Supplementary Materials for

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

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Other Supplementary Materials for this manuscript include the following:

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_2O_2/0.5%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 *pT1UciMP 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 *pT1UciMP 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-thiouera (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% Trinton-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_2O_2 (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% Tirton-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 β subunit was carried out as described in (*15*). Briefly, using a capillary approximately 1µl of 2 µg/µl of Alexa Fluor-conjugated cholera toxin β 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 ImspectorPro 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^2, 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 µm, 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 μm 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 ± 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

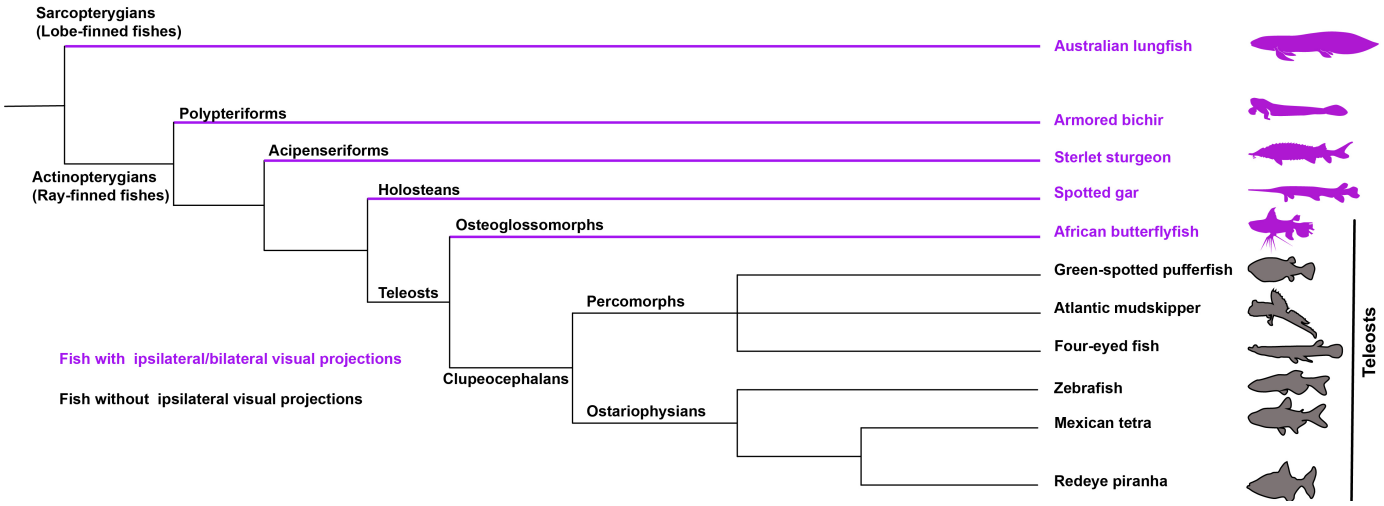


Fig. S1. Simplified chart of fish taxonomy indicating the species analyzed in this study.
Fish with bilateral/ipsilateral visual projections appear in magenta and fish with only contralateral visual projections appear in grey.

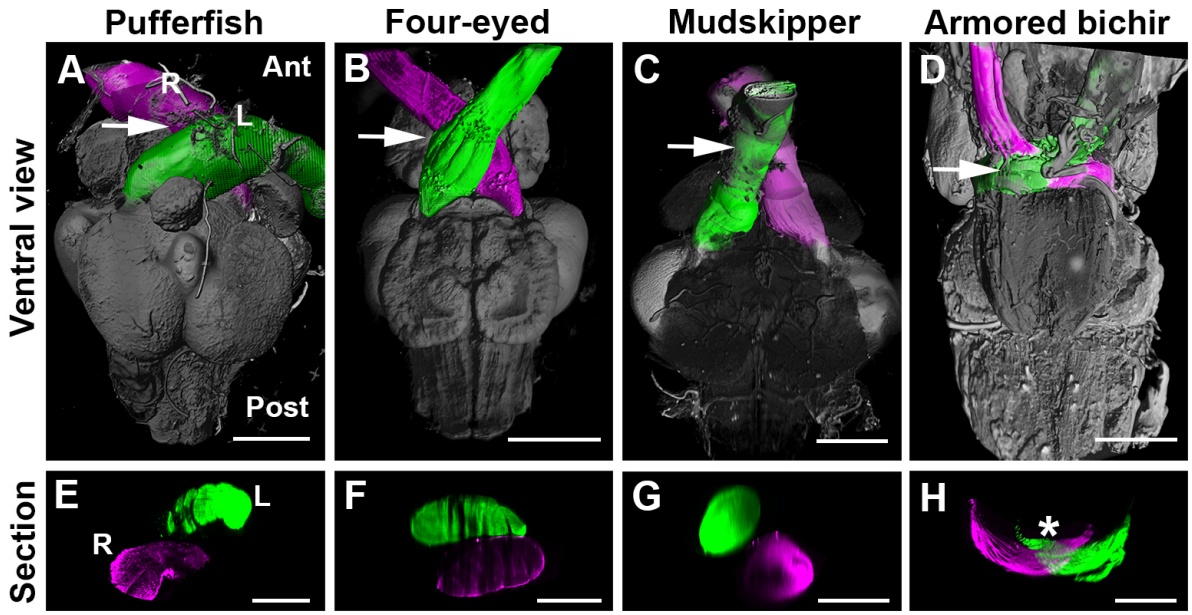


Fig. S2. Two types of optic nerve crossing modalities in ray-finned fishes.
Ventral views (**A** to **D**) and coronal optical sections (**E** to **H**) at the level of the optic chiasm of iDISCO-cleared brains and optic nerves. A surface rendering with normal shading (Imaris) was applied to generate 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 Muskipper (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 μm in (G), 600 μm in (E, H).

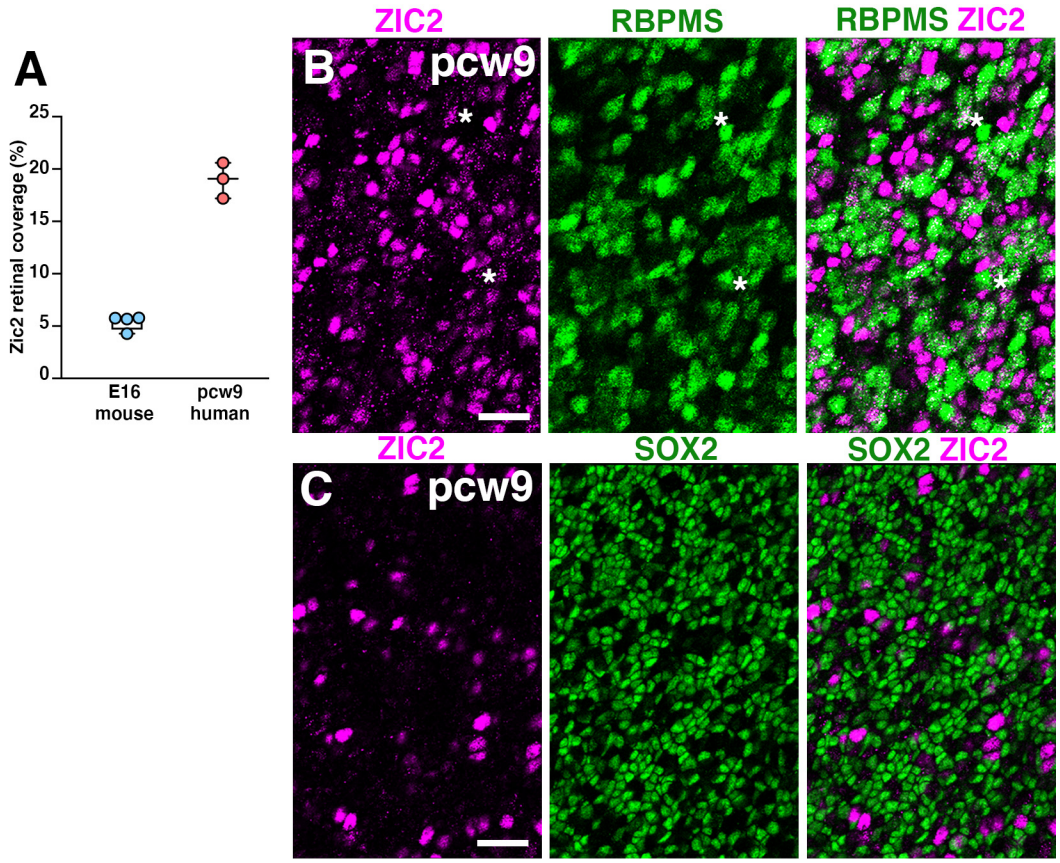


Fig. S3. ZIC2 expression pattern in human embryo retina.
(**A**) Box and whiskers representation of the ZIC2-positive surface in E16 mouse and pcw9 human retinas. (**B** and **C**) flat-mount pcw9 human retina labeled for ZIC2 and RBPMS (B) or SOX2 (C). (B) In the most superficial (basal side) regions 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 μm in (B and C).

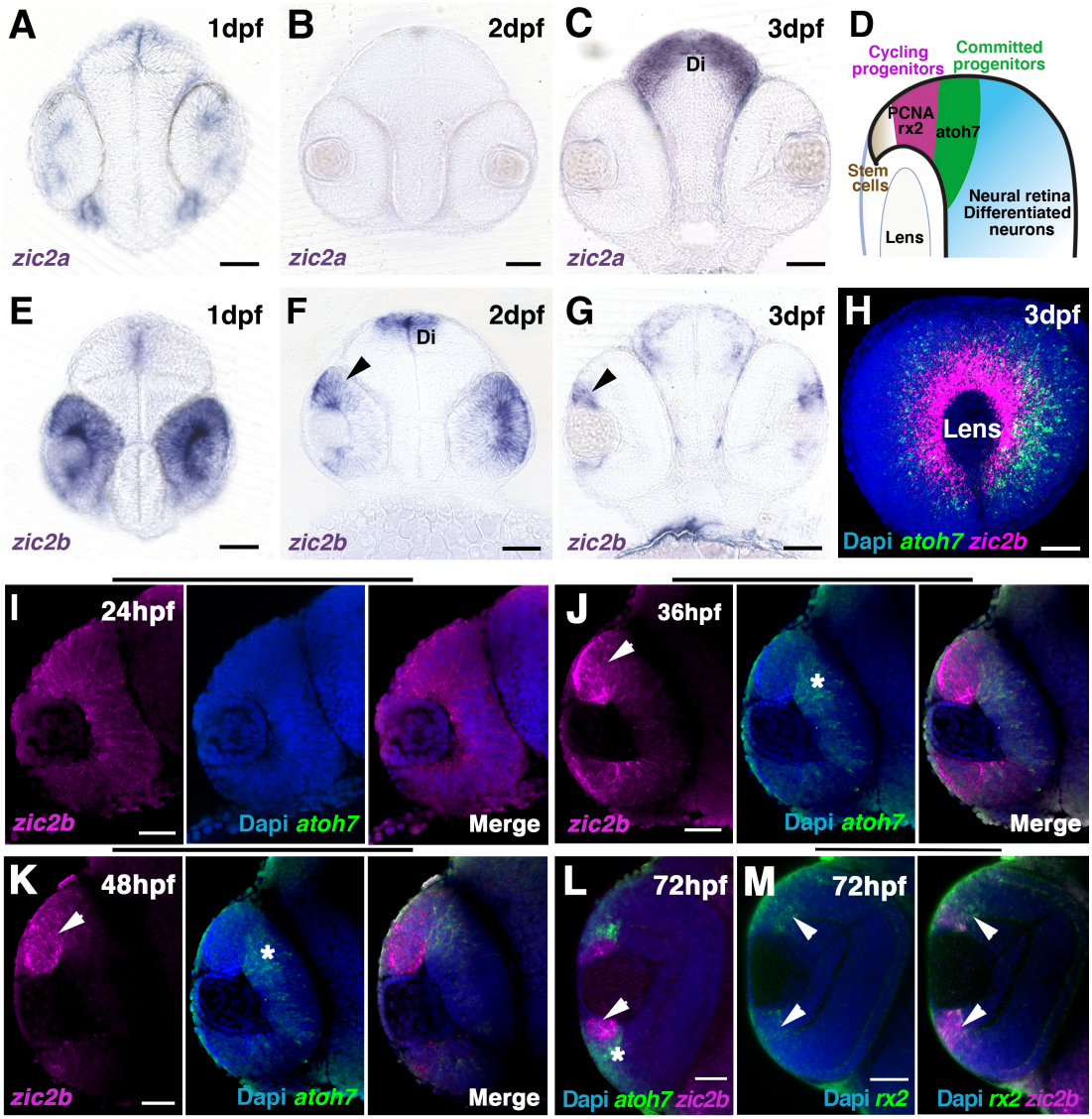
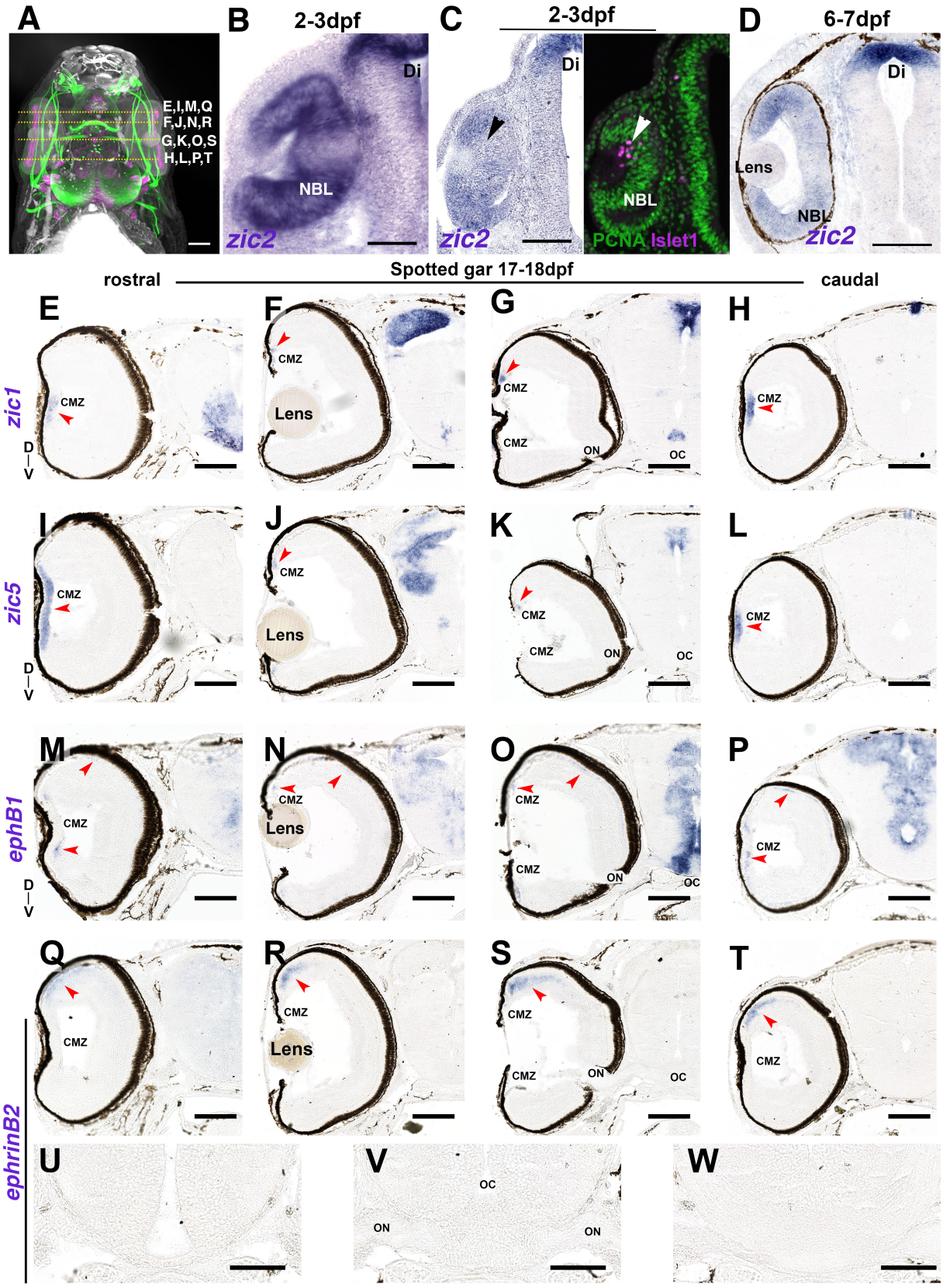


Fig. S4. Zic2 ortholog expression in zebrafish is restricted to the ciliary margin zone.
(**A** to **H**) Whole-mount *in situ* hybridization of zebrafish embryos for *zic2a* at 1 day post fertilization (1 dpf; A), 2 dpf (B) and 3 dpf (C) as well as *zic2b* at 1 dpf (E), 2 dpf (F) and 3 dpf (G). Zic2b 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 or dividing progenitors and stem cells in the CMZ). *Zic2b* expression overlaps with the expression of the *rx2* (arrowheads). All retinae were counterstained with the nuclear marker DAPI. Scale bars are 50 µm (A to C and E to H) and 40 µm (I to M).


A
E,I,M,Q
F,J,N,R
G,K,O,S
H,L,P,T
B
2-3dpf
Di
NBL
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C
2-3dpf
Di
NBL
zic2
PCNA Islet1
D
6-7dpf
Di
Lens
NBL
zic2
Spotted gar 17-18dpf
rostral
caudal
zic1
zic5
ephB1
ephrinB2
D
V
CMZ
Lens
ON
OC
E
F
G
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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 μm; B to D, 50 μm; C,U to W, 100 μm; D to T, 250 μm.

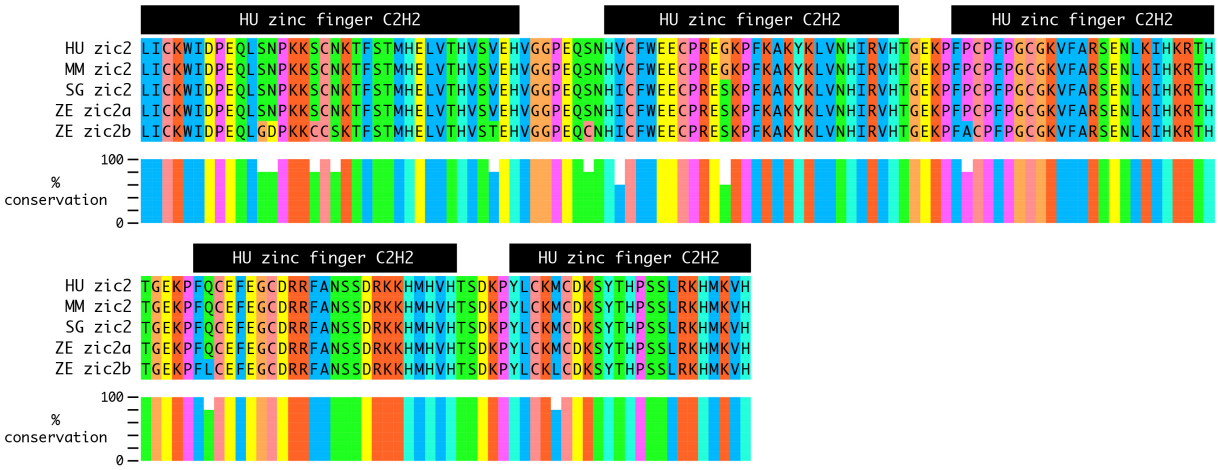

Fig. S6. Alignment between the amino acid sequences of the Zic2 protein zing finger domains of fish and mammals.

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.

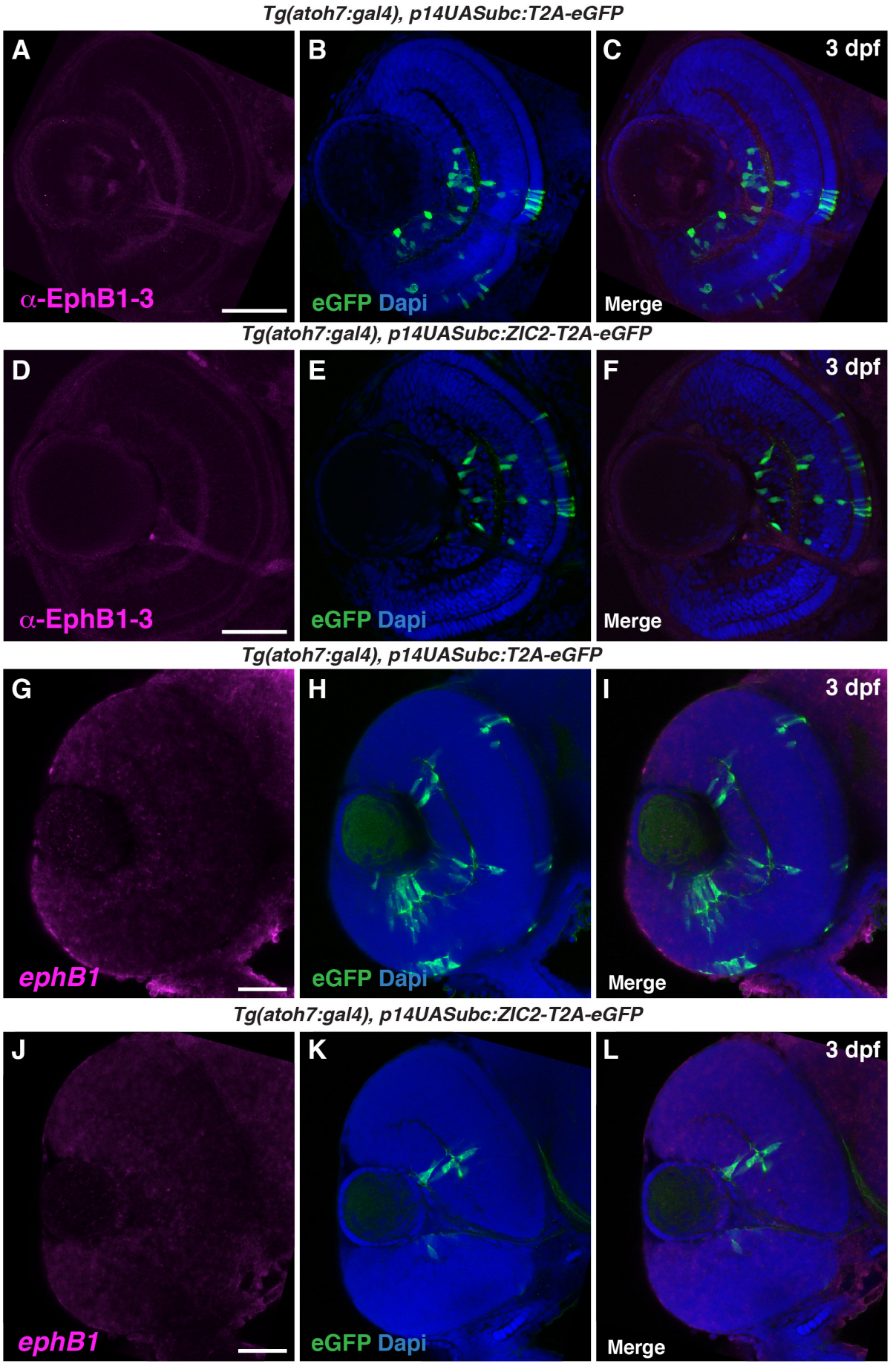
Tg(atoh7:gal4), p14UASubc:T2A-eGFP
A
B
C
3 dpf
α-EphB1-3
eGFP Dapi
Merge
Tg(atoh7:gal4), p14UASubc:ZIC2-T2A-eGFP
D
E
F
3 dpf
α-EphB1-3
eGFP Dapi
Merge
Tg(atoh7:gal4), p14UASubc:T2A-eGFP
G
H
I
3 dpf
ephB1
eGFP Dapi
Merge
Tg(atoh7:gal4), p14UASubc:ZIC2-T2A-eGFP
J
K
L
3 dpf
ephB1
eGFP Dapi
Merge

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 μm.

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	CCTGCCGCGATGTTCACATTTA			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	ACAACAATCTGTCGCCTTCCTC			n/a	1:200	whole-mount
zf-zic2a rev	ACAAATGCCCCTGTTTAGCCC			n/a	1:200	whole-mount
zf-zic2b fwd	TCTTCCGCTACATGCGACAAC			n/a	1:200	whole-mount
zf-zic2b rev	GCAACACCGACATGCTGAGAAC			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	GGAGAAGTTTGAGAGTGCTATGCGG			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 fwd	CCATCGACTGAATGTGCTCCTTGG			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 (a-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).