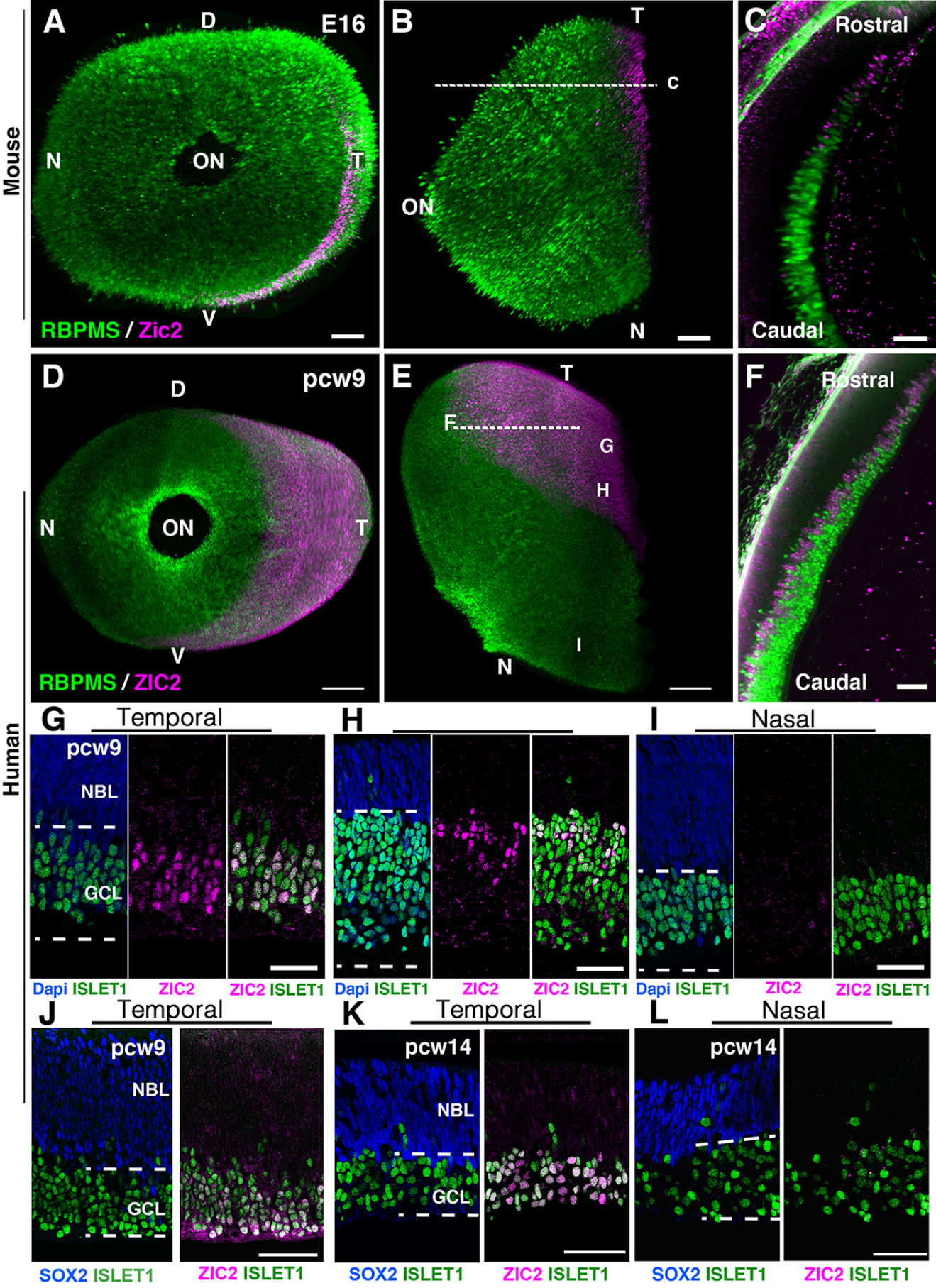
## 585 **List of supplementary materials**

586 Materials and Methods  
587 Figs. S1 to S7  
588 Table S1  
589 Movies S1 to S5

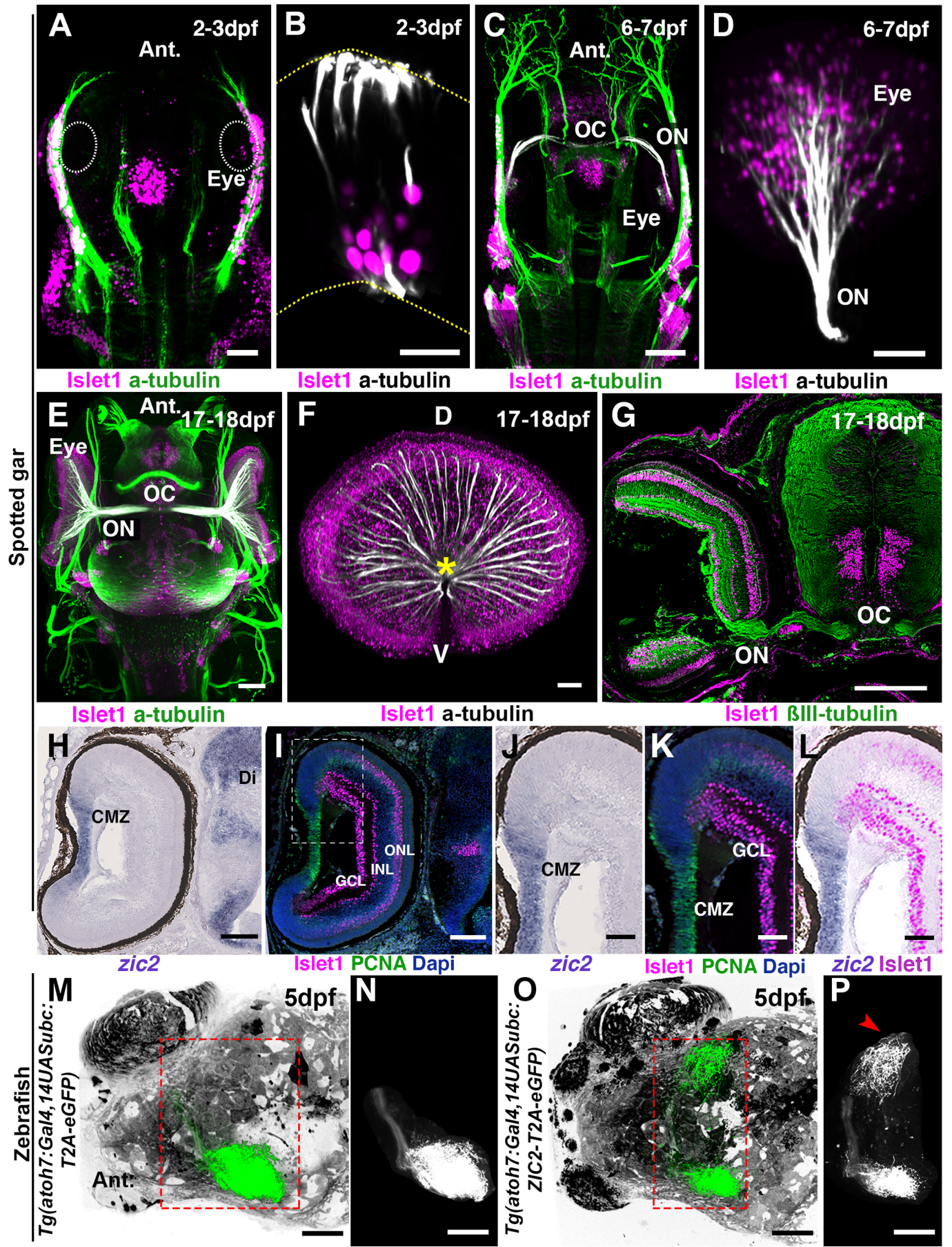












## Supplementary Materials for

Bilateral visual projections exist in non-teleost bony fish and predate the emergence of tetrapods

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**This PDF file includes:**

Materials and Methods  
Figs. S1 to S7  
Table S1  
Captions for Movies S1 to S5

**Other Supplementary Materials for this manuscript include the following:**

Movies S1 to S5

## **Material and Methods**

### Animals

Juvenile Mexican tetra (San Solomon Spring, Balmorhea State Park, Texas, USA) were maintained at 26°C (surface fish) on a 12:12 h light:dark cycle. Juvenile zebrafish and embryos were maintained at 28.5°C on a 14 h light/10 h dark cycle. Juvenile Australian lungfish (10.2-13.5 cm body length; Jardini Pty Ltd, Brisbane, Australia) were on freshwater at 26°C on a 12:12 h light:dark cycle. Juvenile armored bichir, sterlet sturgeon, African butterflyfish, redeye piranha, atlantic mudskipper, green puffer fish and four-eyed fish, were acquired from commercial vendors. Spotted gar embryos were spawned at Nicholls State University in Louisiana and then raised and maintained at Michigan State University as previously described (65). Embryos were raised at 18°C which leads to a comparatively slow progression through the Long & Ballard stages of gar development (48). Sizes of each specimen were recorded for future analysis. Juvenile specimens of either sex were used. All animal procedures were performed under the in accordance with protocols approved by Sorbonne Université and Institut Curie (EU0143-21323 and APAFIS #6031-2016070822342309), Queensland Brain Institute (#QBI/041/20/France) and Michigan State University (#10/16-179-00).

### Human eye samples

Human fetal eyes from terminated pregnancies were obtained from the INSERM-funded Human Developmental Cell Atlas collection (HuDeCA, <https://hudeca.genouest.org/>). All tissues were collected with appropriate maternal consent and approval from the French National Biomedicine agency (N° PFS19-012).

### In Situ Hybridization

Spotted gar sections were hybridized with digoxigenin-labeled riboprobes as described in (66). Briefly, tissue sections were postfixed for 10 min in 4% paraformaldehyde (PFA) before being treated with Proteinase K (10 µg/ml; Invitrogen, #03115852001) for 2 min and subsequently postfixed for 5 min in 4% PFA. Sections were then acetylated and permeabilized in PBS, 1% Triton X-100. Sections were first homogenized with hybridization buffer (50% formamide (VWR #24311.291), 5× SSC (Euromedex, #EU0300-A), 1× Denhardt's, 250 µg/ml yeast tRNA, and 500 µg/ml herring sperm DNA, pH 7.4) for 2 h at RT and then hybridized overnight at 72°C with riboprobes (1/200), see Table S1 for probe sequences. The next day, sections were rinsed for 2 h in 2× SSC at 72°C, and blocked in 0.1 M Tris, pH 7.5, 0.15 M NaCl (B1) containing 10% normal goat serum (NGS) for 1 h at RT. After blocking, slides were incubated o/n at 4°C with anti-DIG antibody conjugated with the alkaline phosphatase (1/5000, Roche Diagnostics) or anti-DIG antibody conjugated with peroxidase in B1 containing 1% NGS. After washing in B1 buffer, the alkaline phosphatase activity was detected by using nitroblue tetrazolium chloride (337.5 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (175 µg/ml) (Roche Diagnostics). The peroxidase activity was detected by using Tyramide Signal Amplification (TSA) (PerkinElmer, #NEL741001KT) and incubated with Fluorescein fluorophore Tyramide diluted at 1:50 in TSA. Sections were mounted in Mowiol (Calbiochem/Merck, Carlstadt, Germany).

Whole-mount in situ hybridization were carried out on zebrafish as previously described (67). Embryos were then embedded in gelatin/albumin with 4% of glutaraldehyde and sectioned (20 µm) on a VT1000 S vibrating blade microtome (Leica). Slides were scanned with either a Nanozoomer (Hamamatsu) or laser scanning confocal microscope (Olympus, FV1000).

### Fluorescent in Situ Hybridization



To generate anti-sense probes, DNA fragments were obtained by PCR using Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, #F530L) with the primers listed in Table S1. Total cDNA from 1 to 5 dpf zebrafish were used as a template. PCR fragments were cloned into the pCRII-TOPO vector (Invitrogen, #K280040) according to manufacturer's instructions. All plasmids used were sequenced for confirmation. Anti-sense DIG or fluorescein-labeled riboprobes were *in vitro* transcribed using the RNA labeling kit (Roche, #11685619910 or #11277073910) according to manufacturer's instructions. De-chorionated embryos at the appropriate developmental stages were fixed in fresh 4% PFA in 1X PBS (pH7.4) containing 0.1% Tween20 (PBSTw) for 4 h at RT and stored o/n in 100% methanol. Embryos were re-hydrated by immersing them in subsequent baths of 50% methanol/PBSTw (Sigma, #34860) and then twice in PBSTw, baths followed by a 10 min incubation in a 3% H<sub>2</sub>O<sub>2</sub>/0.5%KOH (Sigma, #P5958) solution. Embryos were then rinsed in 50% methanol and post-fixed in 100% methanol at -20°C for 2 h. Embryos were then re-hydrated in methanol/PBSTw (75%/50%/25%) followed by treatment in 10 µg/ml proteinase K at RT (1 dpf = 5 min, 2 dpf = 15 min, 3 dpf = 20 min), and post-fixed for 20 min in 4% PFA in PBSTw. Embryos were pre-hybridized at 68°C, and hybridized with either a fluorescein-labelled probe or DIG-labelled probe or both probes for dFISH assays o/n at 68°C with gentle shaking. Embryos were then rinsed at 68°C in 50% formamide/2XSSC/0.1%Tween-20 twice, 2XSSC/0.1%Tween-20, 0.2XSSC/0.1% Tween-20 twice and finally in TNT buffer (0.1 M Tris pH7.5, 0.15 M NaCl, 0.1% Tween-20). Blocking was done in TNB buffer (2% DIG block (Roche, #11096176001) in TNT) for 2 h at RT and incubated o/n with anti-Fluo-Fab-POD (Roche, #11426346910) diluted at 1:50 in TNB buffer at 4°C. All steps were performed in the dark. Embryos were then washed several times in TNT, rinsed using 100 µl Tyramide Signal Amplification (TSA) (PerkinElmer, #NEL741001KT) and incubated with Fluorescein fluorophore Tyramide diluted at 1:50 in TSA.

The reaction was stopped by 5 rapid washes of TNT. For dFISH assays, the DIG-labelled probe was then revealed by carrying out a 20 min incubation in 1% $H_2O_2$ /TNT (Sigma, #18312-1L), then washed several times in TNT. A second blocking step was carried out for 1 h in TNB buffer prior to incubating embryos in anti-DIG-POD (Roche, #11207733910) diluted at 1:100 in TNB buffer o/n at 4°C. Revelation was done with Cy3 Fluorophore Tyramide solution (PerkinElmer, NEL#744001KT), washed with TNT and processed for imaging upon DAPI staining.

### Molecular cloning

*14xUAS:ubc-ZIC2-T2A-GFP-pA* or *14xUAS:ubc-T2A-GFP-pA* were obtained via Gibson assembly using the *pTIUciMP Toll* (Addgene, #62215) destination vector described by (68). *Toll*mRNA was synthesized from the plasmid (Addgene, #61388) digested by NotI (NEB, #R3189S) and retro-transcribed with SP6 RNA polymerase (Roche, #10810274001). Human *ZIC2* (*hZIC2*), *GFP*, and *T2A* were amplified via PCR from pCAG-hZIC2 and *pUAS:Cas9T2AGFP;U6:sgRNA1;U6sgRNA2* (Addgene, #74009) respectively using the NEBuilder HiFi DNA Assembly Cloning kit (NEB, #E5520). Appropriate sequences were inserted after the *UBC* intron of the *pTIUciMP Toll* destination vector opened by restriction digest with NcoI-HF (NEB).

### Alignment between the amino acid sequences of the Zic2 proteins zing finger domains

A multiple sequence alignment was performed for the region covering the ZIC2 zinc finger domains of NCBI Reference Sequence proteins of mouse (NP\_033600), human (NP\_009060) and zebrafish (*ZIC2a* NP\_571633, *ZIC2b* NP\_001001820), as well as for genome-predicted ZIC2 proteins the spotted gar (XP\_006638968). The UniProtKB/Swiss-Prot curated zinc finger sequences of human ZIC2 (O95409) were used to delineate the domain positions within the

alignment. Protein sequence alignment was performed using MUSCLE version 3.8.31 (69), the amino acid conservation at each aligned position visualised using BIS2Analyzer (70).

### Eye enucleation

The transgenic line *Tg(atoh7:gal4-vp16)* (RRID: ZFIN\_ZDB-GENO-130306-1) was used. Prior to eye enucleation, fish were selected for the *atoh7* expression in green. At 2 dpf, eye enucleation was performed. The embryos were anesthetized in 0.004% tricaine MS222 in a 2% agarose gel solution (Life technologies, #16520050). One eye was surgically removed using a pulled capillary and mouth pipetting. Embryos were then transferred into fish medium (egg medium with penicillin/streptomycin (Life Technologies, #15140122) and 0.003% 1-phenyl-2-thiourea (Sigma, #189235) until 5 dpf, for whole-mount immunohistochemistry.

### Immunohistochemistry

#### Cryosections

Spotted gar embryos were fixed by immersion in 4% PFA in 0.12 M phosphate buffer (VWR, 28028.298 and 28015.294), pH 7.4 (PFA) o/n at 4°C. Following three washes in 1XPBS, the samples were incubated in 10% sucrose (VWR, 27478.296) in 0.12 M phosphate buffer o/n at 4°C. The next day, samples were transferred to a 30% sucrose solution in 0.12 M phosphate buffer o/n at 4°C. Samples were then embedded in 0.12 M phosphate containing 7.5% gelatin (Sigma, 62500) and 10% sucrose, frozen in isopentane at -40°C and then cut at 16 µm with a cryostat (Leica, CM3050S). Sections were blocked in PBS containing 0.2% gelatin (VWR) and 0.25% Triton-X100 (PBS-GT) for 1 h at RT. Following the blocking, sections were incubated with primary antibodies (see Table S1) diluted in a PBS-GT solution o/n at RT. Following three washes in PBST (0.05% Triton-X100) secondary antibodies coupled to the appropriate fluorophore (see Table S1) were diluted in PBS-GT and incubated for 2 h at RT. Sections were

counterstained with Hoechst (Sigma, B2883, 1:1000) or DAPI (Life Technologies, D3571, 1/500). For PCNA staining, an antigen retrieval step was performed by boiling sections in a 1X Sodium Citrate solution pH 6.0 for 5 min using a microwave. This step was skipped when the samples were first used for an *in-situ* hybridization assay. Slides were scanned with either a Nanozoomer (Hamamatsu) or laser scanning confocal microscope (Olympus, FV1000).

#### Whole-mount Immunohistochemistry

Zebrafish whole-mount immunohistochemistry was adapted from (61). Briefly, embryos were fixed in 4% PFA diluted in PBS containing 0.1% Tween-20 (VWR, #0777-1L) (PBSTw) for 4 h at RT and stored o/n in 100% methanol. After re-hydration, embryos were incubated for 20 min at -20°C in already pre-chilled acetone (Sigma, #650501). The embryos were rinsed several times with PBSTw and blocked for 2 h in blocking solution (10% bovine serum albumin (BSA) (Euromedex, #04-100-812-C), 10% normal goat serum (LifeTechnologies, #1000C), 1% DMSO (Sigma Aldrich, #D8418) in PBSTw). The primary antibodies were incubated o/n at 4°C in 1% BSA, 1% normal goat serum, 0.1% DMSO in PBSTw according to the dilutions in Table S1. After several washes in PBSTw, the secondary antibodies were incubated o/n at 4°C. The next day, embryos were rinsed in PBSTw and processed for imaging.

Whole-mount immunostaining on spotted gar embryos was carried out as previously described (15). Briefly, embryos were depigmented in a solution of 11% H<sub>2</sub>O<sub>2</sub> (VWR, 216763) at 70 rpm exposed to an 11W warm white Light-Emitting Diode (LED) (3000° Kelvin) for 1-3 days. Samples were then blocked and permeabilized before being incubated with the primary antibodies for 7 days at RT (see Table S1) in a solution containing: 0.5% Triton-X100, 5% donkey normal serum, 20% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal. The samples were further labeled with secondary antibodies (see Table S1) for 2 days at RT under agitation.

#### Retinal flat-mounts

For retinal flat mounts, human eyes were harvested and fixed in 4% PFA, followed by three

washes in 1XPBS. Eyes were then de-pigmented using the EyeDISCO protocol as previously described (15). For immunohistochemistry, retinas were permeabilized and blocked in a solution containing 0.5% Triton-X100, 5% donkey normal serum, 1XPBS, 0.1 g/L thimerosal for 1 day at RT under agitation. Primary antibodies (see Table S1) were diluted in a solution containing 0.5% Triton-X100, 5% donkey normal serum, 20% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal for 3 days at RT under agitation. The retinas were then washed for 1 day in PBST (1XPBS, 0.5% Triton-X100). The secondary antibodies (see Table S1) were diluted in the same solution as primary antibodies and left for 2 days. After washing retinas for 1 day, they were mounted on slides and imaged using a scanning confocal microscope (Olympus, FV1000).

#### Tracing of visual projections

All fish were anesthetized with 0,04% MS222, tricaine-methanesulfonate (Sigma, #E10521) diluted in fish water. Australian lungfish were anesthetized with 0.05% clove oil in fresh water. Injection of cholera toxin  $\beta$  subunit was carried out as described in (15). Briefly, using a capillary approximately 1 $\mu$ l of 2  $\mu$ g/ $\mu$ l of Alexa Fluor-conjugated cholera toxin  $\beta$  subunit (Thermo Fischer, Alexa Fluor555-CTb C22843 and Alexa Fluor647-CTb C34778) was injected intravitreally. 72-96 h following CTb injection, specimens were transcardially perfused with 4%PFA and the heads and/or brains were dissected for tissue clearing.

#### Tissue clearing and imaging

##### Clearing

Prior to clearing, spotted gar *embryos* were embedded in 1.5% agarose (Roth) in 1X TAE (Life Technologies). Clearing was carried out as previously described (15). Briefly, samples were gently de-hydrated in ascending baths of methanol (1.5 h). Samples were further treated with a

solution containing 2/3 Dichloromethane (DCM, Sigma) 1/3 methanol o/n. The next day, samples were placed in DCM for 30 min prior to being immersed in Di-benzyl Ether (DBE, Sigma).

### Imaging

Acquisitions were performed by using an UltraMicroscope I (Miltenyi Biotec, Germany) or UltraMicroscope Blaze (Miltenyi Biotec, Germany) with the InspectorPro software (Miltenyi Biotec, Germany, 5.1.328 version). The light sheet was generated by a laser (wavelength 488, 561, 647 Coherent Sapphire Laser, LaVision BioTec, Miltenyi Biotec, Germany) or a second-generation laser beam combiner (wavelengths 488 nm, 561 nm and 647 nm; LaVision BioTec, Miltenyi Biotec, Germany). All light sheets were matched within their Rayleigh lengths for optimal illumination at the sample site. Either a binocular stereomicroscope (Olympus, MXV10) with a 2x objective (Olympus, MVPLAPO) was used Or a MI Plan 1.1x (NA = 0.1), a MI Plan 4x (NA = 0.35), and a MI Plan 12x (NA = 0.53) objectives were used (Miltenyi Biotec, Germany). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec, Miltenyi Biotec) filled with DBE and illuminated from the side by the laser light. A Zyla sCMOS camera (Andor, Oxford Instrument; 2,048 × 2,048, 6.5 x 6.5 μm<sup>2</sup>, peak QE 82%) was used to acquire images. The step size between each image was fixed at 1 or 2 μm (NA = 0.5, 150 ms time exposure). All tiff images are generated in 16-bit.

### Confocal microscopy

Whole-mount 5 dpf zebrafish larvae were mounted in a labtex plates (LabTex) in 2.5% agarose or in 1% low-melting agarose on FluoroDish Cell Culture dish (FD3510-100, World Precision Instruments). For imaging, a scanning inverted confocal microscope (FV1200, Olympus) was used with a 30x objective (Olympus, UPLSAPO30XS, NA = 1.05, WD = 0.8 mm) as well as the LSM780 and LSM880 scanning inverted confocal microscopes (Zeiss) for high resolution microscopy. 40x water immersion objective for whole mount dFISH stained zebrafish embryos

and 63x oil objective for zebrafish retinal cryosections were used and a 10x air objective was used to image the spotted gar cryosections.

### Image Processing

3D rendering of light sheet and confocal stacks were converted to an Imaris file (.ims) using ImarisFileConverter (Bitplane, 9.5.1 version) and then visualized using the Imaris x64 software (Bitplane, 9.5.1). To quantify ipsilateral territories, entire tectum volume and ipsilateral projections were automatically segmented with a surface detail of 5.00  $\mu\text{m}$ , automatic threshold. Volumes were extracted from the surface. Movies were generated using the animation tool on Imaris x64 software (Bitplane, version 9.1.2) and movie reconstruction with .tiff series were done using ImageJ (1.50e, Java 1.8.0\_60, 64-bit). All movie editing (text and transitions) was performed using iMovie (Apple Inc., version 10.1.1).

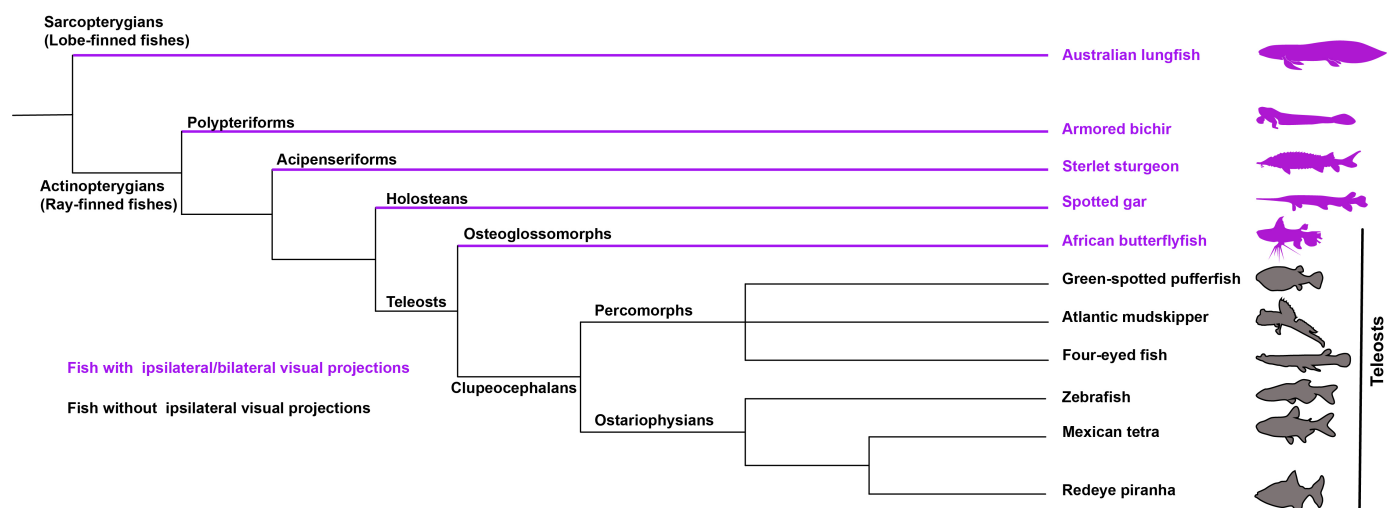
To quantify the ipsilateral projections in the hZIC2 overexpression experiments, a fixed region of interest was identified for each zebrafish (corresponding to the ipsilateral and contralateral optic tecta). Retinal projections were segmented with a surface detail of 0.5  $\mu\text{m}$  using an automatic threshold. Ipsilateral and contralateral volumes were extracted and summed to constitute the “total visual projections” using Imaris x64 software (Bitplane, version 9.1.2). The volume of ipsilateral projections was isolated as a ratio of ipsilateral projections:total projections.

### Statistical analyses

All data are described are listed as biological replicates (n) and all experiments (N) were carried out at least in triplicates unless indicated otherwise). An observer blinded to the experimental conditions realized all the quantifications. No data were excluded from the statistical analyses. All data are represented as mean values  $\pm$  SEM. Statistical significance was estimated using two-tailed unpaired tests for non-parametric tendencies (Kruskall-Wallis or Mann-Whitney),

two-way ANOVA and Bonferroni's multiple comparison test. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ . All statistical measurements were carried out using GraphPad Prism 7.

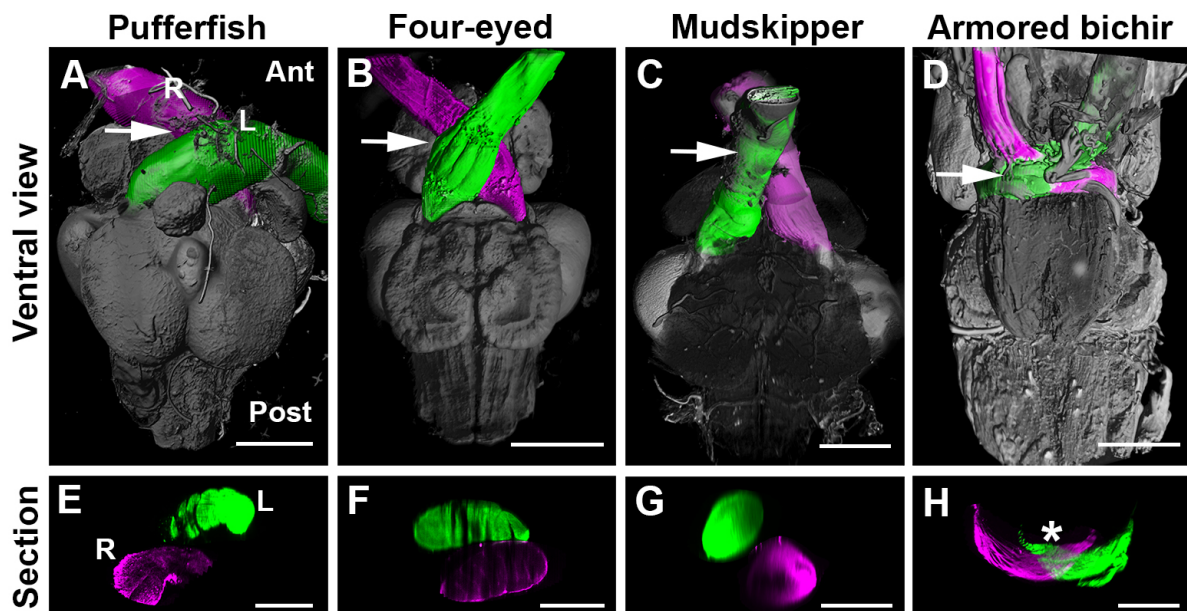
## Supplementary Figures



**Fig. S1. Simplified chart of fish taxonomy indicating the species analyzed in this study.**

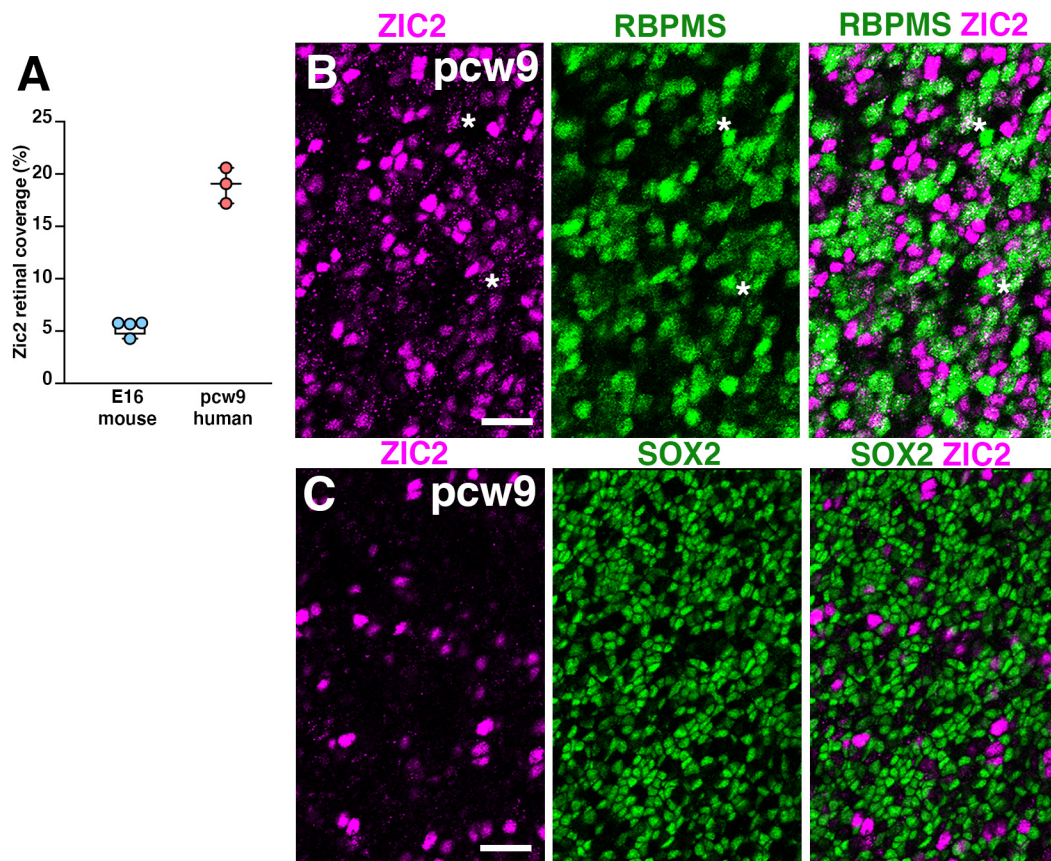
Fish with bilateral/ipsilateral visual projections appear in magenta and fish with only contralateral visual projections appear in grey.





**Fig. S2. Two types of optic nerve crossing modalities in ray-finned fishes.**

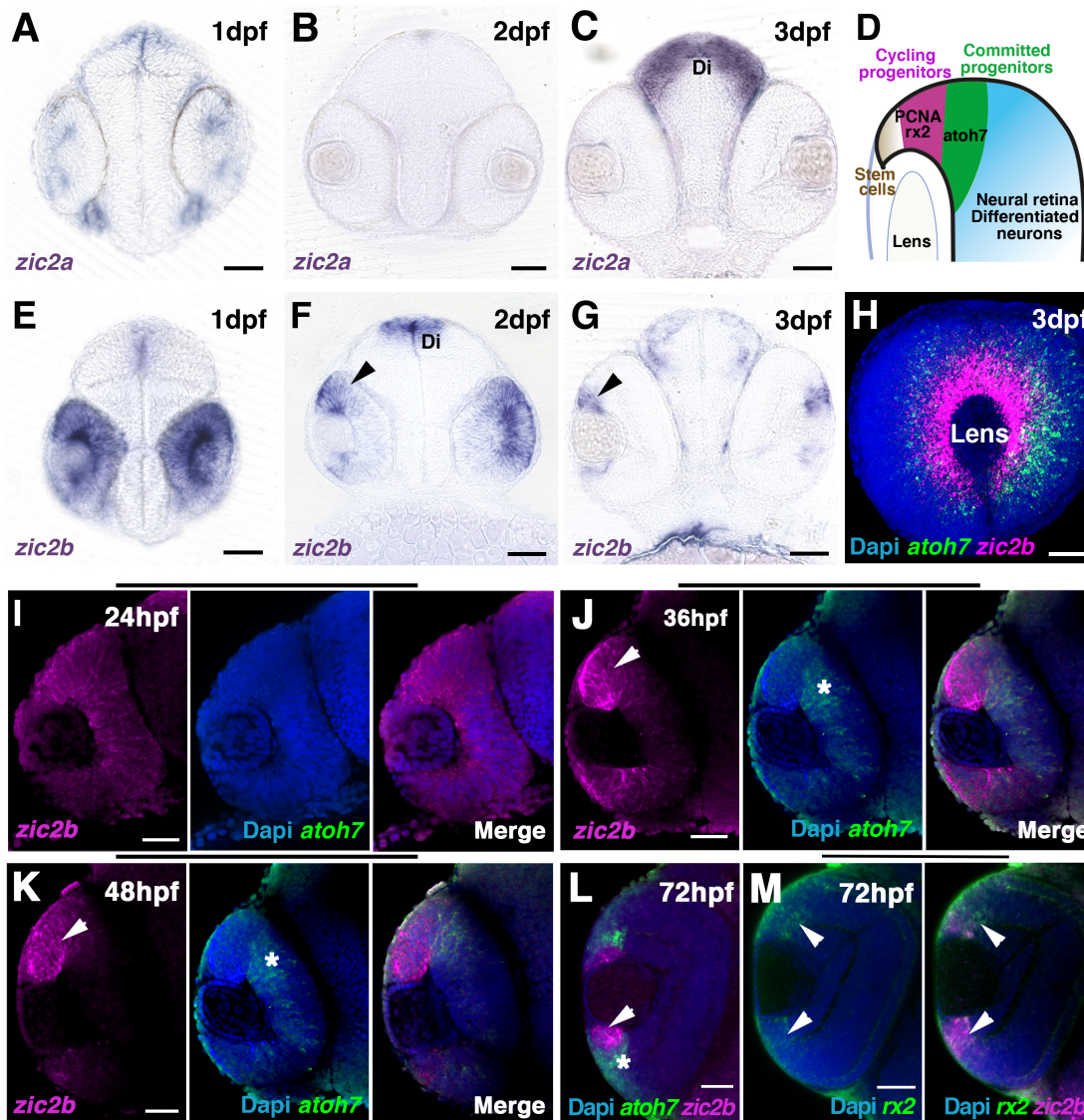
Ventral views (A to D) and coronal optical sections (E to H) at the level of the optic chiasm of iDISCO-cleared brains and optic nerves. A surface rendering with normal shading (Imaris) was applied to generate the ventral view images. The arrowheads (A to D) indicate the level of the chiasm optical section in (E to H). In all fishes, one eye was injected with Alexa Fluor-555-conjugated CTb and the other one with Alexa Fluor-647-conjugated CTb. The right (R) and left (L) optic nerves were pseudo-colored in magenta and green respectively. In Pufferfish (A and E), Four-eyed (B and F) and Mudskipper (C and G), the two optic nerves pass over and overlap at the chiasm but remain separated up to the brain. By contrast, in the Armored bichir (D and H), the right and left nerves meet at the chiasm and retinal ganglion cell axons from both eyes interweave during crossing (asterisk). Abbreviations: Ant, anterior; Post, Posterior. Scale bars are: 2 mm in (B), 1 mm in (A, C, D, F), 800  $\mu\text{m}$  in (G), 600  $\mu\text{m}$  in (E, H).



**Fig. S3. ZIC2 expression pattern in human embryo retina.**

(A) Box and whiskers representation of the ZIC2-positive surface in E16 mouse and pcw9 human retinas. (B and C) flat-mount pcw9 human retina labeled for ZIC2 and RBPMS (B) or SOX2 (C). (B) In the most superficial (basal side) regions of the temporal retina, ganglion cells expressing low levels of ZIC2 and RBPMS (arrowheads) are seen but ZIC2 and RBPMS are mostly exclusive. (C) image at the level of the interface between the neuroblastic layer showing that ZIC2+ cells are not SOX2+. Scale bars are: 50  $\mu$ m in (B and C).

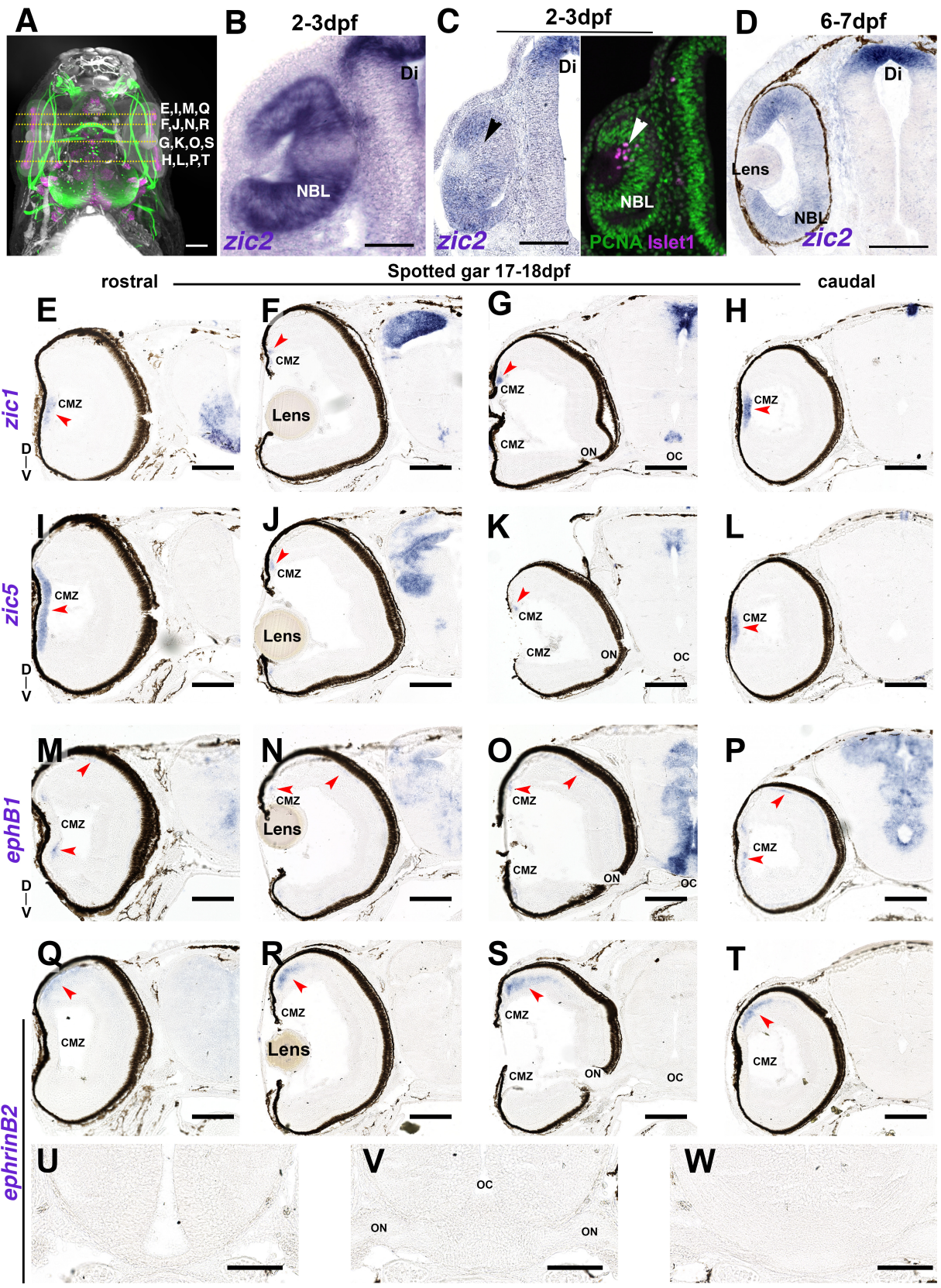




**Fig. S4. Zic2 ortholog expression in zebrafish is restricted to the ciliary margin zone.**

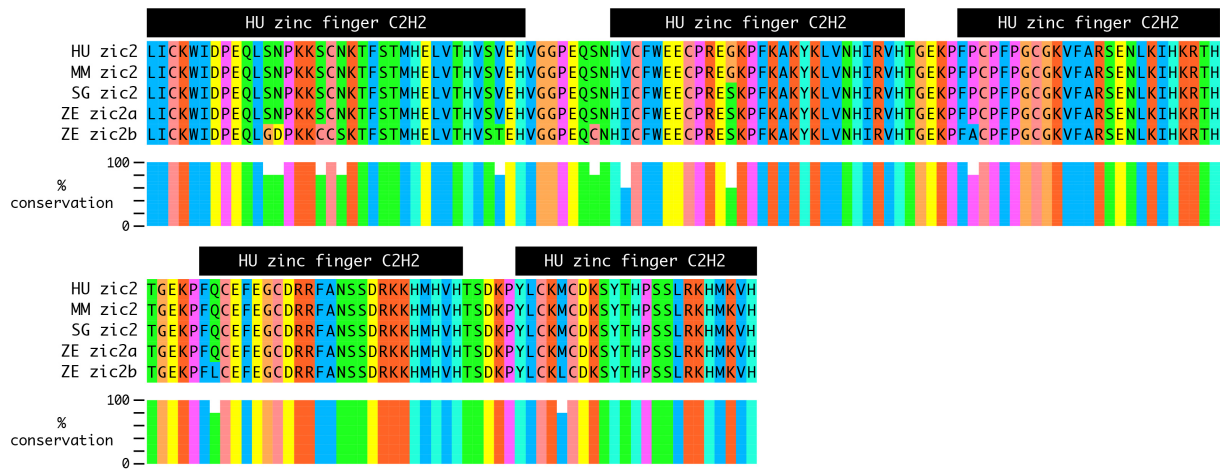
(A to H) Whole-mount *in situ* hybridization of zebrafish embryos for *zic2a* at 1 day post fertilization (1 dpf; A), 2 dpf (B) and 3 dpf (C) as well as *zic2b* at 1 dpf (E), 2 dpf (F) and 3 dpf (G). *Zic2b* is expressed in the ciliary marginal zone (CMZ, arrowheads in F and G) and in the dorsal diencephalon (Di). (D) Schematic drawing of the zebrafish CMZ in the developing retina showing spatial distribution of stem cells, cycling progenitors, committed progenitors and differentiated neurons. (H), Lateral view of whole-mount double fluorescent *in situ* hybridization for *zic2b* and *atoh7* on 3 dpf zebrafish embryos with DAPI counterstaining. (I to L) Confocal sections through the central retina of wild-type embryos hybridized with antisense RNA probes for *zic2b* and *atoh7*. At 24 hpf, *zic2b* is expressed in the entire proliferative neuroepithelium and later from a central to peripheral wave-like manner (arrowheads) in complementarity to the neurogenic transient expression of *atoh7* (asterisks) as shown here for 36, 48 and 72 hpf. (L to M) Confocal sections through the central retina of 72 hpf wild-type zebrafish embryos hybridized with antisense RNA probes for *zic2b* and *retinal homeo- box transcription factor2 (rx2)*, a marker of dividing progenitors and stem cells in the CMZ). *Zic2b* expression overlaps with the expression of the *rx2* (arrowheads). All retinæ were counterstained with the nuclear marker DAPI. Scale bars are 50  $\mu\text{m}$  (A to C and E to H) and 40  $\mu\text{m}$  (I to M).





**Fig. S5. Mammalian ipsilateral markers are not expressed in the spotted gar visual system.**

(A) 3D light-sheet fluorescence microscopy images of iDISCO-cleared 17-18 dpf spotted gar indicating with dotted lines the anatomical levels of the cryosections. (B to D) *In situ* hybridization for *zic2* on retinal cryosections of the developing spotted gar at 2-3 dpf (B), 6-7 dpf (C, left panel), 17-18 dpf (D). Only proliferating cells in the neuroblastic layer (NBL) express *zic2*. The right panel in (C) is an immunostaining for PCNA and Islet1. The arrowheads in (C) indicate the region where the first ganglion cells (Islet1+) are present at this stage in the retina. *zic2* is also found in the diencephalon (Di). (E to T) Rostral-to-caudal coronal cryosections from 17-18 dpf spotted gar. *zic1* (E to H) and *zic5* (I to L) are only expressed in the ciliary marginal zone (CMZ; arrow). (M to P) *ephB1* is absent from the retina and weakly expressed in the CMZ. (Q to T) *ephrinB2* is expressed in the dorsal retina (arrow). (U to W) Cryosections of the diencephalon of a 17-18dpf spotted gar hybridized for *ephrinB2*. *ephrinB2* is absent from the optic chiasm (asterisk). Immuno-reactive regions are highlighted (arrowhead). Abbreviations: NBL, Neuroblastic layer; ON, Optic nerve, OC, Optic chiasm; GCL, Ganglion cell layer; INL, Inner nuclear layer; ONL, Outer nuclear layer. Scale bars: A, 200  $\mu\text{m}$ ; B to D, 50  $\mu\text{m}$ ; C,U to W, 100  $\mu\text{m}$ ; D to T, 250  $\mu\text{m}$ .

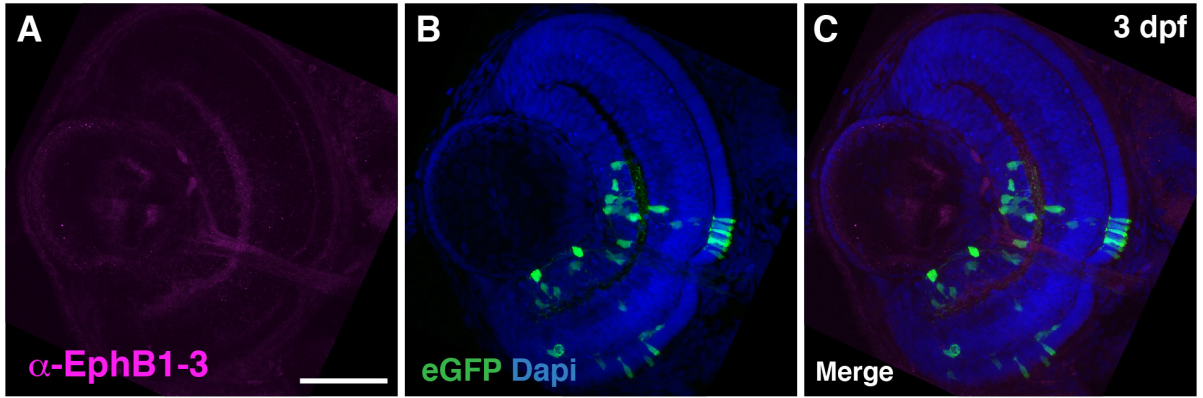


**Fig. S6. Alignment between the amino acid sequences of the Zic2 protein zinc finger domains of fish and mammals.**

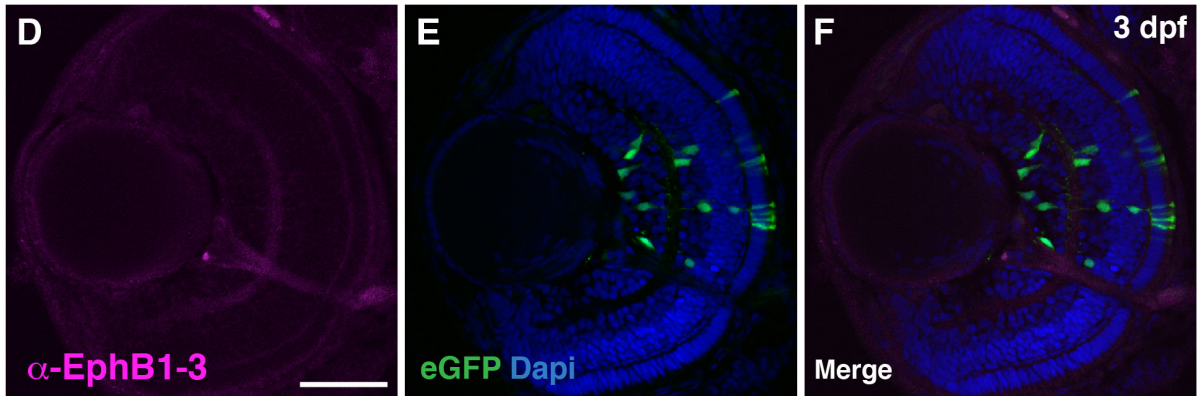
Alignment of Zic proteins across the zinc finger domains shows the high level of conservation between ray-finned fish and mammals. Amino acids are color coded according to the physiochemical class they belong to. Abbreviations: HU, human; MM, mouse; SG, spotted gar; ZE, zebrafish.



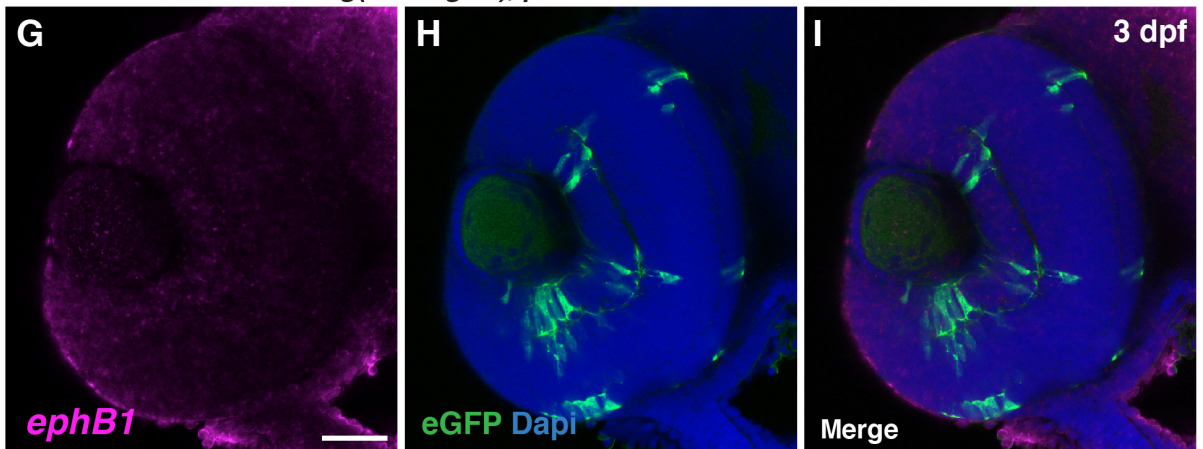
*Tg(atoh7:gal4), p14UASubc:T2A-eGFP*



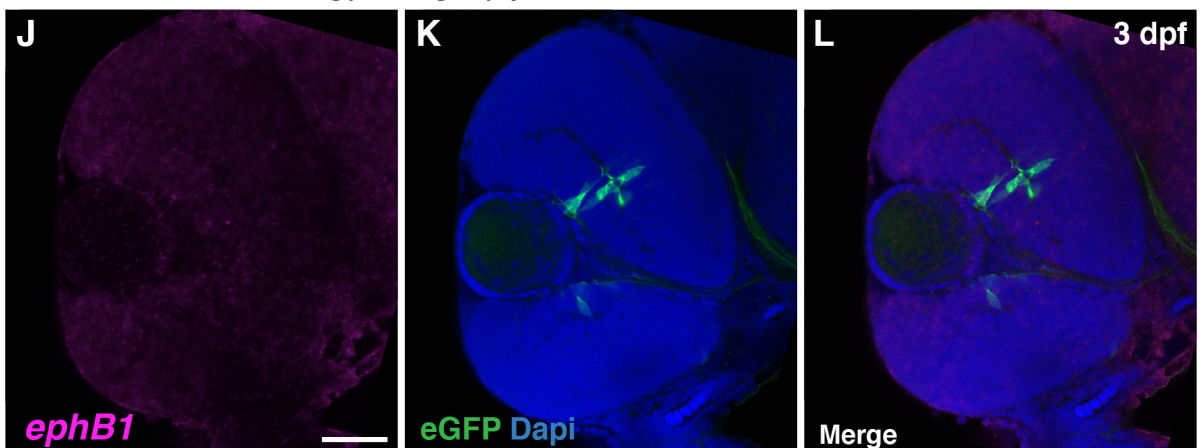
*Tg(atoh7:gal4), p14UASubc:ZIC2-T2A-eGFP*



*Tg(atoh7:gal4), p14UASubc:T2A-eGFP*



*Tg(atoh7:gal4), p14UASubc:ZIC2-T2A-eGFP*



**Fig.S7. Ectopic *ZIC2* expression in *atoh7* retinal progenitor cells does not induce EphB1 expression in retinal ganglion cells.**

Confocal images of cryostat (A-F) or optical (G-L) sections of 3 dpf retinae from *Tg(atoh7:gal4)* embryos injected at 1-cell stage with either a *p14UASubc:T2A-eGFP* (A to C and G to I) control construct or a *p14UASubc:ZIC2-T2A-eGFP* construct (D to F and J to L). No signal for EphB1 in GFP-positive cells is detectable in all injected retinae either double stained with anti-EphB1 antibody (A to F) or hybridized with an *ephB1* antisense riboprobe (G to L). All retinae were counterstained with the nuclear marker DAPI. Scale bars are 50  $\mu\text{m}$ .



**Table S1. Comprehensive table summarizing the antibodies and probes sequences.**

In situ probes						
Name	Sequence	RRID	Dilution	In situ hybridization		
L-zic1 fwd	ACCTCCAGACATCACTCAAC	n/a	1:200	Cryosections		
L-zic1 rev	GGAACACTCTTCCCAGAAAC	n/a	1:200	Cryosections		
L-zic2 fwd	AAACTTAACCACGACCTCTCTC	n/a	1:200	Cryosections		
L-zic2 rev	CTCGTGCATTGTGCTGAAAG	n/a	1:200	Cryosections		
L-zic5 fwd	CTTTGAGCAAGAGGAATCCGGC	n/a	1:200	Cryosections		
L-zic5 rev	CCTGCCGCGATGTTACATTTA	n/a	1:200	Cryosections		
L-efnb2 fwd	TCCCCATTATGAGAAGGTGAGCGG	n/a	1:200	Cryosections		
L-efnb2 rev	ACAGGCTACCACTTCAGAAGGCAG	n/a	1:200	Cryosections		
L-epfb1 fwd	AGAACCTGAACACAATCCGCAC	n/a	1:200	Cryosections		
L-epfb1 rev	ACAGTTTAATGGGCACGTCCAC	n/a	1:200	Cryosections		
zf-zic2a fwd	ACAACAATCTGTCGCCTTCTCTC	n/a	1:200	whole-mount		
zf-zic2a rev	ACAAATGCCCTGTTTAGCCC	n/a	1:200	whole-mount		
zf-zic2b fwd	TCTTCCGCTACATGCCACAAC	n/a	1:200	whole-mount		
zf-zic2b rev	GCAACACCACATGCTGAGAAC	n/a	1:200	whole-mount		
zf-epfb1 fwd	CGCGTGTGGATGGATTACGG	n/a	1:200	whole-mount		
zf-epfb1 rev	CATCCCCACCAGCTGGATCA	n/a	1:200	whole-mount		
zf-atoh7 fwd	GGAGAAGTTTGAGAGTCTATGCGG	n/a	1:200	whole-mount		
zf-atoh7 rev	CGACTTTGAGCTGAGCACACACC	n/a	1:200	whole-mount		
zf-rx2 fwd	GATACCATGAACATGGTGGACGATGG	n/a	1:200	whole-mount		
zf-rx2 rev	CCATCGACTGAATGTGCTCTTGG	n/a	1:200	whole-mount		
Primary antibodies						
Antigen	Species	Catalog #	Company	RRID	Dilution	Immunohistochemistry
Islet1	Rabbit	GTX128201	GeneTex	Ab_2868422	1:300	Cryosections/whole-mount
Acetylated-tubulin	Mouse	T6793	Sigma	Ab_477585	1:300	Cryosections/whole-mount
PCNA	Mouse	P8825	Sigma	Ab_477413	1:500	Cryosections
Islet1+2	Mouse	39.4D5	DSHB	Ab_2314683	1:50	Cryosections
GFP	Chicken	GTX13970	GeneTex	Ab_371416	1:5000	whole-mount
Rbpms	Guinea Pig	ABN1376	Millipore	Ab_2687403	1:400	Cryosections/flat-mount/ whole-
Zic2	Rabbit	Ab150404	Abcam	Ab_2868423	1:300	Cryosections/flat-mount/ whole-
Sox2	Goat	Sc17320	Santa-Cruz	Ab_2286684	1:300	flat-mount
EphB1	Mouse	MAb Efb1-3	DSBH	Ab_2314357	1:5	Cryosection
Secondary antibodies						
Anti-Rabbit cy3	Donkey	711-165-152	Jackson ImmunoResearch	Ab_2307443	1:500	cryosections/whole-mount
Anti-Rabbit Alexa Fluor 647	Donkey	711-605-152	Jackson ImmunoResearch	Ab_2492288	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 488	Donkey	A11055	Life Technologies	Ab_2534102	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 555	Donkey	A21432	Life Technologies	Ab_2535853	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 647	Bovine	805-605-180	Jackson ImmunoResearch	AB_2340885	1:600	cryosections
Anti-Goat cy3	Donkey	705-165-147	Jackson ImmunoResearch	Ab_2307351	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 488	Donkey	A21202	Life Technologies	Ab_141607	1:500	cryosections/Flat-mount/whole-mount
Anti-Guinea-Pig Alexa cy3	Donkey	706-165-148	Jackson ImmunoResearch	Ab_2340460	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 647	Donkey	715-605-150	Jackson ImmunoResearch	Ab_2340862	1:500	cryosections/Flat-mount/whole-mount
Anti-Mouse, Alexa Fluor 635	Goat	A31574	Life Technologies	Ab_2536184	1:500	cryosections
Anti-Rabbit, Alexa Fluor 568	Goat	A11036	Life Technologies	Ab_10563566	1:500	cryosections
Anti-Mouse, Alexa Fluor 568	Goat	A11004	Life Technologies	Ab_2534072	1:500	cryosections
Alexa Fluor 488 anti-chicken	Goat	A11039	Life Technologies	Ab_142924	1:500	cryosections
Tracers						
Cholera toxin subunit B-AlexaFluor555	n/a	C22843	Life technologies	n/a	2 µg/µl	Whole-mount
Cholera toxin subunit B-AlexaFluor647	n/a	C34778	Life technologies	n/a	2 µg/µl	Whole-mount

## **Movie S1.**

### **Visual projections in teleosts.**

Whole brain rendering of visual projections in 5 teleosts, the zebrafish, Mexican tetra, green-spotted pufferfish, mudskipper and butterflyfish. All species shows a complete decussation of retinal projections except the butterflyfish. All fish had bilateral eye injections of CTb coupled to either an Alexa Fluor-555 or and Alexa Fluor-647.

## **Movie S2.**

### **Bilateral visual projections in non teleosts.**

Whole brain rendering of visual projections in spotted gar, sterlet and armored bichir. Ipsilateral projections are seen in all species observed. All fish had bilateral eye injections of CTb coupled to either an Alexa Fluor-555 or and Alexa Fluor-647.

## **Movie S3.**

### **The Australian lungfish possesses non-segregated ipsilateral projections.**

Whole brain rendering of visual projections in the Australian lungfish, a sarcopterygian, injected with either an Alexa Fluor-555 or an Alexa Fluor-647. Many ipsilateral projections are observed, with a major component in the optic tectum. Ipsilateral projections are intermingled with contralateral projections in the optic tectum.

## **Movie S4.**

### **ZIC2 expression is evolutionarily conserved in Humans.**

Whole-mount immunohistochemistry of pcw9 human eyes using EyeDISCO clearing and labeled for the ipsilateral transcription factor ZIC2 (magenta) and the pan-retinal ganglion cell marker RBPMS (green). A large ZIC2-positive region can be seen in the temporal retina.

#### **Movie S5.**

#### **Development of the *Lepisosteus oculatus* visual system.**

3D rendering of 2-3 dpf, 6-7 dpf, and 17-18 dpf spotted gar embryos using EyeDISCO clearing and light-sheet fluorescence microscopy. Spotted gar embryos were labeled with the pan-neuronal marker acetylated tubulin ( $\alpha$ -tubulin, green) and the LIM/homeodomain family of transcription factor Islet1, which is critical for the proper specification of retinal ganglion cells and motor neurons (magenta).

## Supplementary Materials for

Bilateral visual projections exist in non-teleost bony fish and predate the emergence of tetrapods

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### **This PDF file includes:**

Materials and Methods  
Figs. S1 to S7  
Table S1  
Captions for Movies S1 to S5

### **Other Supplementary Materials for this manuscript include the following:**

Movies S1 to S5

## **Material and Methods**

### Animals

Juvenile Mexican tetra (San Solomon Spring, Balmorhea State Park, Texas, USA) were maintained at 26°C (surface fish) on a 12:12 h light:dark cycle. Juvenile zebrafish and embryos were maintained at 28.5°C on a 14 h light/10 h dark cycle. Juvenile Australian lungfish (10.2-13.5 cm body length; Jardini Pty Ltd, Brisbane, Australia) were on freshwater at 26°C on a 12:12 h light:dark cycle. Juvenile armored bichir, sterlet sturgeon, African butterflyfish, redeye piranha, atlantic mudskipper, green puffer fish and four-eyed fish, were acquired from commercial vendors. Spotted gar embryos were spawned at Nicholls State University in Louisiana and then raised and maintained at Michigan State University as previously described (65). Embryos were raised at 18°C which leads to a comparatively slow progression through the Long & Ballard stages of gar development (48). Sizes of each specimen were recorded for future analysis. Juvenile specimens of either sex were used. All animal procedures were performed under the in accordance with protocols approved by Sorbonne Université and Institut Curie (EU0143-21323 and APAFIS #6031-2016070822342309), Queensland Brain Institute (#QBI/041/20/France) and Michigan State University (#10/16-179-00).

### Human eye samples

Human fetal eyes from terminated pregnancies were obtained from the INSERM-funded Human Developmental Cell Atlas collection (HuDeCA, <https://hudeca.genouest.org/>). All tissues were collected with appropriate maternal consent and approval from the French National Biomedicine agency (N° PFS19-012).

### In Situ Hybridization

Spotted gar sections were hybridized with digoxigenin-labeled riboprobes as described in (66). Briefly, tissue sections were postfixed for 10 min in 4% paraformaldehyde (PFA) before being treated with Proteinase K (10 µg/ml; Invitrogen, #03115852001) for 2 min and subsequently postfixed for 5 min in 4% PFA. Sections were then acetylated and permeabilized in PBS, 1% Triton X-100. Sections were first homogenized with hybridization buffer (50% formamide (VWR #24311.291), 5× SSC (Euromedex, #EU0300-A), 1× Denhardt's, 250 µg/ml yeast tRNA, and 500 µg/ml herring sperm DNA, pH 7.4) for 2 h at RT and then hybridized overnight at 72°C with riboprobes (1/200), see Table S1 for probe sequences. The next day, sections were rinsed for 2 h in 2× SSC at 72°C, and blocked in 0.1 M Tris, pH 7.5, 0.15 M NaCl (B1) containing 10% normal goat serum (NGS) for 1 h at RT. After blocking, slides were incubated o/n at 4°C with anti-DIG antibody conjugated with the alkaline phosphatase (1/5000, Roche Diagnostics) or anti-DIG antibody conjugated with peroxidase in B1 containing 1% NGS. After washing in B1 buffer, the alkaline phosphatase activity was detected by using nitroblue tetrazolium chloride (337.5 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (175 µg/ml) (Roche Diagnostics). The peroxidase activity was detected by using Tyramide Signal Amplification (TSA) (PerkinElmer, #NEL741001KT) and incubated with Fluorescein fluorophore Tyramide diluted at 1:50 in TSA. Sections were mounted in Mowiol (Calbiochem/Merck, Carlstadt, Germany).

Whole-mount in situ hybridization were carried out on zebrafish as previously described (67). Embryos were then embedded in gelatin/albumin with 4% of glutaraldehyde and sectioned (20 µm) on a VT1000 S vibrating blade microtome (Leica). Slides were scanned with either a Nanozoomer (Hamamatsu) or laser scanning confocal microscope (Olympus, FV1000).

### Fluorescent in Situ Hybridization

To generate anti-sense probes, DNA fragments were obtained by PCR using Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, #F530L) with the primers listed in Table S1. Total cDNA from 1 to 5 dpf zebrafish were used as a template. PCR fragments were cloned into the pCRII-TOPO vector (Invitrogen, #K280040) according to manufacturer's instructions. All plasmids used were sequenced for confirmation. Anti-sense DIG or fluorescein-labeled riboprobes were *in vitro* transcribed using the RNA labeling kit (Roche, #11685619910 or #11277073910) according to manufacturer's instructions. De-chorionated embryos at the appropriate developmental stages were fixed in fresh 4% PFA in 1X PBS (pH7.4) containing 0.1% Tween20 (PBSTw) for 4 h at RT and stored o/n in 100% methanol. Embryos were re-hydrated by immersing them in subsequent baths of 50% methanol/PBSTw (Sigma, #34860) and then twice in PBSTw, baths followed by a 10 min incubation in a 3% H<sub>2</sub>O<sub>2</sub>/0.5%KOH (Sigma, #P5958) solution. Embryos were then rinsed in 50% methanol and post-fixed in 100% methanol at -20°C for 2 h. Embryos were then re-hydrated in methanol/PBSTw (75%/50%/25%) followed by treatment in 10 µg/ml proteinase K at RT (1 dpf = 5 min, 2 dpf = 15 min, 3 dpf = 20 min), and post-fixed for 20 min in 4% PFA in PBSTw. Embryos were pre-hybridized at 68°C, and hybridized with either a fluorescein-labelled probe or DIG-labelled probe or both probes for dFISH assays o/n at 68°C with gentle shaking. Embryos were then rinsed at 68°C in 50% formamide/2XSSC/0.1%Tween-20 twice, 2XSSC/0.1%Tween-20, 0.2XSSC/0.1% Tween-20 twice and finally in TNT buffer (0.1 M Tris pH7.5, 0.15 M NaCl, 0.1% Tween-20). Blocking was done in TNB buffer (2% DIG block (Roche, #11096176001) in TNT) for 2 h at RT and incubated o/n with anti-Fluo-Fab-POD (Roche, #11426346910) diluted at 1:50 in TNB buffer at 4°C. All steps were performed in the dark. Embryos were then washed several times in TNT, rinsed using 100 µl Tyramide Signal Amplification (TSA) (PerkinElmer, #NEL741001KT) and incubated with Fluorescein fluorophore Tyramide diluted at 1:50 in TSA.

The reaction was stopped by 5 rapid washes of TNT. For dFISH assays, the DIG-labelled probe was then revealed by carrying out a 20 min incubation in 1% $H_2O_2$ /TNT (Sigma, #18312-1L), then washed several times in TNT. A second blocking step was carried out for 1 h in TNB buffer prior to incubating embryos in anti-DIG-POD (Roche, #11207733910) diluted at 1:100 in TNB buffer o/n at 4°C. Revelation was done with Cy3 Fluorophore Tyramide solution (PerkinElmer, NEL#744001KT), washed with TNT and processed for imaging upon DAPI staining.

### Molecular cloning

*14xUAS:ubc-ZIC2-T2A-GFP-pA* or *14xUAS:ubc-T2A-GFP-pA* were obtained via Gibson assembly using the *pTIUciMP Toll* (Addgene, #62215) destination vector described by (68). *Toll* mRNA was synthesized from the plasmid (Addgene, #61388) digested by NotI (NEB, #R3189S) and retro-transcribed with SP6 RNA polymerase (Roche, #10810274001). Human *ZIC2* (*hZIC2*), *GFP*, and *T2A* were amplified via PCR from pCAG-hZIC2 and *pUAS:Cas9T2AGFP;U6:sgRNA1;U6sgRNA2* (Addgene, #74009) respectively using the NEBuilder HiFi DNA Assembly Cloning kit (NEB, #E5520). Appropriate sequences were inserted after the *UBC* intron of the *pTIUciMP Toll* destination vector opened by restriction digest with NcoI-HF (NEB).

### Alignment between the amino acid sequences of the Zic2 proteins zing finger domains

A multiple sequence alignment was performed for the region covering the ZIC2 zinc finger domains of NCBI Reference Sequence proteins of mouse (NP\_033600), human (NP\_009060) and zebrafish (*ZIC2a* NP\_571633, *ZIC2b* NP\_001001820), as well as for genome-predicted ZIC2 proteins the spotted gar (XP\_006638968). The UniProtKB/Swiss-Prot curated zinc finger sequences of human ZIC2 (O95409) were used to delineate the domain positions within the



alignment. Protein sequence alignment was performed using MUSCLE version 3.8.31 (69), the amino acid conservation at each aligned position visualised using BIS2Analyzer (70).

### Eye enucleation

The transgenic line *Tg(atoh7:gal4-*vp16*)* (RRID: ZFIN\_ZDB-GENO-130306-1) was used. Prior to eye enucleation, fish were selected for the *atoh7* expression in green. At 2 dpf, eye enucleation was performed. The embryos were anesthetized in 0.004% tricaine MS222 in a 2% agarose gel solution (Life technologies, #16520050). One eye was surgically removed using a pulled capillary and mouth pipetting. Embryos were then transferred into fish medium (egg medium with penicillin/streptomycin (Life Technologies, #15140122) and 0.003% 1-phenyl-2-thiourea (Sigma, #189235) until 5 dpf, for whole-mount immunohistochemistry.

### Immunohistochemistry

#### Cryosections

Spotted gar embryos were fixed by immersion in 4% PFA in 0.12 M phosphate buffer (VWR, 28028.298 and 28015.294), pH 7.4 (PFA) o/n at 4°C. Following three washes in 1XPBS, the samples were incubated in 10% sucrose (VWR, 27478.296) in 0.12 M phosphate buffer o/n at 4°C. The next day, samples were transferred to a 30% sucrose solution in 0.12 M phosphate buffer o/n at 4°C. Samples were then embedded in 0.12 M phosphate containing 7.5% gelatin (Sigma, 62500) and 10% sucrose, frozen in isopentane at -40°C and then cut at 16 µm with a cryostat (Leica, CM3050S). Sections were blocked in PBS containing 0.2% gelatin (VWR) and 0.25% Triton-X100 (PBS-GT) for 1 h at RT. Following the blocking, sections were incubated with primary antibodies (see Table S1) diluted in a PBS-GT solution o/n at RT. Following three washes in PBST (0.05% Triton-X100) secondary antibodies coupled to the appropriate fluorophore (see Table S1) were diluted in PBS-GT and incubated for 2 h at RT. Sections were

counterstained with Hoechst (Sigma, B2883, 1:1000) or DAPI (Life Technologies, D3571, 1/500). For PCNA staining, an antigen retrieval step was performed by boiling sections in a 1X Sodium Citrate solution pH 6.0 for 5 min using a microwave. This step was skipped when the samples were first used for an *in-situ* hybridization assay. Slides were scanned with either a Nanozoomer (Hamamatsu) or laser scanning confocal microscope (Olympus, FV1000).

#### Whole-mount Immunohistochemistry

Zebrafish whole-mount immunohistochemistry was adapted from (61). Briefly, embryos were fixed in 4% PFA diluted in PBS containing 0.1% Tween-20 (VWR, #0777-1L) (PBSTw) for 4 h at RT and stored o/n in 100% methanol. After re-hydration, embryos were incubated for 20 min at -20°C in already pre-chilled acetone (Sigma, #650501). The embryos were rinsed several times with PBSTw and blocked for 2 h in blocking solution (10% bovine serum albumin (BSA) (Euromedex, #04-100-812-C), 10% normal goat serum (LifeTechnologies, #1000C), 1% DMSO (Sigma Aldrich, #D8418) in PBSTw). The primary antibodies were incubated o/n at 4°C in 1% BSA, 1% normal goat serum, 0.1% DMSO in PBSTw according to the dilutions in Table S1. After several washes in PBSTw, the secondary antibodies were incubated o/n at 4°C. The next day, embryos were rinsed in PBSTw and processed for imaging.

Whole-mount immunostaining on spotted gar embryos was carried out as previously described (15). Briefly, embryos were depigmented in a solution of 11% H<sub>2</sub>O<sub>2</sub> (VWR, 216763) at 70 rpm exposed to an 11W warm white Light-Emitting Diode (LED) (3000° Kelvin) for 1-3 days. Samples were then blocked and permeabilized before being incubated with the primary antibodies for 7 days at RT (see Table S1) in a solution containing: 0.5% Triton-X100, 5% donkey normal serum, 20% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal. The samples were further labeled with secondary antibodies (see Table S1) for 2 days at RT under agitation.

#### Retinal flat-mounts

For retinal flat mounts, human eyes were harvested and fixed in 4% PFA, followed by three

washes in 1XPBS. Eyes were then de-pigmented using the EyeDISCO protocol as previously described (15). For immunohistochemistry, retinas were permeabilized and blocked in a solution containing 0.5% Triton-X100, 5% donkey normal serum, 1XPBS, 0.1 g/L thimerosal for 1 day at RT under agitation. Primary antibodies (see Table S1) were diluted in a solution containing 0.5% Triton-X100, 5% donkey normal serum, 20% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal for 3 days at RT under agitation. The retinas were then washed for 1 day in PBST (1XPBS, 0.5% Triton-X100). The secondary antibodies (see Table S1) were diluted in the same solution as primary antibodies and left for 2 days. After washing retinas for 1 day, they were mounted on slides and imaged using a scanning confocal microscope (Olympus, FV1000).

#### Tracing of visual projections

All fish were anesthetized with 0,04% MS222, tricaine-methanesulfonate (Sigma, #E10521) diluted in fish water. Australian lungfish were anesthetized with 0.05% clove oil in fresh water. Injection of cholera toxin  $\beta$  subunit was carried out as described in (15). Briefly, using a capillary approximately 1 $\mu$ l of 2  $\mu$ g/ $\mu$ l of Alexa Fluor-conjugated cholera toxin  $\beta$  subunit (Thermo Fischer, Alexa Fluor555-CTb C22843 and Alexa Fluor647-CTb C34778) was injected intravitreally. 72-96 h following CTb injection, specimens were transcardially perfused with 4%PFA and the heads and/or brains were dissected for tissue clearing.

#### Tissue clearing and imaging

##### Clearing

Prior to clearing, spotted gar *embryos* were embedded in 1.5% agarose (Roth) in 1X TAE (Life Technologies). Clearing was carried out as previously described (15). Briefly, samples were gently de-hydrated in ascending baths of methanol (1.5 h). Samples were further treated with a

solution containing 2/3 Dichloromethane (DCM, Sigma) 1/3 methanol o/n. The next day, samples were placed in DCM for 30 min prior to being immersed in Di-benzyl Ether (DBE, Sigma).

### Imaging

Acquisitions were performed by using an UltraMicroscope I (Miltenyi Biotec, Germany) or UltraMicroscope Blaze (Miltenyi Biotec, Germany) with the InspectorPro software (Miltenyi Biotec, Germany, 5.1.328 version). The light sheet was generated by a laser (wavelength 488, 561, 647 Coherent Sapphire Laser, LaVision BioTec, Miltenyi Biotec, Germany) or a second-generation laser beam combiner (wavelengths 488 nm, 561 nm and 647 nm; LaVision BioTec, Miltenyi Biotec, Germany). All light sheets were matched within their Rayleigh lengths for optimal illumination at the sample site. Either a binocular stereomicroscope (Olympus, MXV10) with a 2x objective (Olympus, MVPLAPO) was used Or a MI Plan 1.1x (NA = 0.1), a MI Plan 4x (NA = 0.35), and a MI Plan 12x (NA = 0.53) objectives were used (Miltenyi Biotec, Germany). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec, Miltenyi Biotec) filled with DBE and illuminated from the side by the laser light. A Zyla sCMOS camera (Andor, Oxford Instrument; 2,048 × 2,048, 6.5 x 6.5 μm<sup>2</sup>, peak QE 82%) was used to acquire images. The step size between each image was fixed at 1 or 2 μm (NA = 0.5, 150 ms time exposure). All tiff images are generated in 16-bit.

### Confocal microscopy

Whole-mount 5 dpf zebrafish larvae were mounted in a labtex plates (LabTex) in 2.5% agarose or in 1% low-melting agarose on FluoroDish Cell Culture dish (FD3510-100, World Precision Instruments). For imaging, a scanning inverted confocal microscope (FV1200, Olympus) was used with a 30x objective (Olympus, UPLSAPO30XS, NA = 1.05, WD = 0.8 mm) as well as the LSM780 and LSM880 scanning inverted confocal microscopes (Zeiss) for high resolution microscopy. 40x water immersion objective for whole mount dFISH stained zebrafish embryos

and 63x oil objective for zebrafish retinal cryosections were used and a 10x air objective was used to image the spotted gar cryosections.

### Image Processing

3D rendering of light sheet and confocal stacks were converted to an Imaris file (.ims) using ImarisFileConverter (Bitplane, 9.5.1 version) and then visualized using the Imaris x64 software (Bitplane, 9.5.1). To quantify ipsilateral territories, entire tectum volume and ipsilateral projections were automatically segmented with a surface detail of 5.00  $\mu\text{m}$ , automatic threshold. Volumes were extracted from the surface. Movies were generated using the animation tool on Imaris x64 software (Bitplane, version 9.1.2) and movie reconstruction with .tiff series were done using ImageJ (1.50e, Java 1.8.0\_60, 64-bit). All movie editing (text and transitions) was performed using iMovie (Apple Inc., version 10.1.1).

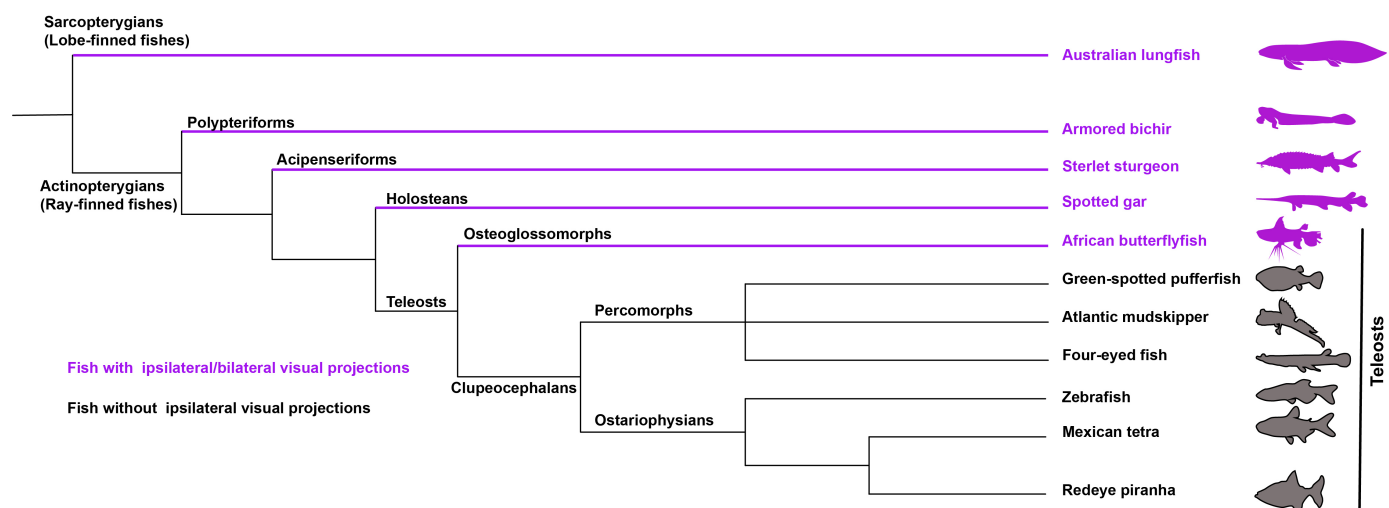
To quantify the ipsilateral projections in the hZIC2 overexpression experiments, a fixed region of interest was identified for each zebrafish (corresponding to the ipsilateral and contralateral optic tecta). Retinal projections were segmented with a surface detail of 0.5  $\mu\text{m}$  using an automatic threshold. Ipsilateral and contralateral volumes were extracted and summed to constitute the “total visual projections” using Imaris x64 software (Bitplane, version 9.1.2). The volume of ipsilateral projections was isolated as a ratio of ipsilateral projections:total projections.

### Statistical analyses

All data are described are listed as biological replicates (n) and all experiments (N) were carried out at least in triplicates unless indicated otherwise). An observer blinded to the experimental conditions realized all the quantifications. No data were excluded from the statistical analyses. All data are represented as mean values  $\pm$  SEM. Statistical significance was estimated using two-tailed unpaired tests for non-parametric tendencies (Kruskall-Wallis or Mann-Whitney),

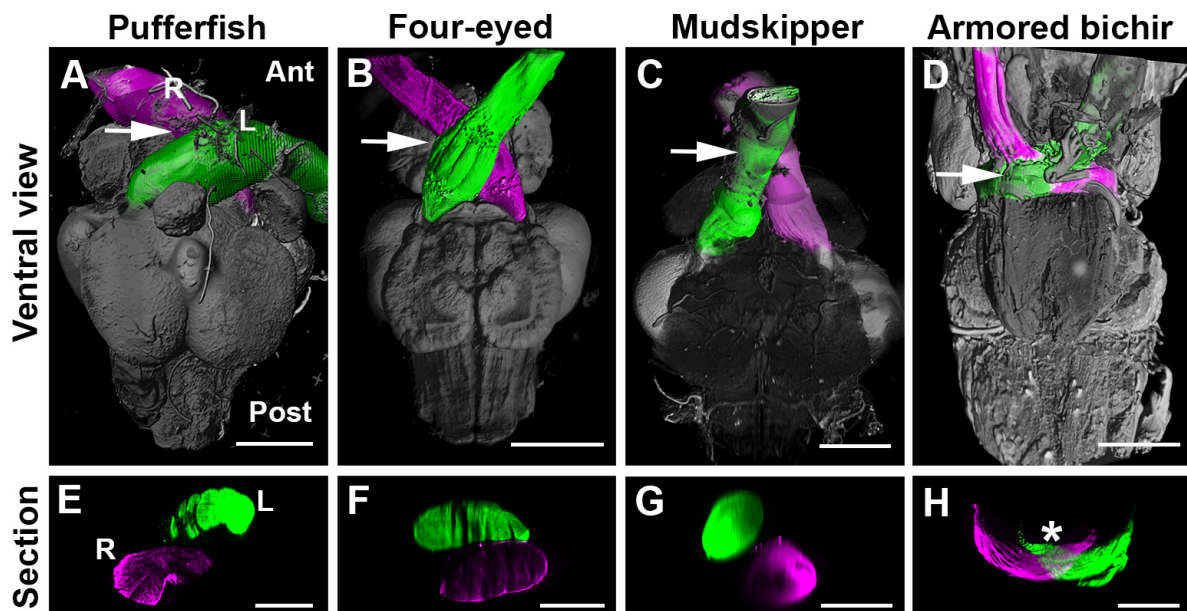
two-way ANOVA and Bonferroni's multiple comparison test. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ . All statistical measurements were carried out using GraphPad Prism 7.

## Supplementary Figures



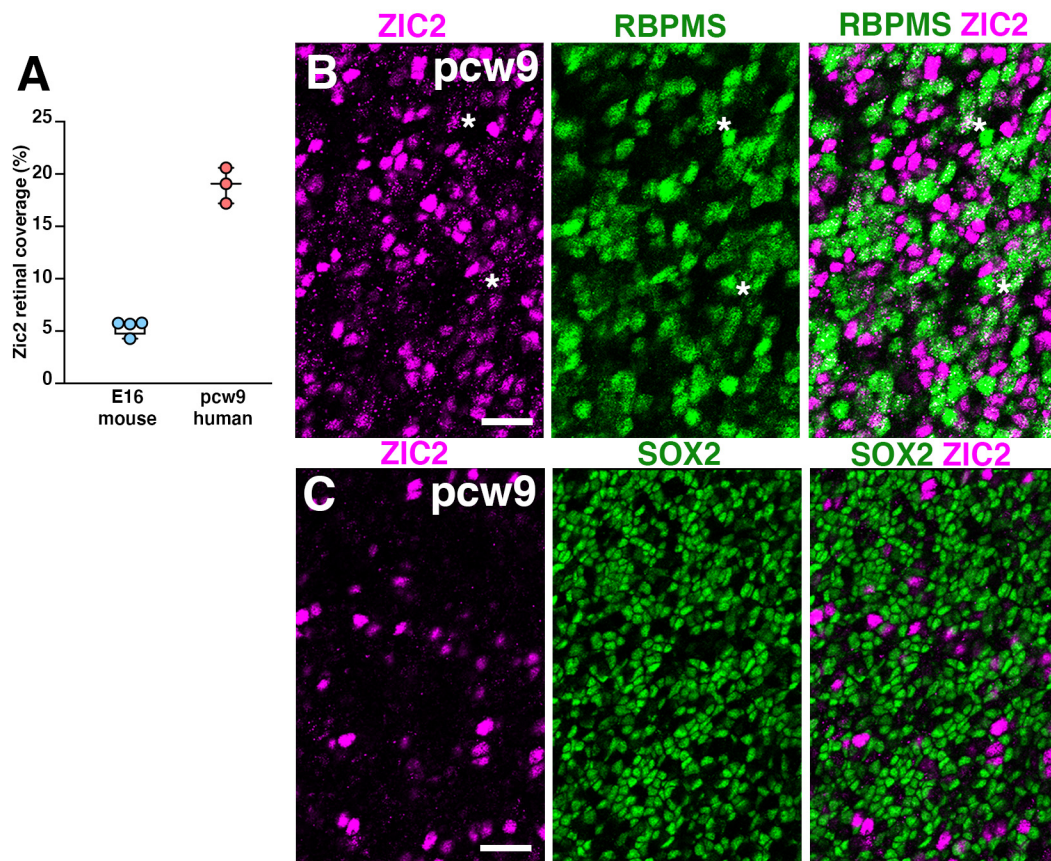
**Fig. S1. Simplified chart of fish taxonomy indicating the species analyzed in this study.**

Fish with bilateral/ipsilateral visual projections appear in magenta and fish with only contralateral visual projections appear in grey.



**Fig. S2. Two types of optic nerve crossing modalities in ray-finned fishes.**

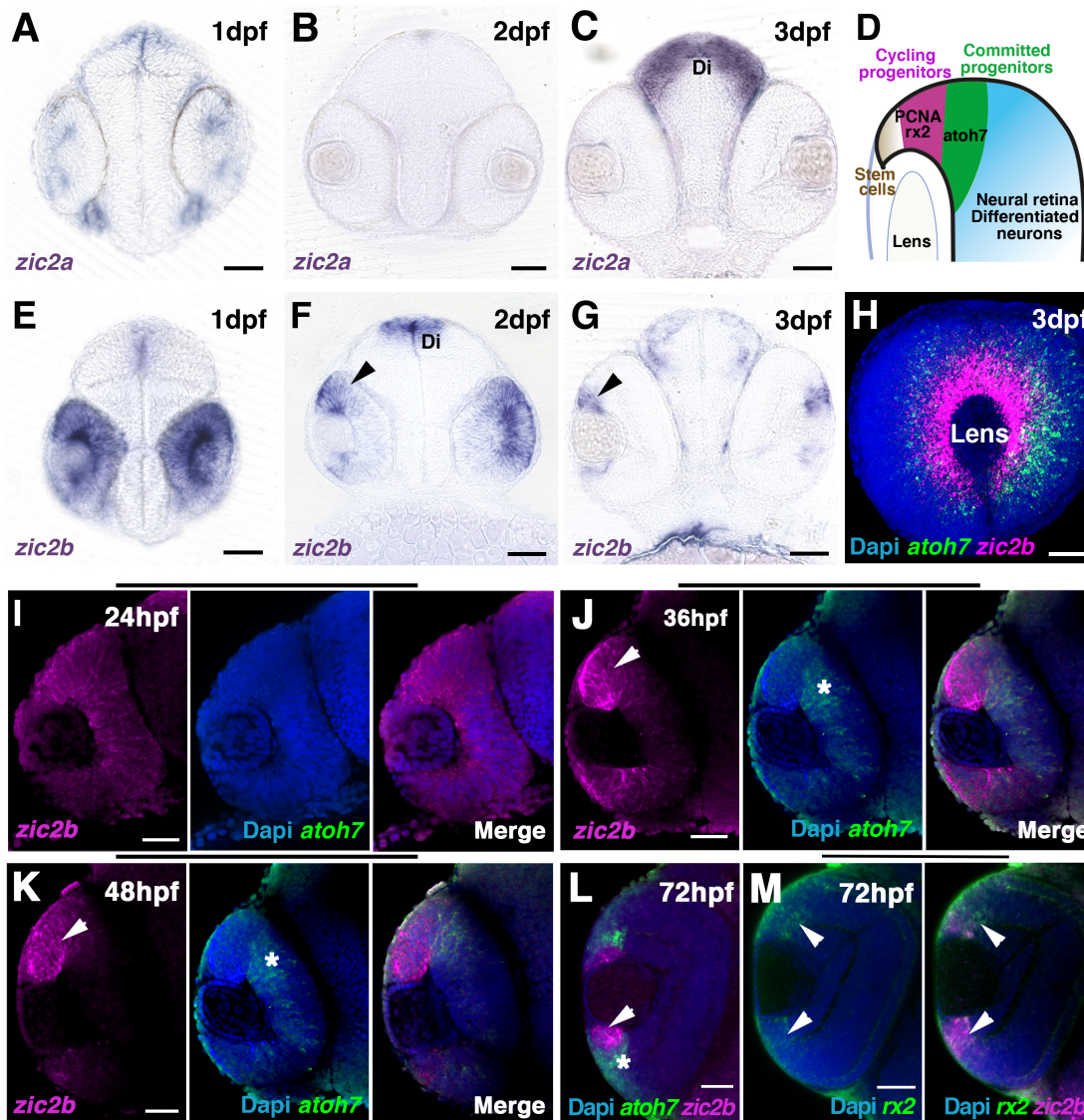
Ventral views (A to D) and coronal optical sections (E to H) at the level of the optic chiasm of iDISCO-cleared brains and optic nerves. A surface rendering with normal shading (Imaris) was applied to generate the ventral view images. The arrowheads (A to D) indicate the level of the chiasm optical section in (E to H). In all fishes, one eye was injected with Alexa Fluor-555-conjugated CTb and the other one with Alexa Fluor-647-conjugated CTb. The right (R) and left (L) optic nerves were pseudo-colored in magenta and green respectively. In Pufferfish (A and E), Four-eyed (B and F) and Mudskipper (C and G), the two optic nerves pass over and overlap at the chiasm but remain separated up to the brain. By contrast, in the Armored bichir (D and H), the right and left nerves meet at the chiasm and retinal ganglion cell axons from both eyes interweave during crossing (asterisk). Abbreviations: Ant, anterior; Post, Posterior. Scale bars are: 2 mm in (B), 1 mm in (A, C, D, F), 800  $\mu\text{m}$  in (G), 600  $\mu\text{m}$  in (E, H).



**Fig. S3. ZIC2 expression pattern in human embryo retina.**

(A) Box and whiskers representation of the ZIC2-positive surface in E16 mouse and pcw9 human retinas. (B and C) flat-mount pcw9 human retina labeled for ZIC2 and RBPMS (B) or SOX2 (C). (B) In the most superficial (basal side) regions of the temporal retina, ganglion cells expressing low levels of ZIC2 and RBPMS (arrowheads) are seen but ZIC2 and RBPMS are mostly exclusive. (C) image at the level of the interface between the neuroblastic layer showing that ZIC2+ cells are not SOX2+. Scale bars are: 50  $\mu$ m in (B and C).

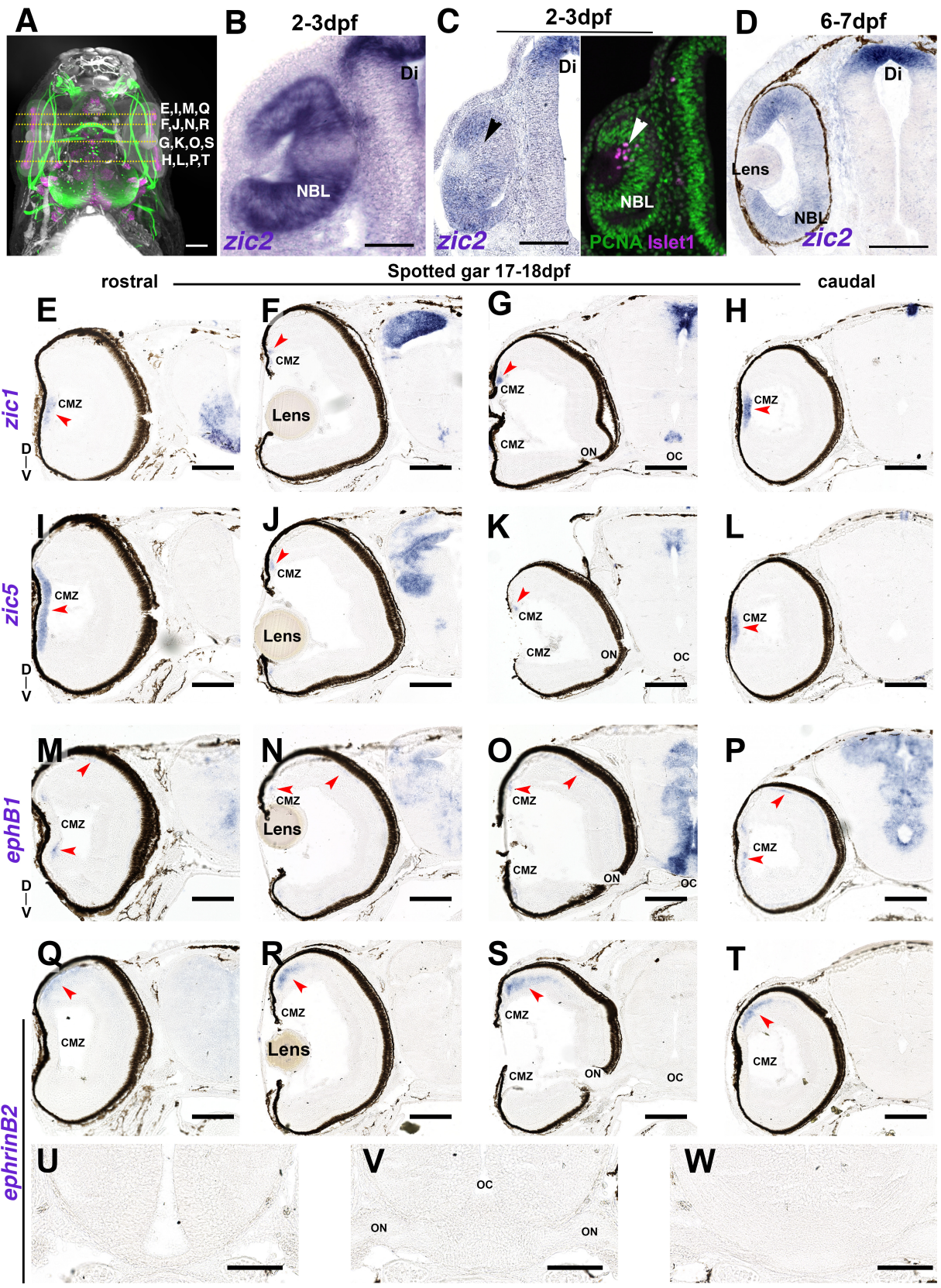




**Fig. S4. Zic2 ortholog expression in zebrafish is restricted to the ciliary margin zone.**

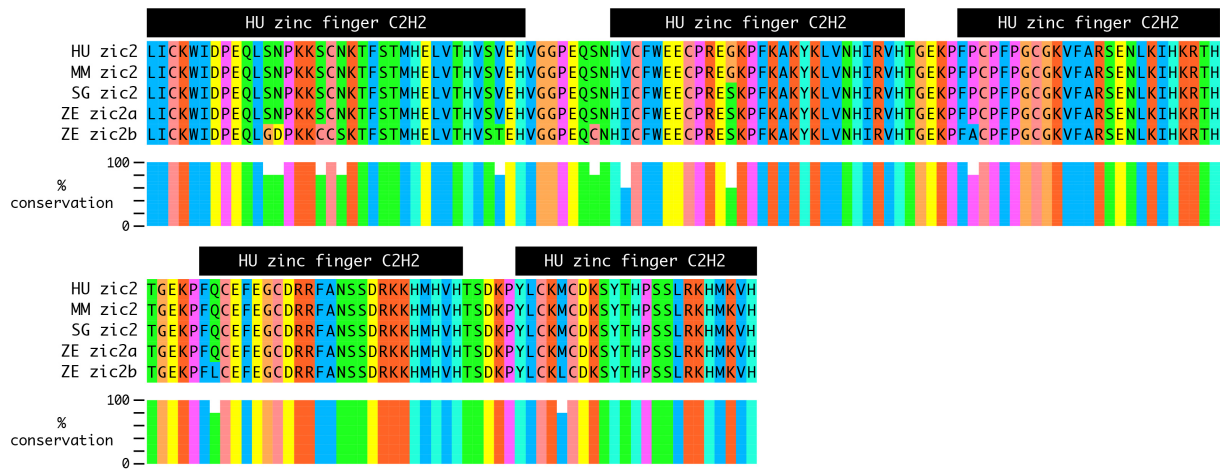
(A to H) Whole-mount *in situ* hybridization of zebrafish embryos for *zic2a* at 1 day post fertilization (1 dpf; A), 2 dpf (B) and 3 dpf (C) as well as *zic2b* at 1 dpf (E), 2 dpf (F) and 3 dpf (G). *Zic2b* is expressed in the ciliary marginal zone (CMZ, arrowheads in F and G) and in the dorsal diencephalon (Di). (D) Schematic drawing of the zebrafish CMZ in the developing retina showing spatial distribution of stem cells, cycling progenitors, committed progenitors and differentiated neurons. (H), Lateral view of whole-mount double fluorescent *in situ* hybridization for *zic2b* and *atoh7* on 3 dpf zebrafish embryos with DAPI counterstaining. (I to L) Confocal sections through the central retina of wild-type embryos hybridized with antisense RNA probes for *zic2b* and *atoh7*. At 24 hpf, *zic2b* is expressed in the entire proliferative neuroepithelium and later from a central to peripheral wave-like manner (arrowheads) in complementarity to the neurogenic transient expression of *atoh7* (asterisks) as shown here for 36, 48 and 72 hpf. (L to M) Confocal sections through the central retina of 72 hpf wild-type zebrafish embryos hybridized with antisense RNA probes for *zic2b* and *retinal homeo- box transcription factor2* (*rx2*), a marker of dividing progenitors and stem cells in the CMZ). *Zic2b* expression overlaps with the expression of the *rx2* (arrowheads). All retinæ were counterstained with the nuclear marker DAPI. Scale bars are 50  $\mu$ m (A to C and E to H) and 40  $\mu$ m (I to M).





**Fig. S5. Mammalian ipsilateral markers are not expressed in the spotted gar visual system.**

(A) 3D light-sheet fluorescence microscopy images of iDISCO-cleared 17-18 dpf spotted gar indicating with dotted lines the anatomical levels of the cryosections. (B to D) *In situ* hybridization for *zic2* on retinal cryosections of the developing spotted gar at 2-3 dpf (B), 6-7 dpf (C, left panel), 17-18 dpf (D). Only proliferating cells in the neuroblastic layer (NBL) express *zic2*. The right panel in (C) is an immunostaining for PCNA and Islet1. The arrowheads in (C) indicate the region where the first ganglion cells (Islet1+) are present at this stage in the retina. *zic2* is also found in the diencephalon (Di). (E to T) Rostral-to-caudal coronal cryosections from 17-18 dpf spotted gar. *zic1* (E to H) and *zic5* (I to L) are only expressed in the ciliary marginal zone (CMZ; arrow). (M to P) *ephB1* is absent from the retina and weakly expressed in the CMZ. (Q to T) *ephrinB2* is expressed in the dorsal retina (arrow). (U to W) Cryosections of the diencephalon of a 17-18dpf spotted gar hybridized for *ephrinB2*. *ephrinB2* is absent from the optic chiasm (asterisk). Immuno-reactive regions are highlighted (arrowhead). Abbreviations: NBL, Neuroblastic layer; ON, Optic nerve, OC, Optic chiasm; GCL, Ganglion cell layer; INL, Inner nuclear layer; ONL, Outer nuclear layer. Scale bars: A, 200  $\mu\text{m}$ ; B to D, 50  $\mu\text{m}$ ; C,U to W, 100  $\mu\text{m}$ ; D to T, 250  $\mu\text{m}$ .

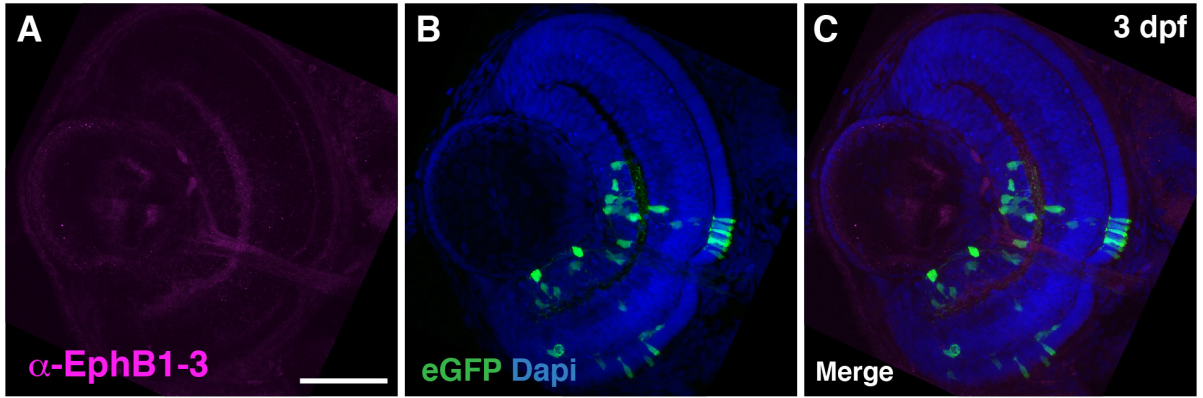


**Fig. S6. Alignment between the amino acid sequences of the Zic2 protein zinc finger domains of fish and mammals.**

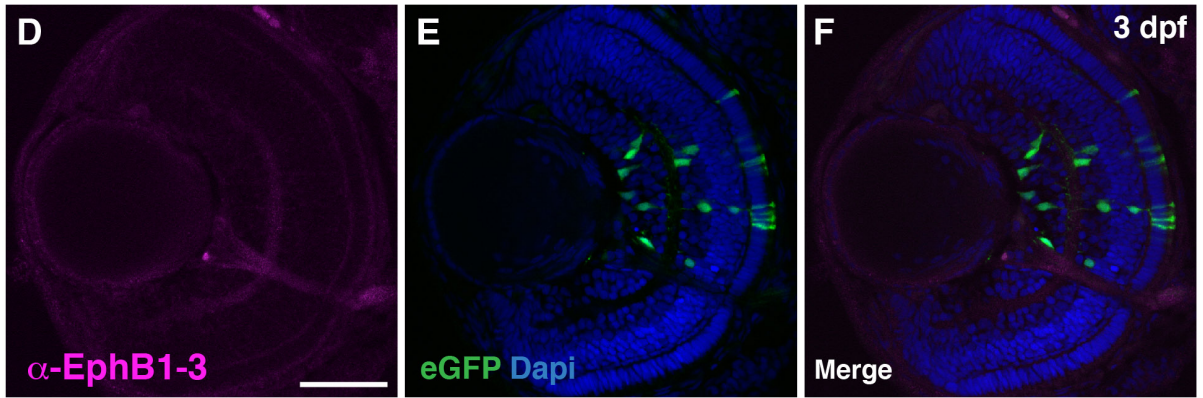
Alignment of Zic proteins across the zinc finger domains shows the high level of conservation between ray-finned fish and mammals. Amino acids are color coded according to the physiochemical class they belong to. Abbreviations: HU, human; MM, mouse; SG, spotted gar; ZE, zebrafish.



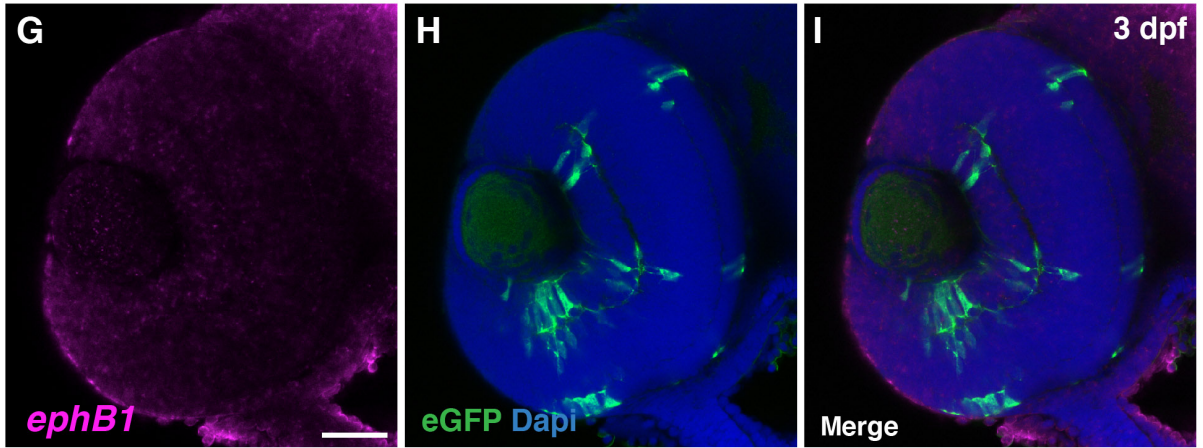
*Tg(ato7:gal4), p14UASubc:T2A-eGFP*



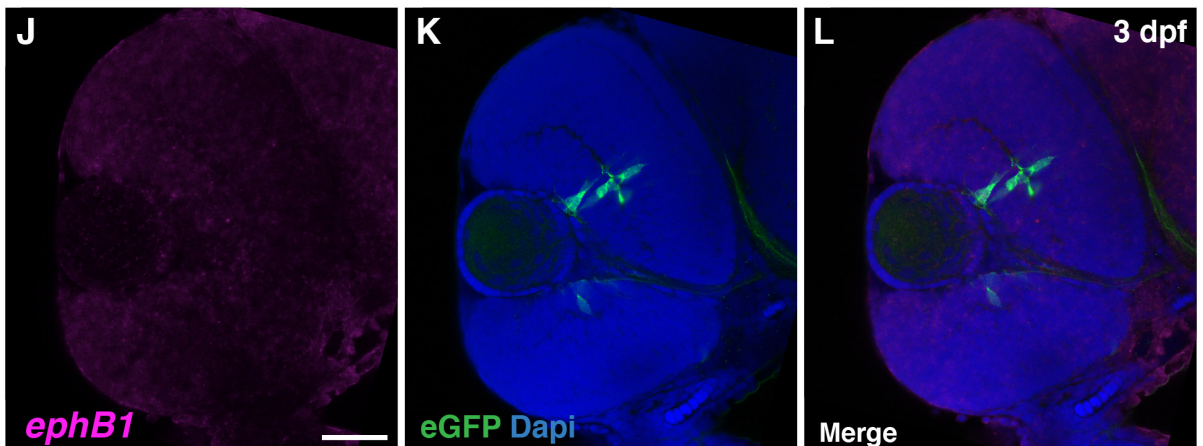
*Tg(ato7:gal4), p14UASubc:ZIC2-T2A-eGFP*



*Tg(ato7:gal4), p14UASubc:T2A-eGFP*



*Tg(ato7:gal4), p14UASubc:ZIC2-T2A-eGFP*



**Fig.S7. Ectopic *ZIC2* expression in *atoh7* retinal progenitor cells does not induce EphB1 expression in retinal ganglion cells.**

Confocal images of cryostat (A-F) or optical (G-L) sections of 3 dpf retinae from *Tg(atoh7:gal4)* embryos injected at 1-cell stage with either a *p14UASubc:T2A-eGFP* (A to C and G to I) control construct or a *p14UASubc:ZIC2-T2A-eGFP* construct (D to F and J to L). No signal for EphB1 in GFP-positive cells is detectable in all injected retinae either double stained with anti-EphB1 antibody (A to F) or hybridized with an *ephB1* antisense riboprobe (G to L). All retinae were counterstained with the nuclear marker DAPI. Scale bars are 50  $\mu\text{m}$ .

**Table S1. Comprehensive table summarizing the antibodies and probes sequences.**

In situ probes						
Name	Sequence		RRID	Dilution	In situ hybridization	
L-zic1 fwd	ACCTCCAGACATCACTCAAC		n/a	1:200	Cryosections	
L-zic1 rev	GGAACACTCTTCCCAGAAAC		n/a	1:200	Cryosections	
L-zic2 fwd	AAACTTAACCACGACCTCTCTC		n/a	1:200	Cryosections	
L-zic2 rev	CTCGTGCATTGTGCTGAAAG		n/a	1:200	Cryosections	
L-zic5 fwd	CTTTGAGCAAGAGGAATCCGGC		n/a	1:200	Cryosections	
L-zic5 rev	CCTGCCGCGATGTTACATTTA		n/a	1:200	Cryosections	
L-efnb2 fwd	TCCCCATTATGAGAAGGTGAGCGG		n/a	1:200	Cryosections	
L-efnb2 rev	ACAGGCTACCACTTCAGAAGGCAG		n/a	1:200	Cryosections	
L-epfb1 fwd	AGAACCTGAACACAATCCGCAC		n/a	1:200	Cryosections	
L-epfb1 rev	ACAGTTTAATGGGCACGTCCAC		n/a	1:200	Cryosections	
zf-zic2a fwd	ACAACAATCTGTCGCCTTCTCTC		n/a	1:200	whole-mount	
zf-zic2a rev	ACAAATGCCCTGTTTAGCCC		n/a	1:200	whole-mount	
zf-zic2b fwd	TCTTCCGCTACATGCGACAAC		n/a	1:200	whole-mount	
zf-zic2b rev	GCAACACCACATGCTGAGAAC		n/a	1:200	whole-mount	
zf-epfb1 fwd	CGCGTGTGGATGGATTACGG		n/a	1:200	whole-mount	
zf-epfb1 rev	CATCCCCACCAGCTGGATCA		n/a	1:200	whole-mount	
zf-atoh7 fwd	GGAGAAGTTTGAGAGTCTATGCGG		n/a	1:200	whole-mount	
zf-atoh7 rev	CGACTTTGAGCTGAGCACACACC		n/a	1:200	whole-mount	
zf-rx2 fwd	GATACCATGAACATGGTGGACGATGG		n/a	1:200	whole-mount	
zf-rx2 rev	CCATCGACTGAATGTGCTCTTGG		n/a	1:200	whole-mount	
Primary antibodies						
Antigen	Species	Catalog #	Company	RRID	Dilution	Immunohistochemistry
Islet1	Rabbit	GTX128201	GeneTex	Ab_2868422	1:300	Cryosections/whole-mount
Acetylated-tubulin	Mouse	T6793	Sigma	Ab_477585	1:300	Cryosections/whole-mount
PCNA	Mouse	P8825	Sigma	Ab_477413	1:500	Cryosections
Islet1+2	Mouse	39.4D5	DSHB	Ab_2314683	1:50	Cryosections
GFP	Chicken	GTX13970	GeneTex	Ab_371416	1:5000	whole-mount
Rbpms	Guinea Pig	ABN1376	Millipore	Ab_2687403	1:400	Cryosections/flat-mount/ whole-
Zic2	Rabbit	Ab150404	Abcam	Ab_2868423	1:300	Cryosections/flat-mount/ whole-
Sox2	Goat	Sc17320	Santa-Cruz	Ab_2286684	1:300	flat-mount
EphB1	Mouse	MAb Efb1-3	DSBH	Ab_2314357	1:5	Cryosection
Secondary antibodies						
Anti-Rabbit cy3	Donkey	711-165-152	Jackson ImmunoResearch	Ab_2307443	1:500	cryosections/whole-mount
Anti-Rabbit Alexa Fluor 647	Donkey	711-605-152	Jackson ImmunoResearch	Ab_2492288	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 488	Donkey	A11055	Life Technologies	Ab_2534102	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 555	Donkey	A21432	Life Technologies	Ab_2535853	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 647	Bovine	805-605-180	Jackson ImmunoResearch	AB_2340885	1:600	cryosections
Anti-Goat cy3	Donkey	705-165-147	Jackson ImmunoResearch	Ab_2307351	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 488	Donkey	A21202	Life Technologies	Ab_141607	1:500	cryosections/Flat-mount/whole-mount
Anti-Guinea-Pig Alexa cy3	Donkey	706-165-148	Jackson ImmunoResearch	Ab_2340460	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 647	Donkey	715-605-150	Jackson ImmunoResearch	Ab_2340862	1:500	cryosections/Flat-mount/whole-mount
Anti-Mouse, Alexa Fluor 635	Goat	A31574	Life Technologies	Ab_2536184	1:500	cryosections
Anti-Rabbit, Alexa Fluor 568	Goat	A11036	Life Technologies	Ab_10563566	1:500	cryosections
Anti-Mouse, Alexa Fluor 568	Goat	A11004	Life Technologies	Ab_2534072	1:500	cryosections
Alexa Fluor 488 anti-chicken	Goat	A11039	Life Technologies	Ab_142924	1:500	cryosections
Tracers						
Cholera toxin subunit B-AlexaFluor555	n/a	C22843	Life technologies	n/a	2 µg/µl	Whole-mount
Cholera toxin subunit B-AlexaFluor647	n/a	C34778	Life technologies	n/a	2 µg/µl	Whole-mount

## **Movie S1.**

### **Visual projections in teleosts.**

Whole brain rendering of visual projections in 5 teleosts, the zebrafish, Mexican tetra, green-spotted pufferfish, mudskipper and butterflyfish. All species shows a complete decussation of retinal projections except the butterflyfish. All fish had bilateral eye injections of CTb coupled to either an Alexa Fluor-555 or and Alexa Fluor-647.

## **Movie S2.**

### **Bilateral visual projections in non teleosts.**

Whole brain rendering of visual projections in spotted gar, sterlet and armored bichir. Ipsilateral projections are seen in all species observed. All fish had bilateral eye injections of CTb coupled to either an Alexa Fluor-555 or and Alexa Fluor-647.

## **Movie S3.**

### **The Australian lungfish possesses non-segregated ipsilateral projections.**

Whole brain rendering of visual projections in the Australian lungfish, a sarcopterygian, injected with either an Alexa Fluor-555 or an Alexa Fluor-647. Many ipsilateral projections are observed, with a major component in the optic tectum. Ipsilateral projections are intermingled with contralateral projections in the optic tectum.

## **Movie S4.**

### **ZIC2 expression is evolutionarily conserved in Humans.**

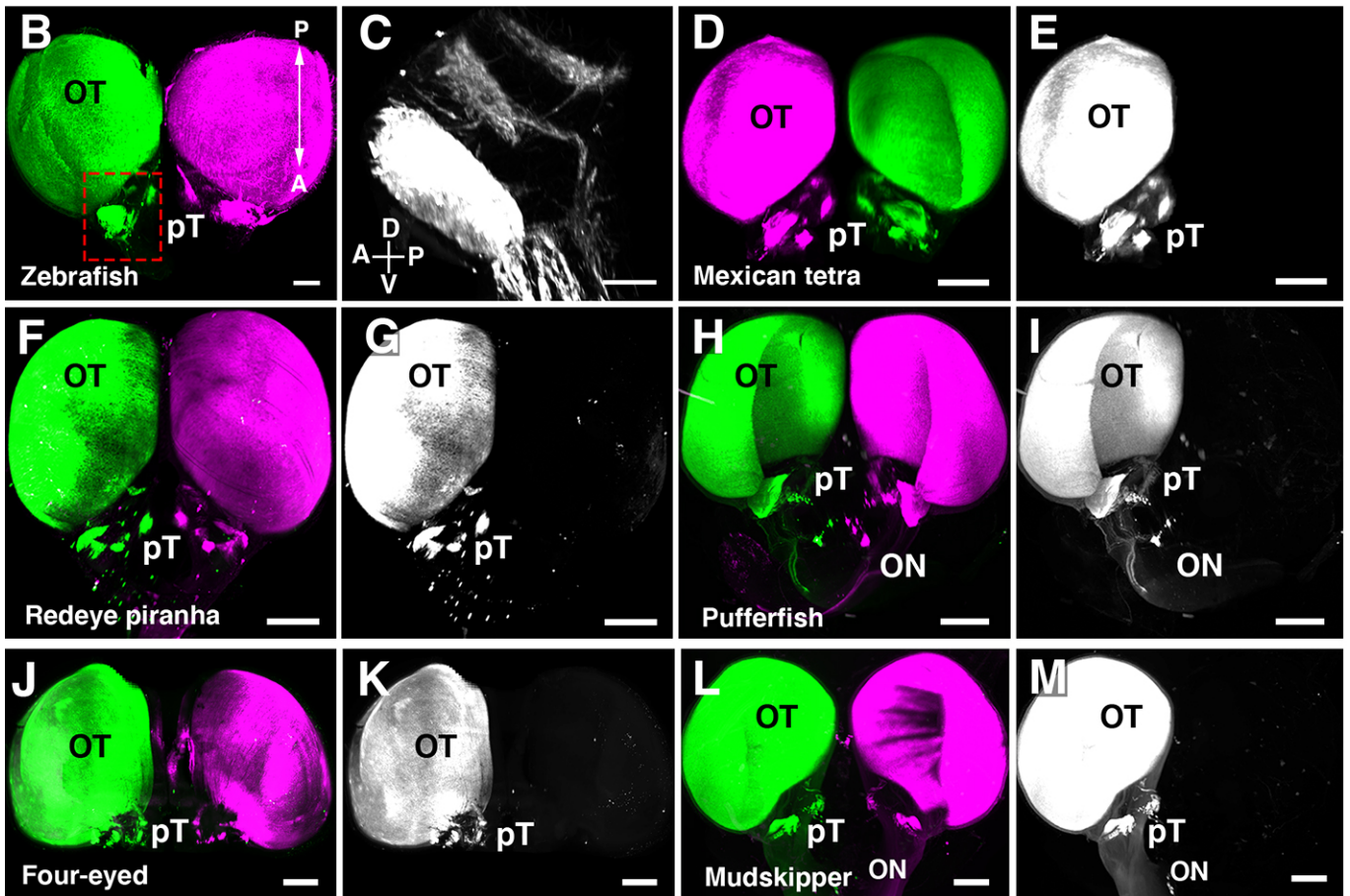
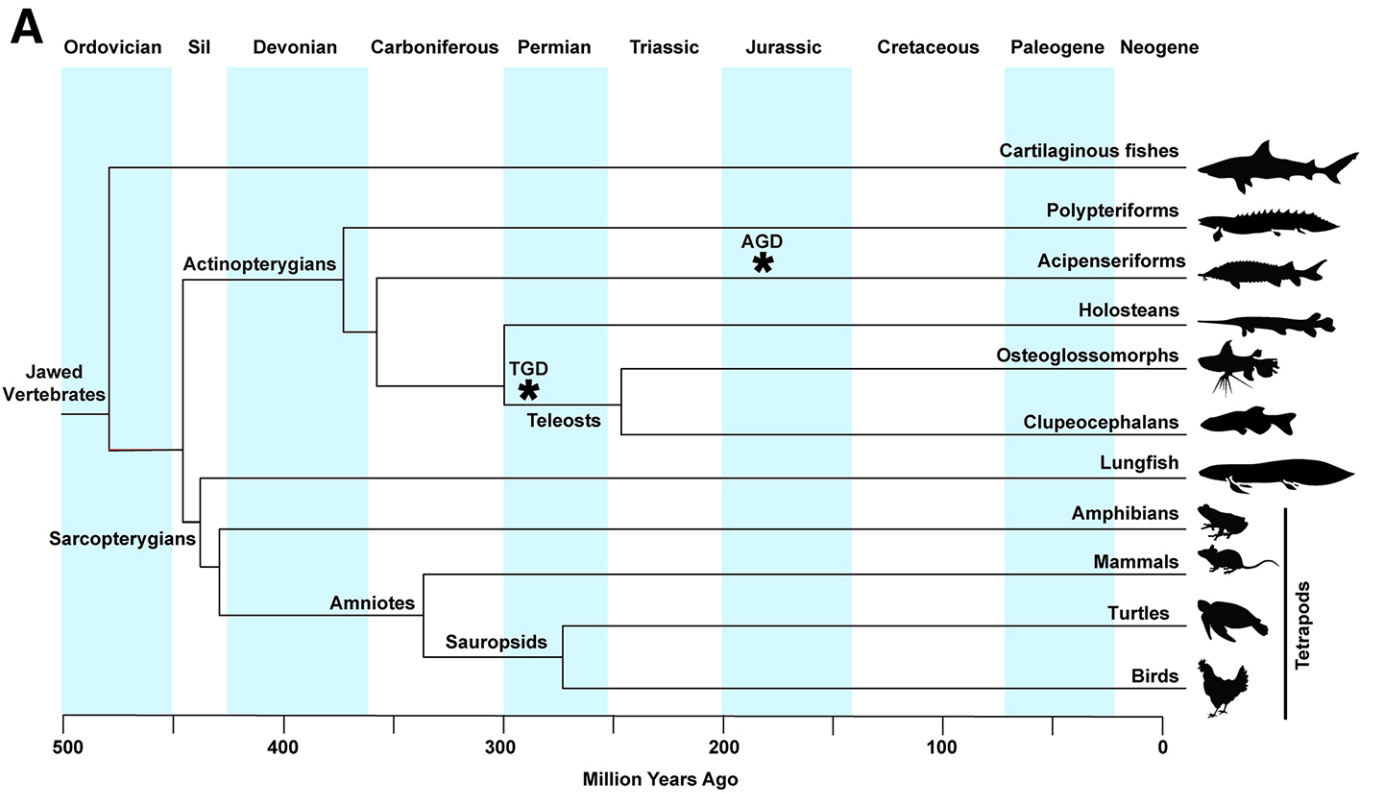


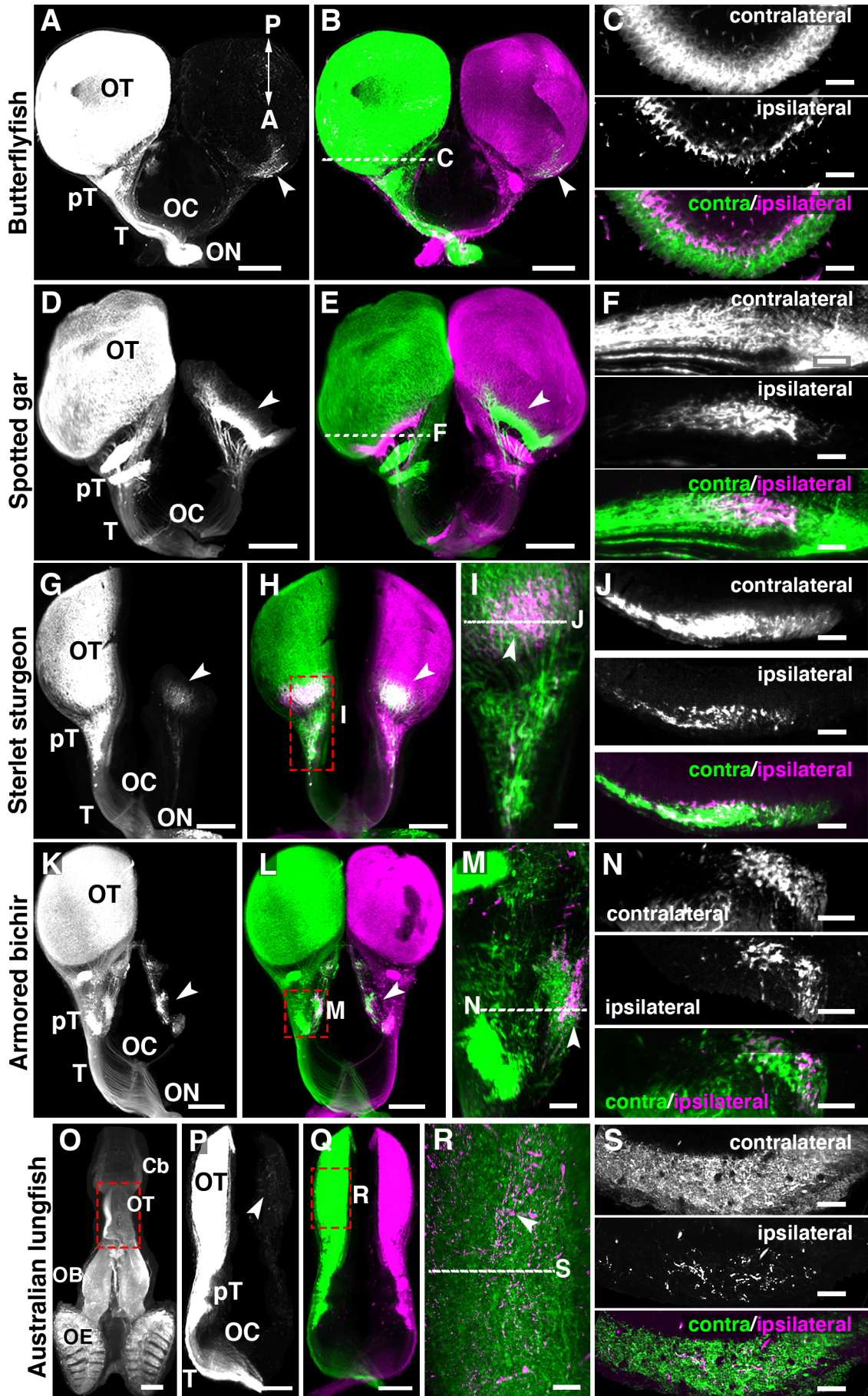
Whole-mount immunohistochemistry of pcw9 human eyes using EyeDISCO clearing and labeled for the ipsilateral transcription factor ZIC2 (magenta) and the pan-retinal ganglion cell marker RBPMS (green). A large ZIC2-positive region can be seen in the temporal retina.

**Movie S5.**

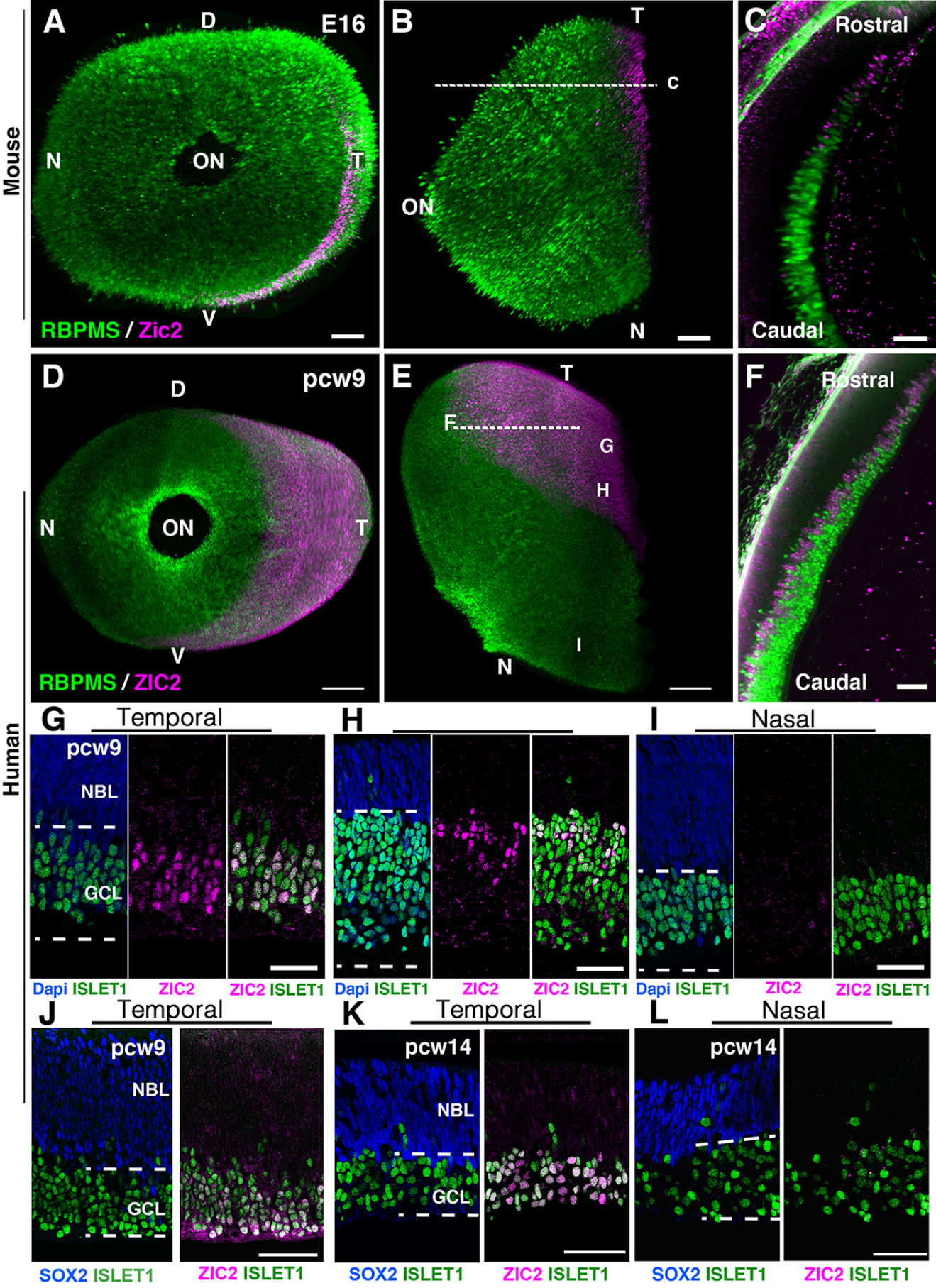
**Development of the *Lepisosteus oculatus* visual system.**

3D rendering of 2-3 dpf, 6-7 dpf, and 17-18 dpf spotted gar embryos using EyeDISCO clearing and light-sheet fluorescence microscopy. Spotted gar embryos were labeled with the pan-neuronal marker acetylated tubulin ( $\alpha$ -tubulin, green) and the LIM/homeodomain family of transcription factor Islet1, which is critical for the proper specification of retinal ganglion cells and motor neurons (magenta).

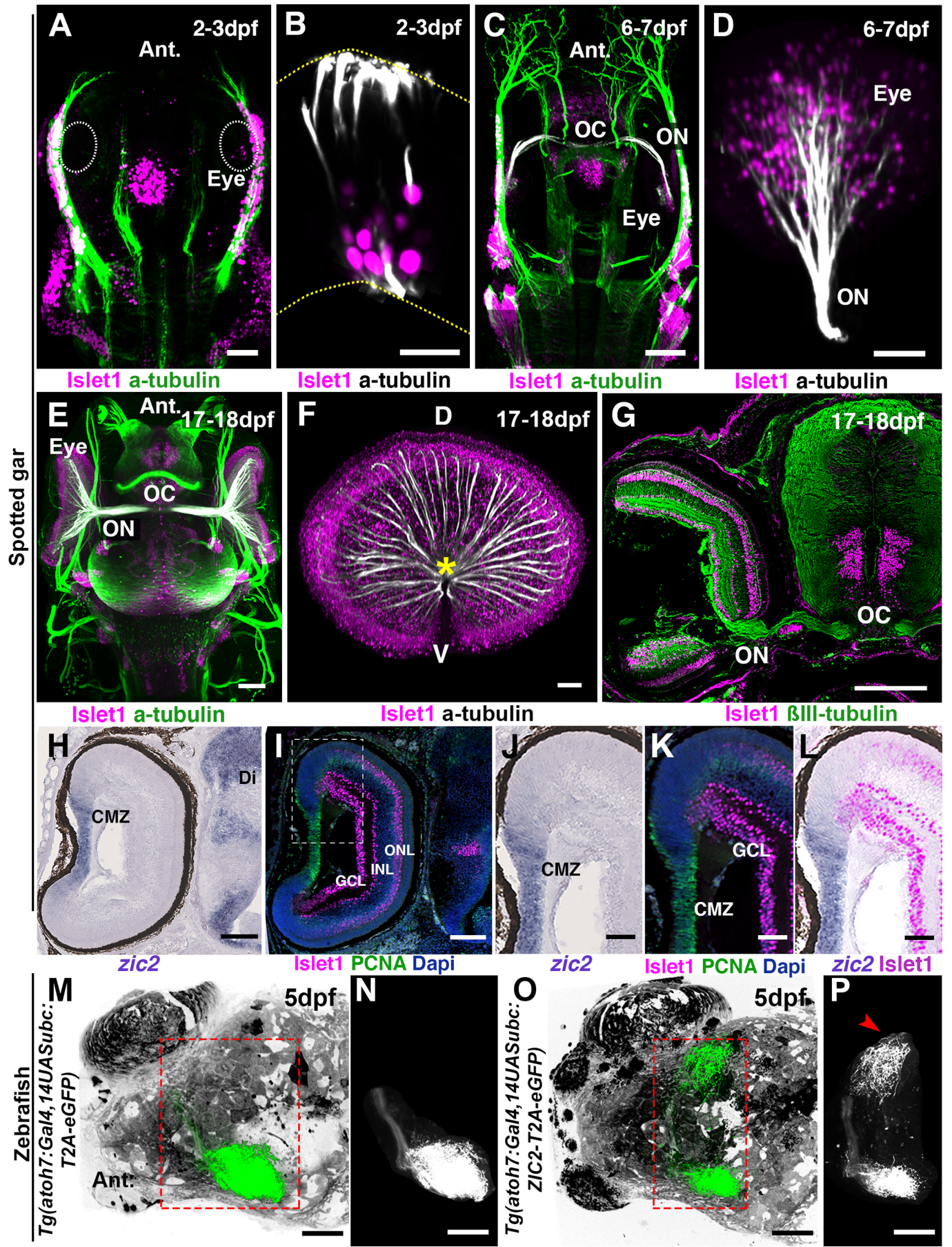












**Table S1. Comprehensive table summarizing the antibodies and probes sequences.**

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Name	Sequence		RRID	Dilution	In situ hybridization	
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L-zic1 rev	GGAACACTCTTCCCAGAAAC		n/a	1:200	Cryosections	
L-zic2 fwd	AAACTTAACCACGACCTCTCTC		n/a	1:200	Cryosections	
L-zic2 rev	CTCGTGCATTGTGCTGAAAG		n/a	1:200	Cryosections	
L-zic5 fwd	CTTTGAGCAAGAGGAATCCGGC		n/a	1:200	Cryosections	
L-zic5 rev	CCTGCCGCGATGTTACATTTA		n/a	1:200	Cryosections	
L-efnb2 fwd	TCCCCATTATGAGAAGGTGAGCGG		n/a	1:200	Cryosections	
L-efnb2 rev	ACAGGCTACCACTTCAGAAGGCAG		n/a	1:200	Cryosections	
L-ephb1 fwd	AGAACCTGAACACAATCCGCAC		n/a	1:200	Cryosections	
L-ephb1 rev	ACAGTTTAATGGGCACGTCCAC		n/a	1:200	Cryosections	
zf-zic2a fwd	ACAACAATCTGTCGCCTTCTCTC		n/a	1:200	whole-mount	
zf-zic2a rev	ACAAATGCCCTGTTTAGCCC		n/a	1:200	whole-mount	
zf-zic2b fwd	TCTTCCGCTACATGCCACAAC		n/a	1:200	whole-mount	
zf-zic2b rev	GCAACACCACATGCTGAGAAC		n/a	1:200	whole-mount	
zf-ephb1 fwd	CGCGTGTGGATGGATTACGG		n/a	1:200	whole-mount	
zf-ephb1 rev	CATCCCCACCAGCTGGATCA		n/a	1:200	whole-mount	
zf-atoh7 fwd	GGAGAAGTTTGAGAGTCTATGCGG		n/a	1:200	whole-mount	
zf-atoh7 rev	CGACTTTGAGCTGAGCACACACC		n/a	1:200	whole-mount	
zf-rx2 fwd	GATACCATGAACATGGTGGACGATGG		n/a	1:200	whole-mount	
zf-rx2 rev	CCATCGACTGAATGTGCTCTTGG		n/a	1:200	whole-mount	
Primary antibodies						
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PCNA	Mouse	P8825	Sigma	Ab_477413	1:500	Cryosections
Islet1+2	Mouse	39.4D5	DSHB	Ab_2314683	1:50	Cryosections
GFP	Chicken	GTX13970	GeneTex	Ab_371416	1:5000	whole-mount
Rbpms	Guinea Pig	ABN1376	Millipore	Ab_2687403	1:400	Cryosections/flat-mount/ whole-
Zic2	Rabbit	Ab150404	Abcam	Ab_2868423	1:300	Cryosections/flat-mount/ whole-
Sox2	Goat	Sc17320	Santa-Cruz	Ab_2286684	1:300	flat-mount
EphB1	Mouse	MAb Efb1-3	DSBH	Ab_2314357	1:5	Cryosection
Secondary antibodies						
Anti-Rabbit cy3	Donkey	711-165-152	Jackson ImmunoResearch	Ab_2307443	1:500	cryosections/whole-mount
Anti-Rabbit Alexa Fluor 647	Donkey	711-605-152	Jackson ImmunoResearch	Ab_2492288	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 488	Donkey	A11055	Life Technologies	Ab_2534102	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 555	Donkey	A21432	Life Technologies	Ab_2535853	1:500	cryosections/Flat-mount/whole-mount
Anti-Goat Alexa Fluor 647	Bovine	805-605-180	Jackson ImmunoResearch	AB_2340885	1:600	cryosections
Anti-Goat cy3	Donkey	705-165-147	Jackson ImmunoResearch	Ab_2307351	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 488	Donkey	A21202	Life Technologies	Ab_141607	1:500	cryosections/Flat-mount/whole-mount
Anti-Guinea-Pig Alexa cy3	Donkey	706-165-148	Jackson ImmunoResearch	Ab_2340460	1:500	cryosections/Flat-mount/whole-mount
Anti-mouse Alexa Fluor 647	Donkey	715-605-150	Jackson ImmunoResearch	Ab_2340862	1:500	cryosections/Flat-mount/whole-mount
Anti-Mouse, Alexa Fluor 635	Goat	A31574	Life Technologies	Ab_2536184	1:500	cryosections
Anti-Rabbit, Alexa Fluor 568	Goat	A11036	Life Technologies	Ab_10563566	1:500	cryosections
Anti-Mouse, Alexa Fluor 568	Goat	A11004	Life Technologies	Ab_2534072	1:500	cryosections
Alexa Fluor 488 anti-chicken	Goat	A11039	Life Technologies	Ab_142924	1:500	cryosections
Tracers						
Cholera toxin subunit B-AlexaFluor555	n/a	C22843	Life technologies	n/a	2 µg/µl	Whole-mount
Cholera toxin subunit B-AlexaFluor647	n/a	C34778	Life technologies	n/a	2 µg/µl	Whole-mount