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RESEARCH ARTICLE

Genetic developmental timing revealed by inter-species transplantations in fish

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ABSTRACT

The path from a fertilised egg to an embryo involves the coordinated formation of cell types, tissues and organs. Developmental modules comprise discrete units specified by self-sufficient genetic programs that can interact with each other during embryogenesis. Here, we have taken advantage of the different span of embryonic development between two distantly related teleosts, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) (3 and 9 days, respectively), to explore modularity principles. We report that inter-species blastula transplantations result in the ectopic formation of a retina formed by donor cells – a module. We show that the time taken for the retina to develop follows a genetic program: an ectopic zebrafish retina in medaka develops with zebrafish dynamics. Heterologous transplantation results in a temporal decoupling between the donor retina and host organism, illustrated by two paradigms that require retina-host interactions: lens recruitment and retino-tectal projections. Our results uncover a new experimental system for addressing temporal decoupling along embryonic development, and highlight the presence of largely autonomous but interconnected developmental modules that orchestrate organogenesis.

KEY WORDS: Developmental timing, Genetic chimera, Inter-species transplantation, Organogenesis, Medaka, Zebrafish, Retina, Lens induction, Retino-tectal projection

INTRODUCTION

In vertebrates, organogenesis takes place during embryonic development and follows a stereotypic, species-specific timing. Cases in which two organs of the same type are generated within an organism – eyes, ears, lungs, kidneys, gonads – indicate that these develop in a synchronized manner, despite constituting independent units. The temporal control of organogenesis is of paramount importance to secure the functional coordination of organs within systems, i.e. neurons in a sensory organ should mature and become functional together with their target regions. A long-standing issue in the field is whether neural organs follow an endogenous timing that defines the onset of neurogenesis – autonomous timing – or whether, alternatively, there are global signals that guarantee coordination among cell types, tissues and organs – ontogenic timing.

The vertebrate neural retina constitutes a major model for neurogenesis in the central nervous system (CNS), and it was demonstrated long ago that the different types of retinal neurons are formed in a stereotypic temporal order and arranged in dedicated layers (Livesey and Cepko, 2001). Retinal organoids and aggregates demonstrate that the vertebrate retina is capable of autonomously patterning (Eiraku et al., 2011), earlier suggested by the transplantation of optic vesicles into ectopic regions of the chick and fish (Gestri et al., 2018; Picker and Brand, 2005). Although the self-organizing properties of the neural retina have been demonstrated for other organoids, the inherently artificial conditions needed to develop 3D cultures, or the technical artefacts that accompany the transplantation of an optic cup, might affect the temporal sequence of biological processes on organoids or aggregates (DiStefano et al., 2018). The ideal set-up to explore developmental timing, therefore, should exploit the self-organizing properties of the retina while developing in a homo- or heterochronic physiological environment.

Teleost fish represent the vertebrate clade with most species; these vary hugely in sizes and shapes, and in the duration of their embryonic development (Betancur-R et al., 2017). Among teleost fish, *Danio rerio* and *Oryzias latipes* (zebrafish and medaka, respectively) diverged some 250 million years ago, and belong to two of the most distant subgroups (Schartl et al., 2013). One of the most obvious differences between them is the time that they take to complete embryonic development: 3 days for zebrafish and 9 days for medaka (Fig. 1). Here, we have used zebrafish-medaka inter-species chimeras to report that isochronic transplantation of blastomeres from one species into the other results in the formation of an ectopic retina by donor cells. Our set up is unique, as the entire developmental process from a blastomere to a differentiated retinal neuron (i.e. patterning, morphogenesis and neurogenesis) happens within the host species. Taking advantage of the fact that donor cells do not intermingle with host cells, we follow intrinsic and extrinsic properties of an entire module in a physiological environment. We use fluorescent transgenic reporters, *in vivo* imaging and RNA-seq analysis to show that the ectopic cluster uses host cues to trigger a retinal transcriptional program. This program, however, follows the temporal logic of the donor species and forms heterochronic retinal neurons compared with those in the host retinae. Heterochronic neural development has phenotypic consequences that we illustrate using inter-lineage communication paradigms: the induction of the lens by the retinal vesicle and the navigation of retinal axons to their target optic tectum. Altogether, we present an experimental set up for studying developmental dynamics, use it to report on genetic timing in the execution of the retinal transcriptional program in vertebrates and illustrate the consequences of heterochronic neurogenesis.

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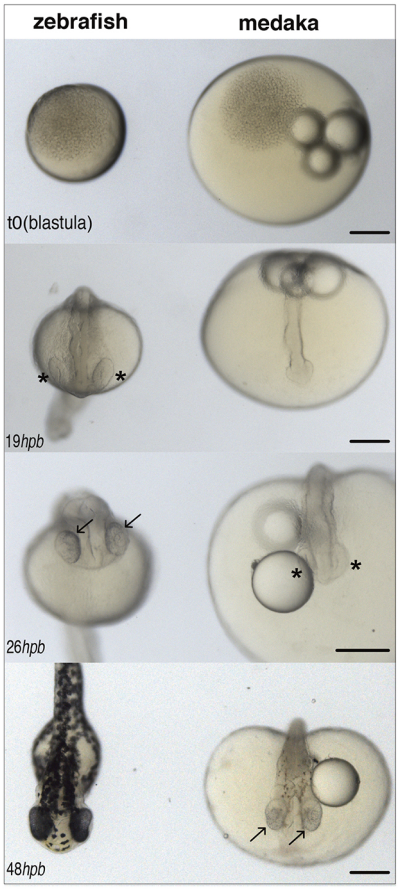

Fig. 1. Different developmental timing for zebrafish and medaka. Images of zebrafish (left) and medaka (right) embryos synchronised at the blastula stage (top, 512 cells, t=0) and at different stages of embryonic development (hours post blastula, hpb). Eye cups (asterisks) are evident in zebrafish by 19 hpb and retinal pigmentation (and neurogenesis) (arrows) by 26 hpb; in medaka, retinal pigmentation does not start before 48 hpb. Scale bars: 200 µm.

RESULTS

Medaka/zebrafish inter-species transplantation results in the formation of ectopic retinae

Genetic chimeras formed by transplanting blastomeres from one fish embryo to another have been extensively used in zebrafish (Fig. 1, left) and medaka (Fig. 1, right), i.e. to define the cell-autonomous versus cell-non-autonomous roles of novel mutant lines, to study lineages during embryonic and post embryonic development, etc. (Centanin et al., 2011; Haas and Gilmour, 2006; Poggi et al., 2005; Vogeli et al., 2006; Winkler et al., 2000). Intra-species transplantations at blastula stage result in the mixing of donor and host cells along the developing embryo. This was indeed the case when we transplanted blastomeres from ubiquitously labelled transgenic lines (*ßactin*::CAAX-EYFP in zebrafish, *Wimbledon* or *Gaudi*$^{LoxP.OUT}$ in medaka) (Centanin et al., 2014, 2011) into non-labelled controls from the same species (Fig. 2A,B) (n>20 transplantation experiments for each species, n>10 embryos per transplantation experiment). However, we have observed that, in trans-species transplantations, donor cells stay clustered together and do not mix with host cells during gastrulation, both for zebrafish to medaka (zebraka, Fig. 2C) and for medaka to zebrafish (medrafish, Fig. 2D) (n=17 transplantation experiments, n>100 chimeras for zebraka; n=33 transplantation experiments, n>100 chimeras for medrafish; a transplantation event is a transplantation experiment performed on a given day with a specific donor-host combination that leads to one or more chimeras of the described phenotype) (Tables S1 and S2). In both zebraka and medrafish, host blastomeres can proceed through gastrulation and a body axis is evident at 19 h post-transplantation (hpt) (Fig. 2C,D). Stunningly, the cluster of transplanted cells often develops into an ectopic organ that resembles a retina, which is formed by EGFP-positive cells (Fig. 3A,B). We have observed ectopic retinae for both trans-species transplantation set-ups; they frequently contain retinal pigmented epithelium (Fig. 3B, see also Fig. 5C″), which indicates that the initial alien cluster follows a developmental program despite the lack of intermingling with host cells during epiboly.

The formation of an ectopic retina in medaka-zebrafish chimera is highly reproducible, having observed the same phenotype consistently on different transplantation experiments using diverse transgenic donors and hosts (16 out of 21 transplantation experiments for zebraka, 29 out of 37 transplantation experiments for medrafish; on a representative experiment using heterozygote transgenic founders, we obtained 23 zebrakas out of 72 transplanted embryos) (Tables S1 and S2). The expression of retinal transcripts in the cluster was confirmed by using transcriptional reporters for retinal progenitors and retinal neurons in medaka, namely *rx2* – retinal homeobox factor 2 (Inoue and Wittbrodt, 2011; Reinhardt et al., 2015; Winkler et al., 2000) and *atoh7* (Del Bene et al., 2007; Kay et al., 2005; Poggi et al., 2005; Souren et al., 2009). When blastomeres from medaka Tg(*rx2*:H2B-RFP) were transplanted into non-labelled zebrafish blastulae, the retinal-like cluster expressed the medaka retinal reporter *rx2* (Fig. 3C) (Table S2). The same result was obtained when we used Tg(*Atoh7*:EGFP) as donors, suggesting that the ectopic cluster has both retinal identity and the potential to trigger neurogenesis (Fig. 3D) (Table S2).

Molecular confirmation and morphological characterisation of ectopic retinae

There are a number of methods available to address species-specific contribution of transcripts in a chimera (Ealba and Schneider, 2013). The low sequence identity of homologous genes between zebrafish and medaka allows the segregation of the bulk transcriptome of chimerae *in silico*, therefore expanding the analysis of the transcriptional profile in ectopic retinae of zebrakas. We compared RNA-seq from zebrafish, medaka and zebraka embryos at 48 hpf, a stage during which neurogenesis has started in zebrafish but it is only about to start in medaka. We selected chimeras with a clear EGFP+ cluster close to one of the endogenous retinae, as these are the most likely to become a retina. As expected, the transcriptome from zebrafish, but not from medaka, aligned to the zebrafish genome (Fig. 3E, Fig. S1). Owing to the large evolutionary distance, only a few RNA-seq reads form the medaka transcriptome mapped on the zebrafish genome (Fig. S1), and these displayed a distinguishable morphology as well as a minimal number of reads (Table S1). In contrast, numerous reads from the transcriptome of zebrakas, consisting of a full medaka transcriptome plus a partial transcriptome from the few zebrafish cells, could be aligned to the zebrafish genome (Fig. 3E, Fig. S1, Table S3). These peaks corresponded to genes that are expressed by the zebrafish retina at the same developmental time (*vsx2*, *rx1* and *rx2* among others, Fig. 3E, Table S4 and Fig. S2). Genes exclusively expressed in other organs at the same stage, and therefore present in the zebrafish transcriptome (i.e. cardiac muscle) were absent in the zebraka transcriptome (Fig. 3E and Table S4). This analysis confirms the molecular retinal identity of the EGFP+ cluster in zebraka chimeras.

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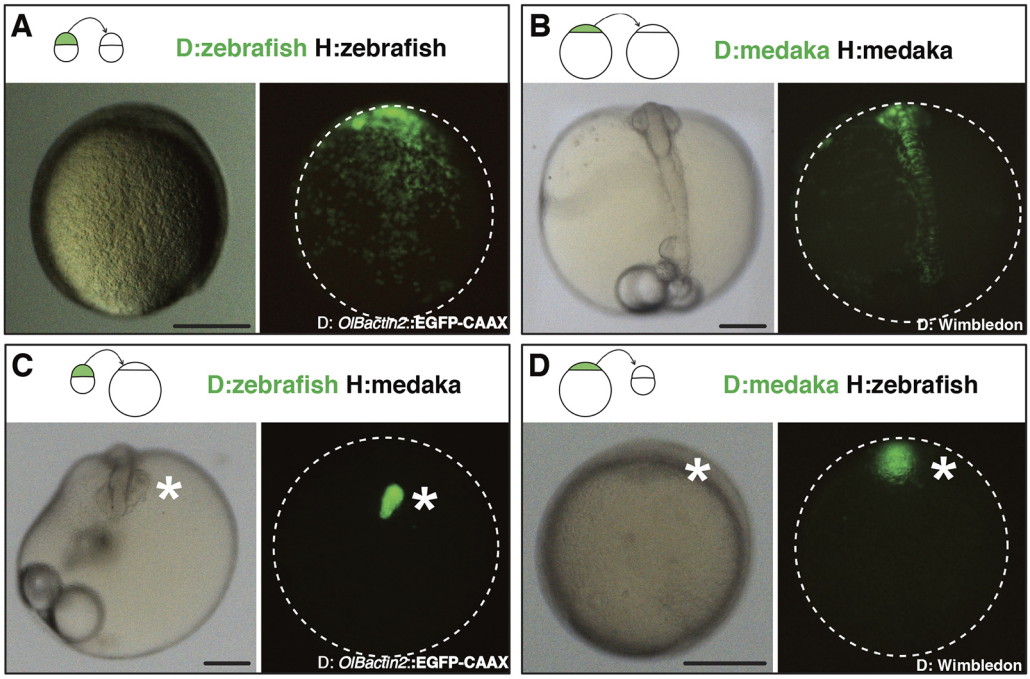

Fig. 2. Intra- and inter-species transplantation of blastocysts in zebrafish and medaka. (A-D) Transmitted (left) and fluorescent (right) images of a non-labelled host embryo that was transplanted at a blastula stage with isochronic EGFP fluorescent cells. Images were taken after completion of initial morphogenesis (90% epiboly stage for zebrafish and early neurula stage for medaka), and transplantation schemes are displayed at the top of each panel. Animal side is up for zebrafish hosts (A,D) and anterior side is up for medaka hosts (B,C). Dispersed green dots in A,B are donor cells intermingled with host cells (*n*>20 transplantation events for each species, *n*>10 embryos per transplantation event; a transplantation event is a transplantation experiment performed on a given day with a specific donor-host combination that leads to one or more chimeras of the described phenotype). EGFP+ clusters in C,D (asterisks) contain donor cells that did not mix with host cells. Representative images were chosen out of *n*=10 zebraka and *n*=12 medrafish. Clusters of the donor cells were found at later developmental stages in *n*=17 transplantation experiments, *n*>100 chimeras for zebraka; *n*=33 transplantation experiments, *n*>100 chimeras for medrafish (see Tables S1 and S2). Scale bars: 200 µm. D, donor; H, host.

The vertebrate retina displays a stereotypic distribution of cell types arranged in defined nuclear layers (Centanin and Wittbrodt, 2014), most evident when analysed in cryosections (Fig. 4A-A‴). To address the layering and cellular organisation of the ectopic retina in both zebraka and medrafish, we grew chimeras until late embryonic stages – 9 dpf for zebraka, 5 dpf for medrafish. Retinae were analysed by DAPI nuclear staining either in whole-mount or in cryosections.

A whole-mount analysis of 9 dpf zebraka revealed that the ectopic retina was not organised in the three classical nuclear layers, but lamination could still be observed on patches within the ectopic cluster (Fig. 4B-C″, white arrows). The examination of ectopic clusters in chimeras where Tg(*atoh7*:EGFP) was used as a donor allowed us to follow the occurrence of RGCs and their axons. Notably, EGFP+ cells usually localised adjacent to each other, as is the case in the endogenous retina, forming pseudo-layers in the ectopic retinae (Fig. 4B‴,C-C″). The internal organisation of cell types is more apparent when analysing cryosections of zebraka and medrafish ectopic retinae using DAPI, membrane labelling and/or cell type-specific antibodies (Fig. 5). A zebraka ectopic retina (Fig. 5A,B, top) from Tg(*βactin*:CAAX-EGFP) donors typically displays a row of nuclei (Fig. 5A′,B′) separated from other cells in the organ by a space filled in by membranes (Fig. 5A″,B′), resembling an outer plexiform layer (compare with Fig. 4A). Staining with an anti-Rx2 antibody (Inoue and Wittbrodt, 2011) that recognises retinal progenitors and photoreceptors (Fig. 4A′-A‴) indicates that these cells indeed express photoreceptor molecular markers. The same distribution of nuclei (Fig. 5C-C″,D) and signal detected using a Rx2 antibody (Fig. 5D″) can be seen in the ectopic retinae of medrafish, which also display an RPE cell layer that becomes pigmented (Fig. 5C″). Further attempts to detect additional cell types in the neural retina, using specific antibodies against cells present in the INL, did not result in any significant staining (Fig. 5D′ and data not shown). Overall, our experiments reveal that in both zebraka and medrafish, the ectopic retinae include patches of lamination and clusters of differentiated RGCs and photoreceptors.

It has long been proposed that neural ectoderm constitutes the default differentiation program for early embryonic cells. Indeed, neural retina was the first organoid produced in 3D cultures from an aggregate of embryonic stem cells (ESCs) (Eiraku et al., 2011), followed by other neural organs (Lancaster et al., 2013; Suga et al., 2011). In zebraka and medrafish chimeras, we have noticed that ectopic retinae inevitably form adjacent to an endogenous retina (Fig. 3A,B, *n*>60 chimerae). We have never observed an ectopic retina in remote locations, which strongly suggests that positional information from the host can be decoded by the transplanted cells – although it does not clarify whether the host anlage has a permissive or an inductive role. Retinal identity is not the only fate that ectopic blastocysts can adopt when transplanted into the foreign species, as we have observed clusters differentiating into vasculature or pigmented cells (Fig. S3) (Hong et al., 2012). The ectopic retina obtained using the current inter-species transplantation protocol, however, is the only organ entirely composed of foreign cells (Figs 3A and 5A″,C′,D′) and, as such, permits a compartmentalised analysis of host and donor organs going through embryonic development in parallel and in the same embryonic environment.

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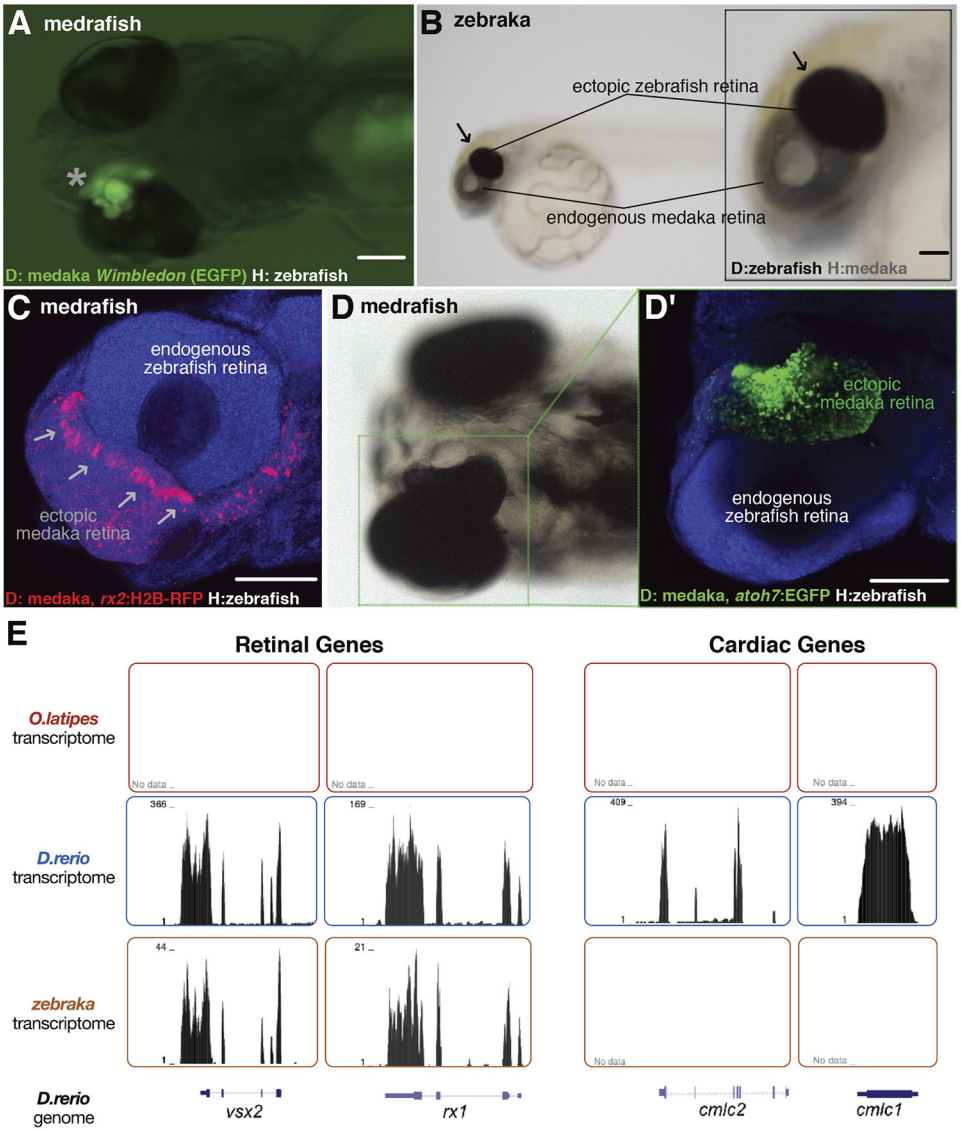

Fig. 3. Transplanted EGFP+ cluster develops into an ectopic retina both in zebraka and medrafish. Images of medrafish (A,C,D) and zebraka (B). The EGFP+ cluster (asterisk, ventral view of a hatch embryo; A) and the pigmented cluster (arrows, lateral view of a hatched embryo; B) develop into an ectopic retina (*n*=45 transplantation events). Confocal images show expression markers of retinal progenitors (arrows in C; *rx2*:H2B-RFP donors in medrafish at 4 dpf) and retinal neurogenesis (D; *atoh7*:EGFP donors in medrafish at 5 dpf) (C,D′, DAPI in blue) (*n*=17 transplantation events). (E) Transcriptomes of medaka (top, *n*=2), zebrafish (middle, *n*=2) and zebraka (bottom, *n*=3) plotted along the zebrafish genome. The zebrafish cells in medrafish display retinal identity (*vsx2* and *rx1*, left two panels) and no cardiac gene markers (*cmlc1* and *cmlc2*, right panels). Scale bars: 100 µm. D, donor; H, host.

Ectopic retinal differentiation follows a genetic timer

The onset of retinal neurogenesis in medaka and zebrafish occurs at different hours post-fertilization (hpf). We decided to use the inter-species chimeras as a paradigm to address developmental timing, i.e. to explore whether retinal neurogenesis follows an intrinsic temporal program (genetic timing) or whether it responds to signals from neighbouring tissues operating as temporal coordinators (ontogenic timing). Using the transcriptome data that we obtained from zebrakas at 48 hpf, we aimed to analyse the relative expression of progenitor and neurogenic genes in the ectopic retinae. We used *rx3* and *vsx2* as retinal progenitor markers and *atoh7*, *ptf1a* and *pou42* as neurogenic/differentiation markers (Barabino et al., 1997; Jusuf and Harris, 2009; Kay et al., 2001; Loosli et al., 2003), and compared their expression ratios in zebrafish, medaka and zebrakas, using as a scaffold published transcriptomes from zebrafish and medaka at different developmental stages (Marlétaz et al., 2018). We noticed that ratios in the medaka transcriptome of zebraka match those of wild-type medakas, whereas ratios in the zebrafish transcriptome of zebrakas are consistently found closer to the zebrafish control (Fig. S4). We have extended this approach to include all retinal genes, by performing PCA analysis using zebrafish and medaka orthologues that are expressed in the retina and using ratios to *rx3* and *vsx2* as internal reference (Fig. 6A,B). Again, we observed that the medaka transcriptome from zebrakas (zebraka:medaka) groups with medaka controls. The zebrafish component of zebraka (zebraka:zebrafish), however, occupies a very different position, falling closer to the zebrafish control. These results indicate that, despite developing in a foreign species, a zebrafish retina in zebraka does not follow the differentiation pace occurring in the host but rather maintains its own differentiation dynamics, resulting in a premature generation of retinal neurons.

To confirm the genetic timing of retinogenesis in zebrakas *in vivo*, we performed transplantations using a Tg(*atoh7*:EGFP) zebrafish as a donor. Retinal ganglion cells (RGCs) represent the first cell type to differentiate in the vertebrate neural retina, and *atoh7* has extensively been used as a marker to follow the onset of RGCs in both zebrafish and medaka (Del Bene et al., 2007; Kay et al., 2005; Poggi et al., 2005; Souren et al., 2009). Fluorescent proteins under the control of an *atoh7* promoter can be detected at 26 hpf in zebrafish and 1 day later (50 hpf) in medaka, in progenitor cells that are poised to generate RGCs (Poggi et al., 2005; Souren et al., 2009). We transplanted transgenic Tg(*atoh7*:EGFP)(*ef1*:LynTomato) zebrafish blastocysts into unlabelled medaka blastula and observed the onset of EGFP expression by 26 hpf, ∼24 h before the expression of the endogenous

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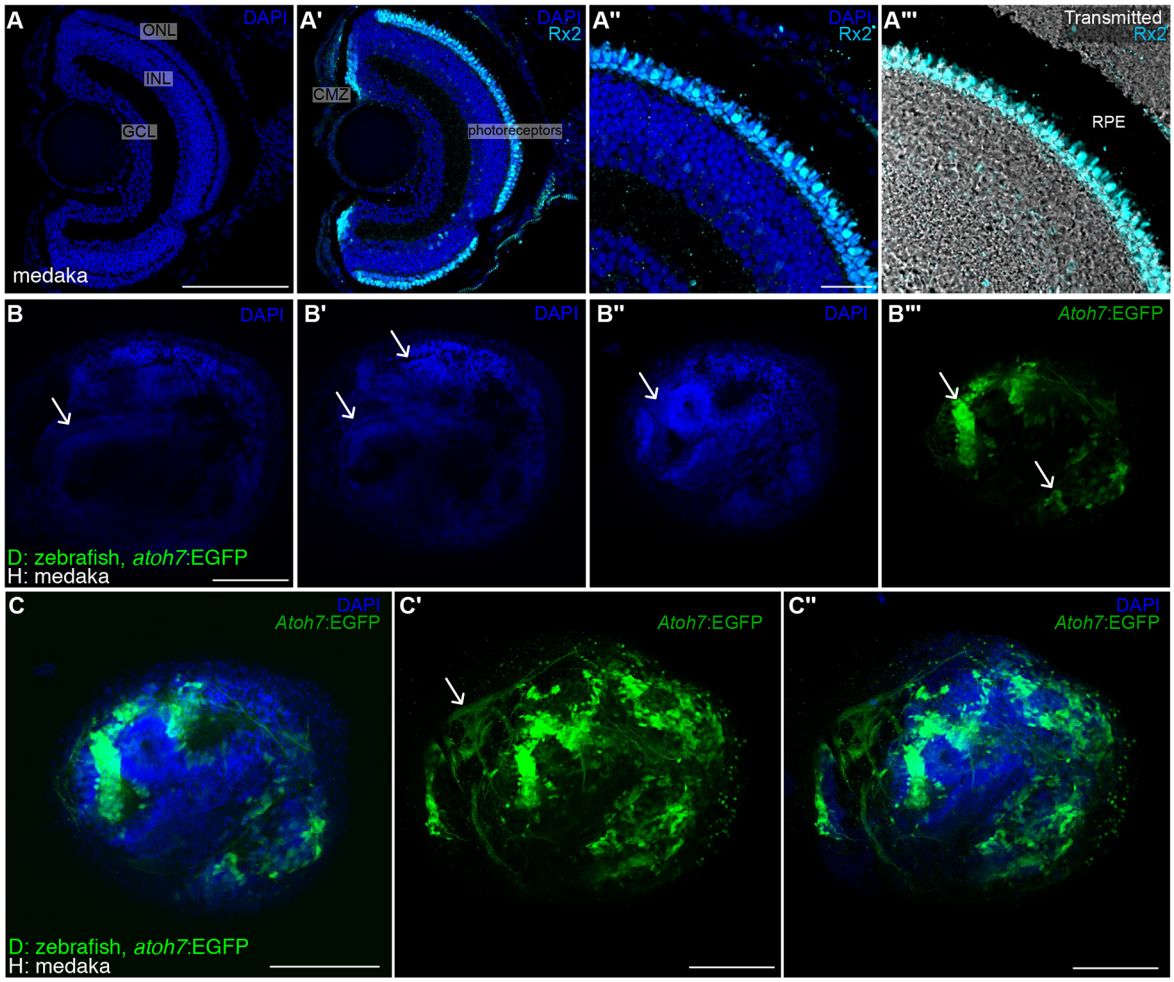

Fig. 4. Topological organisation of retinal ganglion cells in ectopic retina. (A-A‴) DAPI and immunostaining using an anti-Rx2 antibody on a medaka retina cryosection. Retinal ganglion cells localise to the ganglion cell layer (GCL) and Rx2+ photoreceptors (cyan staining in A′-A‴) are found in the outer nuclear layer (ONL), adjacent to the retinal pigmented epithelium (black region in the transmitted channel, A‴). (B-C″) DAPI staining of whole-mount retinae in zebrakas shows conspicuous layering (arrows in B,B′) and clusters of retinal ganglion cells (arrows in B″,B‴) labelled in green using a Tg(*atoh7*:EGFP) zebrafish donor. Single plane (C) and stack (C′,C″) showing RGCs and their axons (arrow in C′) in an ectopic zebraka retina (n=6 retinae in six chimeras). Scale bars: 100 µm in A,A′,B-C″; 20 µm in A″,A‴. GCL, ganglion cell layer; INL, inner nuclear cell layer; ONL, outer nuclear cell layer; CMZ, ciliary marginal zone; RPE, retinal pigmented epithelium; D, donor; H, host.

medaka *atoh7* (n=3) (Fig. 6C-C″). This result confirms and complements our transcriptome analysis, and indicates once again that the trigger for RGC generation follows a genetic timer irrespective of the timing of the neighbouring organs.

Extensive arborisation from zebrafish RGCs in the medaka optic tectum

The generation of premature RGCs by the ectopic EGFP+ cluster has consequences in the host that we revealed by analysing axon innervation of RGCs in zebrakas and medrafish. In vertebrates, RGC projections group in an optic nerve that migrates from the retinae to their target region in the brain: the visual cortex in mammals and the optic tectum in fish. In zebrafish and medaka, each retina projects an optic nerve to the contralateral optic tectum (Baier et al., 1996; Yoda et al., 2004). We noticed that, in zebrakas, where donor cells were either Tg(*atoh7*:EGFP) or Tg(*ßactin*::CAAX-EYFP), zebrafish RGCs generate axons that travel to the medaka optic tectum, despite the ectopic position of the retina (Fig. 6D,E). The RGC axons from one ectopic retina usually innervated both ipsi- and contralateral optic tecta (Fig. 6E). The premature birth of zebrafish RGCs guarantees that their projections arrive at the target tissue earlier than the endogenous optic nerve, which results in a substantial innervation of the medaka tecta by the zebrafish RGC projections (Fig. 6E). Following the same rationale, the medaka RGC projections in medrafish arrive at the zebrafish tecta later than the endogenous nerve, and therefore their impact is reduced (Fig. S5). These observations reveal the hetero-chronic formation of the same cell type within a chimeric embryo, following a genetic timing of differentiation despite sharing the physiological domain.

We observed examples in which the earlier formation of zebrafish RGCs resulted in anomalous axon projections in zebrakas, evidence that RGC projections can indeed hijack ectopic paths that are present at the time of navigation. That was the case for seven zebrakas – using either Tg(*ßactin*::CAAX-EYFP) or Tg(*atoh7*:EGFP) as donor (n=5 and n=2, respectively) – in which the RGCs projected along the lateral line nerve (Fig. 6F), which is present in the embryo before the pathfinding cues reach the optic tecta. These mis-projections usually reached the caudal fin, evidence of a promiscuous behaviour of the zebrafish RGC axons in medaka hosts. As these chimeras also all projected to the optic tecta (Fig. 6F), our interpretation is that the

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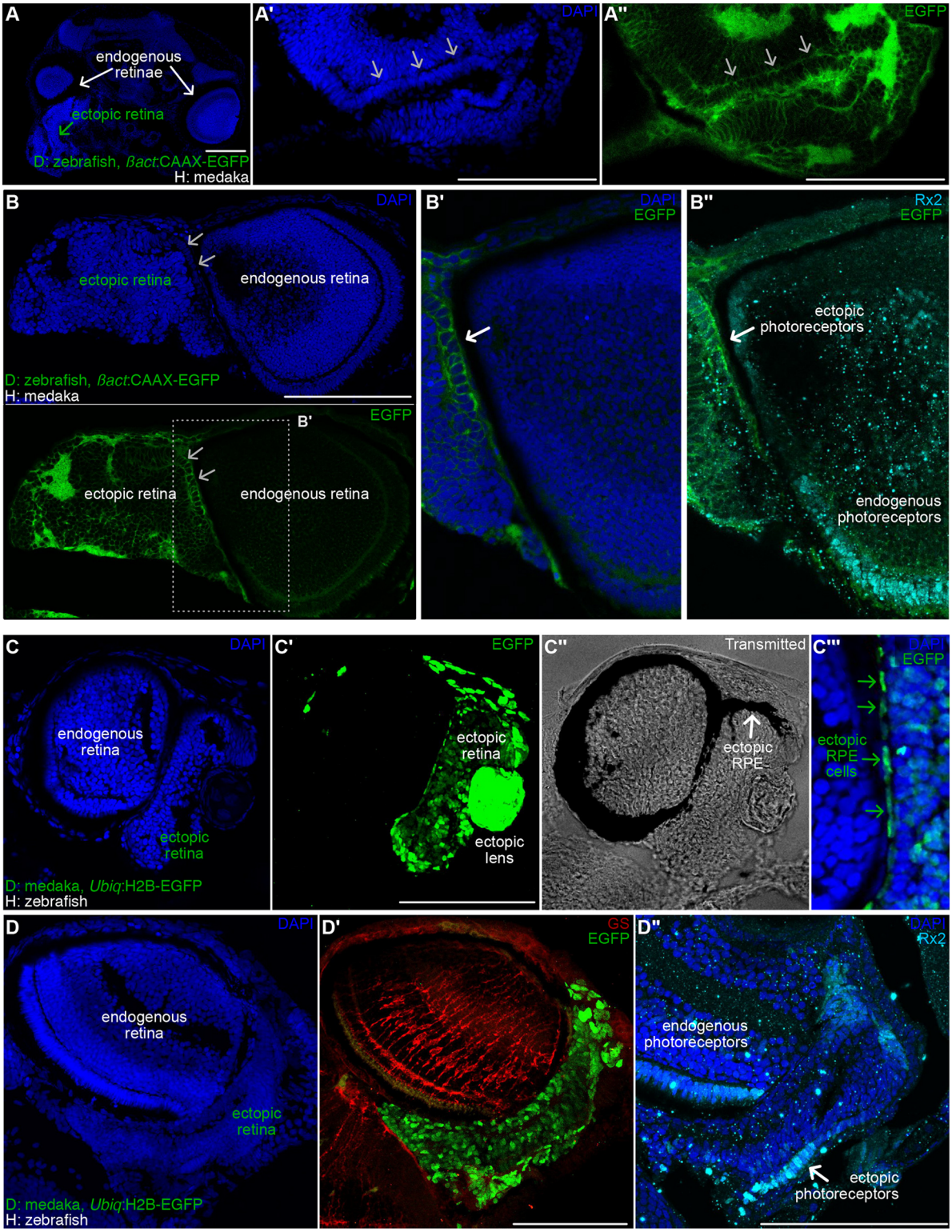

Fig. 5. Partial layering in the ectopic retinae of zebraka and medrafish. (A-B″) DAPI staining on cryosections of zebrakas using Tg(*ßact*:CAAX-EGFP) zebrafish as donors. (A) Cryosection of a transverse plane in a zebraka (dorsal is upwards, anterior is to the front) showing an ectopic retina (green arrow) ventrally adjacent to an endogenous retina (white arrow). Layering is evident in the ectopic retinae both by nuclear morphology (DAPI staining, arrows in A′ and B, top) and by membrane accumulation (CAAX-EGFP, arrows in A″, B, bottom, and B′) (*n*=6 ectopic retinae in 6 zebrakas). Immunostaining using anti-Rx2 Ab reveal photoreceptor identity of cells organised in layers (B″, white arrow) (*n*=3 zebrakas). (C-D″) DAPI staining (C,D) on cryosections of medrafish using Tg(*Ubiq*:H2B-EGFP) medaka as donors. (C′,D′) EGFP signal allows detecting ectopic cells. Transmitted channel (C″) analysis reveals ectopic RPE covering the dorsal part of the ectopic retina (arrow). Merged channels (C‴) showing colocalisation of elongated EGFP+ nuclei and pigmented epithelium (green arrows). (D′) Immunostaining using an anti-GS antibody reveals Muller glia in the endogenous retina and not in the ectopic retina (*n*=4 medrafish). (D″) Immunostaining using an anti-Rx2 antibody label ectopic photoreceptors (arrow) organised in a mononuclear layer (*n*=3 medrafish). Scale bars: 100 µm. D, donor; H, host.

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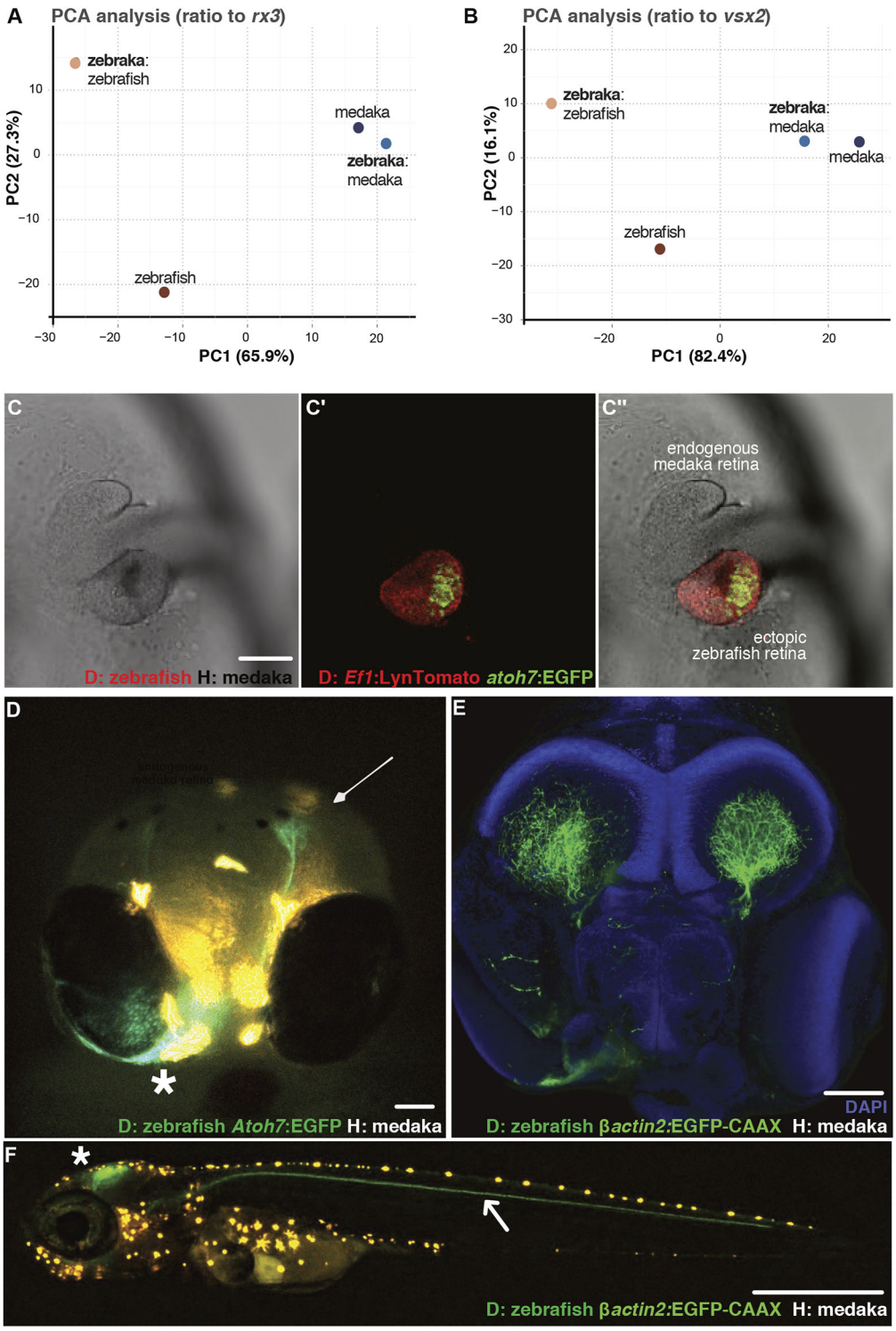

Fig. 6. Retinogenesis follows a genetic developmental time. (A,B) PCA analysis of zebrafish and medaka orthologues expressed in the eye during the 24-48 hpf zebrafish developmental time window. The values are FPKM ratios between retinal genes and retinal progenitor markers *rx3* (A) and *vsx2* (B). (C-C″) Confocal images of the anterior region in a zebraka where the donor is Tg(*Ef1*:LynTomato, *atoh7*:EGFP) (C, transmitted; C′, green and red channels; C″, merged image). EGFP expression in the transplanted cluster (C′,C″) is evident at the vesicle stage of the medaka host (*n*=3 chimeras). (D) Frontal view of a living 5 dpf zebraka where the donor is Tg(*Ef1*:LynTomato, *atoh7*:EGFP). Atoh7+ cells from the ectopic zebrafish retina (asterisk) migrate towards the endogenous medaka tectum (arrow) (*n*=3 transplantation events, *n*=6 chimeras). (E) Confocal image of a fixed 6 dpf zebraka where the donor is Tg(*Olßactin2*:EGFP-CAAX) ubiquitously labelling all donor cell projections (*n*=2 transplantation events, *n*=6 chimeras). The ectopic retina projects to both host tecti. (F) Confocal image of a living zebraka at 9 dpf. A nerve coming from the ectopic zebrafish retina (located in the contralateral side) navigates along the posterior lateral line nerve (arrow). Other projections from the ectopic retina, presumably from later RGCs, project to the tectum (asterisk) (*n*=4 transplantation events, *n*=7 chimeras). Scale bars: 100 µm in C-E; 1 mm in F. D, donor; H, host. Orange dots in D and F correspond to medaka pigments.

earlier development of the lateral line nerve might offer a permissive migratory route. Our results indicate that, even when medaka and zebrafish blastocysts do not intermingle during epiboly and axis formation, differentiated cells can later recognise cues present in the host as being necessary for axonal pathfinding.

Different sources for lens recruitment in zebraka and medrafish

The vertebrate eye is composed of the neuroepithelial derivatives, i.e. the neural retina (NR) and the retinal pigmented epithelium (RPE), which differentiate from a common progenitor pool (Holt et al., 1988; Poggi et al., 2005; Wetts and Fraser, 1988), and additional tissues in the anterior segment of the eye that derive from different germ layers (Soules and Link, 2005). The lens, a distinctive feature of the vertebrate eye, derives from the surface ectoderm and its formation is induced by retinal progenitors during early retinogenesis (Soules and Link, 2005). Lens induction therefore constituted yet another paradigm to assess inter-relations between donor and host tissues in chimeric embryos. When analysing the transcriptomes of zebrakas, we noticed that,

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although retinal genes were represented (Fig. 3E), lens transcripts from the donor were absent in the chimeras (Fig. 7A). This indicate that the lens that is evident in zebraka either expresses a different set of transcripts or, alternatively, is formed using cells from the host.

We performed blastula transplantations using EGFP+ blastomeres from zebrafish into the *cryA*:eCFP line from medaka, which labels the endogenous lenses with a cyan fluorescent protein (Centanin et al., 2014). When generated using this set-up, zebrakas display an ectopic zebrafish retina, the lens of which expresses the medaka *cry*:EGFP transgene (Fig. 7B-B″). This clearly indicates that zebrafish retinal cells recruit medaka host cells to form an ectopic lens. In the context of our previous observations showing that in zebrakas the ectopic retina develops earlier than the host retinae, our results demonstrate that the surface ectoderm has the potential to become a lens before the stage at which it is recruited endogenously in medaka. Surprisingly, this situation differs in the case of medrafish. When we transplanted blastomeres from a medaka *cryA*:ECFP, *Gaudi*$^{\text{LoxPOUT}}$ into unlabelled zebrafish host, we noticed that the ectopic retina displayed a cyan lens (Fig. 7C-C″, see also Fig. 5C′) (n=10 transplantation events). These results reveal that, in contrast to the case in zebrakas, the ectopic medaka neural retina does not induce lens formation from zebrafish surface ectoderm. Therefore, the EGFP+ medaka cluster in medrafish generates both the retina and anterior structures of the eye, such as the lens. It is therefore valid to speculate that, by the time the medaka retina starts the lens induction program, the surface ectoderm in zebrafish is no longer competent to acquire lens identity. Temporal windows for inductive process have been reported in other systems, e.g. the Hensen's node in chicken and the Spemann-Mangold organiser in frogs (Anderson and Stern, 2016; Hensen, 1876; Inagaki and Schoenwolf, 1993; Mangold and Spemann, 1927; Martinez Arias and Steventon, 2018; Spemann and Mangold, 2001; Storey et al., 1992; Waddington, 1934).

DISCUSSION

Trans-species transplantations have been long used in developmental biology to address the most diverse subjects. Examples of such an approach can be found almost one century ago, when Harrison, Twitty and colleagues transplanted eye cups and limbs between two salamander species of different sizes – the large *Ambysioma tigrinum* and the smaller *Ambysioma punctatum* (Harrison, 1929; Twitty and Schwind, 1928, 1931). Their experiments revealed an intrinsic (genetic) control of organ size and evidence for an organ intrinsic growth rate, as the transplanted rudiment (either eyes or limbs) reached the size of the donor species. An *A. tigrinum* eye transplanted into *A. punctatum* outgrows the endogenous eyes at the same rate as the undisturbed control organ in the donor species, while an *A. punctatum* eye transplanted into *A. tigrinum* will remain smaller than its endogenous counterparts (Twitty and Schwind, 1931). During the 1970s, Nicole Le Dourain pioneered the now classical quail-to-chick transplantation as a method for following the lineage of neural crest cells, using distinct nuclear properties between both species that were obvious under a light microscope (Le Douarin, 1973, 2004; Le Douarin and Teillet, 1973). Other examples where pieces of tissues were transplanted from one species to another include graft transplantations from *Planaria dorotocephala* to *Planaria maculata* to demonstrate the potency of regenerating cells (Santos, 1929, 1931) and grafting different plant species to address horizontal genome transfer and speciation (Fuentes et al., 2014).

Inter-species chimeras have also been generated at earlier developmental stages by mixing blastomeres. In fish, this approach has led to the characterisation of primordial germ cell

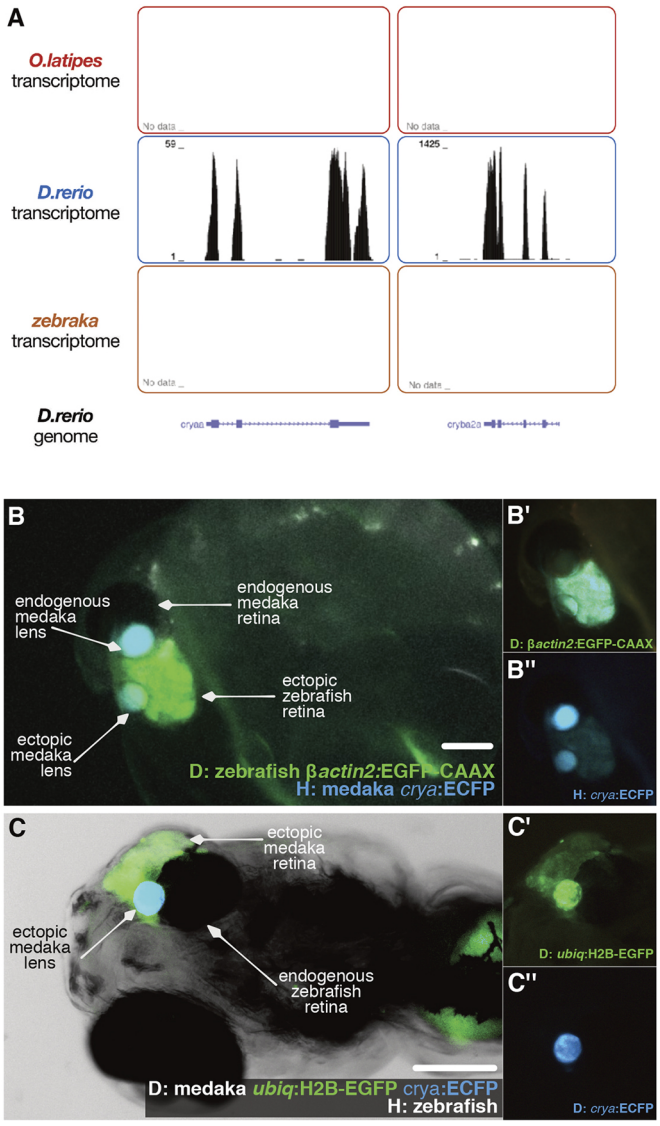

Fig. 7. Ectopic zebrafish retina in zebrakas recruits the lens from the medaka host. (A) Transcriptomes of medaka (upper, n=2), zebrafish (middle, n=2) and zebraka (bottom, n=3) plotted along the zebrafish genome. Lens zebrafish genes (*crystalin a* and *crystalin b a2a*: *cryaa* and *cryba2a*, respectively) are expressed in zebrafish but not in zebrakas. (B-B″) Lateral views of a zebraka where donor Tg(*OlBactin2*:CAAX-EGFP) cells were transplanted into a Tg(*crya*:ECFP) host. Merged (B) and single-channel (EGFP in B′, ECFP in B″) images. Cyan expression in the lens of the ectopic retina reveals its host origin (n=3 transplantation events, n=7 chimeras). (C-C″) Ventral views of a medrafish at 5 dpf, where donor Tg(*ubiq*:H2B-EGFP)(*crya*:ECFP) cells were transplanted into a non-labelled host. Merged (C) and single-channel (EGFP in C′, ECFP in C″) images. Cyan expression in the lens of the ectopic retina reveals its donor origin (n=10 transplantation events, n=23 chimeras). Scale bars: 100 µm. D, donor; H, host.

(PGC) migration to their respective gonad (Saito et al., 2010), providing a valuable resource for saving endangered species. Work using mammals include the early rat-to-mice blastomere transplantation (Gardner and Johnson, 1973), used more recently to address the 'empty niche' hypothesis (Kobayashi et al., 2010). Briefly, the authors used mutant hosts that were unable to generate a defined organ and showed that the transplanted donor blastomeres were able to colonise the empty niche and form the missing organ: a viable mouse with a rat pancreas (Kobayashi et al., 2010). Interestingly, and in contrast to the experiments by Harrison and

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Twitty mentioned earlier, the donor organ adjusts to the size of the host species – a rat pancreas within a mouse has the size of a mouse pancreas – revealing different strategies for organ control in different species; whether these differences are organ or species specific remains an unresolved issue. Inter-species chimerae were also generated using human cells, most famously via heterochronic transplantation into mouse to assess teratoma formation, but also by combining blastomeres with those of other species. Human-to-pig chimerae, for example, were used to explore developmental boundaries of pluripotent stem cell transplantations in different mammals (Wu et al., 2016, 2017). Besides the vast literature on inter-species transplantations, the topic of developmental timing was barely approached. A recent example involves transplanting ESC-derived cortical cells to assess the dynamics of neuronal differentiation, using human cortical neurons (where maturation takes months to years) transplanted into mice (where maturation takes a few weeks) (Linaro et al., 2019). The authors report that transplanted human neurons go through an extended maturation period compared with their mouse counterpart, retaining juvenile properties even when the host mouse was already an adult.

Here, we have developed a new experimental set-up complementary to the previously mentioned methods, where the entire developmental history of an organ unfolds in an alien species – from a blastomere to a retinal neuron. Notably, the entire process happens using host and donor species that are not genetically modified to facilitate or induce grafting. We combined blastomeres of species that diverged 250 My ago and observed that, although initially cells group – or stay grouped – in a species-specific fashion, later the exogenous cluster interacts with the biochemical and/or physical environment of the host. Albeit not participating in the host morphogenesis, the cluster expresses different retinal maker genes and generates retinal cell types. We exploited the fortuitous formation of a retina to explore intrinsic and extrinsic temporal properties of retinogenesis, i.e. the generation of defined cell types (tissue specific) and the interaction with the host for inductive and navigation processes (non-tissue-specific lens formation and optic nerve pathfinding, respectively). We found that the cluster could initially read signals from the host to trigger the retinogenesis program, as ectopic retinae were always found adjacent to one endogenous eye. Yet despite this interaction with the host, ectopic retinae follow a species-specific developmental timespan. On the molecular side, we combined fluorescent reporters, *in vivo* imaging and RNA-seq analysis to show that trans-species retinae were formed by reproducing the dynamics of the well-described transcriptional cascades responsible for retinogenesis in the host species. Finally, the heterochronic retina can still interact with different host tissues to induce the formation of a lens using the host surface ectoderm (in zebrakas) and read molecular cues from the host to navigate axonal outgrowth to the correct endogenous targets. Therefore, our experiments add to the growing evidence on autonomous organisation of biological systems (Eiraku et al., 2011; Sato et al., 2009; van den Brink et al., 2014), with a focus on developmental dynamics, and demonstrate an evolutionarily conserved compatibility between the ectopic retina and the host environment.

It has recently been suggested that, in mammals, developmental timing depends on different biochemical reactions (Matsuda et al., 2020), specifically on protein stability (Rayon et al., 2020), a kinetic parameter that has species-specific features. Whether the same holds true for an entire extrinsic organ developing in a different host species is an attractive hypothesis that needs to be formally tested. Regardless of the molecular nature of the intrinsic timing, our results illustrate the temporal window in which a tissue can be induced to transform into another (i.e. surface ectoderm into a lens), which proved to be far broader than the temporal requirements of embryonic development. Overall, our experiments report a successful chimerism between vertebrate species that differed more than 250 My ago, the highest distance for an isochronic transplantation in such an early embryonic stage. Using this novel inter-species paradigm to tackle temporal aspects of embryonic development, we could reveal some basic principles predicted by the modularity hypothesis (Raff, 1996). Thus, we show that the evolutionarily conserved 'retinal module' can be defined both by its intrinsic genetic identity and its external connectivity to neighbouring modules.

MATERIALS AND METHODS

Fish stocks and transgenic fish lines

Medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) stocks were maintained according to the local animal welfare standards (Tierschutzgesetz §11, Abs. 1, Nr. 1). Animal experiments were performed in accordance with European Union animal welfare guidelines (Tierschutzgesetz 111, Abs. 1, Nr. 1, Haltungserlaubnis AZ35-9185.64 and AZ35-9185.64/BH KIT).

The following zebrafish lines were used in this study: AB zebrafish strain as wild type, *OlBactin2*:EGFP-CAAX (Centanin et al., 2011), *Atoh7*:GFP (Del Bene et al., 2007) and *ef1a*:Lyn-Cherry.

The following medaka lines were used in this study: Cab strain as a wild type, Heino (Albino medaka), Wimbledon DsTrap#6 (Centanin et al., 2011), *rx2*::H2B-mRFP (Inoue and Wittbrodt, 2011), *zFli1*::EGFP (Schaafhausen et al., 2013), Gaudi^{RSG} (contains the integration reporter crya:ECFP that drives ECFP expression in the lens), Gaudi^{LoxPOUT} (Centanin et al., 2014), *Atoh7*::EGFP (Del Bene et al., 2007) and *Atoh7*::lynTdTomato (Lust et al., 2016).

Intra- and inter-species transplantation

Zebrafish crosses were set up at 10:00 a.m., collected after 20 min and kept at room temperature. Medaka couples were maintained together and produced from 8:00 a.m. on. Medaka eggs were collected between 9.00 and 10.30 a.m. and grown at 32°C to synchronize embryos at blastula stage. Blastula stage embryos were dechorionated as previously described using hatching enzyme for medaka and pronase (30 mg/ml) for zebrafish, and placed for transplantation in agarose wells with the proper medium for the host species (E3 medium for zebrafish or ERM for medaka).

One blastula could be used as a donor for three to five hosts, 20-50 cells were transplanted from the animal pole region of the donor to the host animal pole. Transplantations were carried out as previously described (Rembold et al., 2006). Transplanted embryos were kept in growth medium of the host species. In accordance with animal welfare standards, transplantations with zebrafish hosts were maintained up to day 5 of embryonic development and Medaka hosts were grown to day 9 of embryonic development.

Antibodies and staining

Primary antibodies used in this study were rabbit anti-GFP (Life Technologies, 1/750; A11122), chicken anti-GFP (Invitrogen, 1/750; A10262), rabbit α-DsRed (Clontech, 1/500; 632496), rabbit anti-Rx2 (1/200; non-commercial) (Reinhardt et al., 2015), mouse anti-GS (Millipore, 1/100; MAB302) and rabbit anti-Prox1 (Millipore, 1/100; AB5475). Secondary antibodies were Alexa488 anti-rabbit, Alexa546 anti-mouse and Alexa 647 anti-rabbit (Life Technologies, 1/500; A-11034, A-11030 and A-21245, respectively), and Dylight 488 anti-chicken, Dylight 549 anti-rabbit and Cy5 647 anti-mouse (Jackson, 1/500; 703-545-155, 112-505-144 and 715-175-151, respectively). DAPI was used at a final concentration of 5 µg/l. Cryosections were prepared as previously described (Reinhardt et al., 2015).

Imaging

Stained embryos were imaged with a laser-scanning confocal microscope Leica TCS SP8 (20× immersion objective) or a Leica TCS SPE. Imaging was carried out on glass-bottomed dishes (MatTek).

Live embryos were anaesthetised in 1 mg/ml tricaine in the respective fish medium as described by Seleit et al. (2017) and imaged in 3% methylcellulose in ERM or in 0.6% low melting agarose in ERM. Embryos were screened and imaged using a stereomicroscope (Olympus MVX10 MacroView) coupled to a Leica DFC500 camera or at a laser-scanning confocal microscope Leica TCS SP8 (20× immersion objective) or Leica TCS SPE. All subsequent image analysis was performed using Fiji software (Schindelin et al., 2012).

RNA-seq on zebrakas

Zebrakas with an EGFP+ cluster in the head were used to extract total RNA (Trizol) at 50 hfp, together with zebrafish (donor) and medaka (host) embryos that were grown at the same temperature (experiments carried out in triplicate). Libraries were prepared from total RNA followed by a polyA selection (NEBnext PolyA) and sequenced in a NextSeq 500 platform in 85 nt single end reads. The number of duplicates for both zebrafish and medaka samples is two, whereas zebraka experiment was performed in triplicate. Datasets have been deposited in GEO under accession number GSE150009. RNA-seq samples were mapped against both oryLat2 and danRer10 assemblies using Hisat2 (Kim et al., 2015). A summary of the number and percentage of mapped reads on both genomes can be found in Table S3. The aligned SAM files were assembled into transcripts and their abundance was estimated Cufflinks v2.2.1 (Trapnell et al., 2012). The DEG analysis between the zebrafish and zebraka transcriptome was also performed with Cufflinks v2.2.1. For downstream analyses, only the zebraka upregulated genes have been considered, in order to avoid a library size bias produced by the difference in number of mapped reads in the two datasets. GO enrichment was calculated with the tool FishEnrichr (Kuleshov et al., 2016). PCA analysis was performed using a subset of genes selected according the following criteria: (1) genes expressed in the eye between 24 h and 48 h in zebrafish retrieve from ZFIN database (Ruzicka et al., 2019); (2) genes with an expression value >10 FPKM in zebraka; and (3) genes sharing a direct orthologue in medaka. The final list of genes fulfilling all three criteria comprised 817 genes. The inter-species expression differences were normalized for the expression value of the progenitor markers rx3 (rax) and vsx2. Zebraka FPKM ratios between retinal developmental progression genes (*atoh7*, *ptf1a* and *pou4f2*) and retinal progenitor markers (*rx3* and *vsx2*) were compared with the same ratios from wild-type medaka and zebrafish transcriptomes at developmental stages resembling those described by Marlétaz et al. (2018). In the case where both the numerator and the denominator of the ratio were equal to zero, the resulting value was also reported as zero.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.C.; Methodology: J.F.F., L.C.; Software: L.B., J.R.M.-M.; Validation: J.F.F.; Formal analysis: J.F.F., L.B., L.A., J.R.M.-M., L.C.; Resources: J.R.M.-M., L.C.; Writing - original draft: L.C.; Writing - review & editing: J.F.F., J.R.M.-M., L.C.; Visualization: J.F.F., L.B., L.C.; Supervision: L.C.; Project administration: L.C.; Funding acquisition: J.R.M.-M., L.C.

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Data availability

RNA-seq data have been deposited in GEO under accession number GSE150009.

Supplementary information

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