Igf signaling couples retina growth with body growth by modulating progenitor cell division

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Summary statement
Targeted activation of Igf1r signaling in the retinal stem cell niche increases retina size through expanding the progenitor but not stem cell population.

Abstract
How the body and organs balance their relative growth is of key importance for coordinating size and function. This is of particular relevance in organisms, which continue to grow over their entire life span. We addressed this issue in the neuroretina of medaka fish (Oryzias latipes), a well-studied system to address vertebrate organ growth. We reveal that a central growth regulator, Igf1 receptor (Igf1r), is necessary and sufficient for proliferation control in the postembryonic retinal stem cell niche, the ciliary marginal zone (CMZ). Targeted activation of Igf1r signaling in the CMZ uncouples neuroretina growth from body size control, and we demonstrate that Igf1r operates on progenitor cells stimulating their proliferation. Activation of Igf1r signaling increases retinal size while preserving its structural integrity, revealing a modular organization where progenitor differentiation and neurogenesis are self-organized and highly regulated. Our findings position Igf signaling as key module for controlling retinal size and composition with important evolutionary implications.
Introduction

During embryonic development and postembryonic life of multicellular organisms, the proportions of overall body and organ size are actively controlled, being increased or decreased in a species-specific manner. Scaling of overall body and organ size is known to be regulated by systemic signals, which couple nutritional status to growth (Andersen et al., 2013). Conversely, differential growth of organs can be the result of altered sensitivity to systemic signaling (Tang et al., 2011) as well as of variant intrinsic signaling in the respective organ (Bosch et al., 2017; Twitty and Schwind, 1931).

Different eye dimensions of teleost species have been shown to be of functional relevance, since visual acuity is notably correlated with eye size (Caves et al., 2017). Vision and in particular retinal size and relative cell type composition are highly relevant for habitat-specific function and consequently for speciation. Deep sea fish retinae show striking structural differences to the retinae of surface-dwelling fish, accommodating their specific need of increased light sensitivity (Caves et al., 2017; Darwish et al., 2015; de Busserolles et al., 2014; Wagner et al., 1998). How retinal growth is regulated in different fish species to achieve differential eye sizes and function is not understood.

Teleost fish such as medaka (Oryzias latipes) and zebrafish (Danio rerio) are particularly appealing model systems for studying organ size scaling. They display life-long postembryonic growth and each organ harbors distinct stem cell populations, which continuously self-renew, generating progenitor and ultimately differentiated cells (Aghaallaei et al., 2016; Centanin et al., 2014; Furlan et al., 2017). In the eye, growth of lens, retina and cornea is governed by adjacent stem cell niches (Mochizuki et al., 2014; Nowell and Radtke, 2017; Raymond Johns, 1977), and the precise regulation of continuous growth is of utmost importance to ensure the functional consistency of optical parameters such as photoreceptor density, matching of the focal point, photoreceptor to ganglion cell convergence and therefore vision.
Retinal growth in teleosts and amphibians is coordinated by the neuroretinal stem cell niche, the ciliary marginal zone (CMZ) (Hollyfield, 1971; Johns and Easter, 1977; Raymond Johns, 1977; Straznicky and Gaze, 1971). Two proliferating cell populations in the CMZ are responsible for the majority of postembryonic neurogenesis: multipotent, slowly dividing self-renewing stem cells located at the outermost periphery of the CMZ, and rapidly dividing progenitor cells located closer to the differentiated retina (Centanin et al., 2014; Raymond et al., 2006; Tsingos et al., 2019; Wan et al., 2016). Both, stem and progenitor cells represent suitable cell type sources for altering retinal size, since they continuously proliferate and likely possess signal transduction machinery necessary for extrinsic signaling modulation. Which signals, and which of the two CMZ cell types coordinate retinal growth with overall body size could not be addressed due, in part, to the lack of cell-type specific drivers.

A central integrator of organ size within a growing organism is hormonal signaling, translating extrinsic conditions such as nutrient availability into proportionate and coordinated growth (Andersen et al., 2013; Boulan et al., 2015). The Insulin signaling pathway plays a central role in regulating embryonic development and growth and its key function is underlined by a single nucleotide polymorphism in insulin-like growth factor 1 (igf1) as causative alteration in small dog breeds (Sutter et al., 2007). Similarly, mutations in components of the Igf signaling pathway or their knockdown lead to severe dwarfism phenotypes from fish to humans (Baker et al., 1993; Klammt et al., 2008; Liu et al., 1993; Schlueter et al., 2007). Ultimately, final organ size is specified by systemic signals and their organ-specific interpretation (Shingleton and Frankino, 2018). The expression and localization of Igf pathway components during teleost embryonic and larval development has been assessed in a variety of species (Ayaso et al., 2002; Boucher and Hitchcock, 1998; Radaelli et al., 2003a; Radaelli et al., 2003b; Zygar et al., 2005). Consistently, Igf ligands and receptors (Igfr) were found in the developing and adult retina. Igf1 is expressed in the postembryonic goldfish retina, and its receptor is expressed in the CMZ and inner plexiform layer (IPL) (Boucher and Hitchcock, 1998; Otteson et al., 2002). In zebrafish, Igf1r-mediated signaling is required for proper embryonic development, especially of anterior neural structures, and inhibition results in reduced body size, growth arrest and developmental retardation (Eivers et al., 2004; Schlueter et al., 2007).
the expression of its pathway components in the CMZ, the role of Igf1r signaling in the teleost CMZ as well as the consequences of its alteration on size and shape of the continuously growing neuroretina have not been addressed.

In this study, we address the coordination of retinal size with body growth and show that they are coupled by Igf1r signaling in the postembryonic retinal stem cell niche of medaka. We find that global Igf inhibition reduces proliferation in the CMZ. Uncoupling Igf signaling between body and retina by targeted, constitutive activation of Igf1r specifically in stem and progenitor cells of the neuroretina increases proliferation, leads to an increase in eye size and uncouples retinal size from body size. Importantly, the resulting oversized retinae are structurally intact and correctly laminated with expanded thickness of the retinal layers, indicating that progenitor differentiation and neurogenesis are self-organized. We demonstrate that Igf1r activation decreases cell cycle length in the CMZ, expands the progenitor population and ultimately leads to increased neuronal cell numbers. By specifically dissecting the individual contribution of stem cells and progenitor cells to the oversized retinae, we uncover that retinal progenitor, but not stem cells integrate the modulation by Igf1r signaling.

**Results**

**Igf1r signaling regulates proliferation in the medaka CMZ**

The retinal stem cell niche is located in a continuous ring in the CMZ at the periphery of the teleost eye (Fig. 1A). To address the involvement of the Igf signaling pathway in regulating retinal stem and progenitor cell proliferation in the medaka retina, we first assessed expression of receptors and ligands. We find *igf1ra, igf2* and *insrb* expressed in the CMZ, and in specific layers of the differentiated retina (Fig. S1). The activity of receptor tyrosine kinases like Igf1r is mediated by ligand-induced conformational changes and subsequent trans-phosphorylation (Laviola et al., 2007). To assess whether Igf1r-mediated signaling is active in the CMZ, we detected the phosphorylated Igf1r (plgf1r) by immunohistochemistry. Single cells in the progenitor domain of the CMZ (around 2%, estimation based on quantification of Pcna-positive cells in Fig. 4 and Fig. S5) as well as Müller glia cells (MG) in the inner nuclear layer (INL) in the differentiated part of the retina were positive for plgf1r (Fig. 1B). The sparse distribution of plgf1r-positive cells likely reflects a snapshot of the highly
dynamic process of the activation and phosphorylation of receptor tyrosine kinases (Kiyatkin et al., 2020; Varkaris et al., 2013).

Igf1r expression and activity data in the CMZ are consistent with Igf signaling regulating proliferation in the CMZ to scale retinal size relative to the body. To determine the impact of Igf1r signaling on the proliferation of retinal stem and progenitor cells in the CMZ and on MG cells, we employed the widely-used Igf1r inhibitor NVP-AEW541 (Chablais and Jazwinska, 2010; Choi et al., 2013; Huang et al., 2013) and BrdU to detect proliferatively active cells that entered or went through S phase during the incubation time. While the NVP-AEW541 inhibitor displays a high specificity for Igf1r, an impact on Insr and other related kinases cannot be entirely ruled out (Attias-Geva et al., 2011; García-Echeverría et al., 2004). Fish at hatching stage (Iwamatsu stage 40 (Iwamatsu, 2004)) were incubated in 10 µM NVP-AEW541 or DMSO together with BrdU for 24 h, and analyzed subsequently by immunostaining against BrdU (Fig. 1C,D). Inhibitor incubation resulted in a 30% decrease of cells undergoing S phase in the CMZ (Fig. 1E), indicating that Igf/insulin signaling contributes to regulating proliferation in the CMZ. In the absence of MG cell proliferation in the differentiated retina, an impact of the inhibitor NVP-AEW541 was not detectable. Importantly, the 24 hour incubation with the Igf1r inhibitor did not impact on relative eye size (Fig. S2A), nor did average eye diameter or body length differ between inhibitor treated and control groups. Moreover, due to the global nature of the inhibitor assay, a non-cell-autonomous effect of this treatment cannot be excluded.

These results suggest that ligands and receptors of the Igf/insulin signaling cascade expressed in the CMZ contribute to CMZ proliferation.

**Constitutive activation of Igf1r in the CMZ results in increased neuroretinal size**

Our results indicate that Igf1r signaling represents a likely target to coordinate and modulate retina-specific growth. To further test this hypothesis, we established and employed tools for uncoupling Igf1r signaling in the neuroretina from the rest of the body by the precise and constitutive activation of Igf1r signaling in the CMZ.

Activation of the Igf signaling pathway was achieved by a constitutively active cd8a:igf1ra chimeric receptor (caigf1r). We targeted its expression to stem and progenitor cells of the CMZ by employing the rx2 promoter. The cd8a:igf1ra variant
was generated by an in-frame fusion of the extracellular and transmembrane domain of medaka \textit{cd8a} and the intracellular domain of medaka \textit{igf1ra}, as previously described (Carboni et al., 2005). The \textit{rx2} promoter drives expression in stem cells and early multipotent progenitors in the CMZ as well as in MG and photoreceptor (PRC) cells in the differentiated retina (Reinhardt et al., 2015). The potential of the \textit{calgf1r} fusion receptor to induce signaling transduction via the PI3K/Akt axis had been previously validated in several studies by assaying pAkt levels (Carboni et al., 2005; Gusscott et al., 2016; Pappano et al., 2009). Hence, we validated the functionality of the \textit{rx2::caigf1r} transgenic line and addressed the activation of the signaling cascade downstream of Igf1r in \textit{rx2}-positive cells by the phosphorylation status of its downstream effector Akt. In retinal sections of wildtype hatchlings, no pAkt staining was present in the peripheral domain in the CMZ (Fig. S2B). In contrast, \textit{rx2::caigf1r} retinae showed prominent pAkt staining in the CMZ, overlapping with \textit{caigf1r} expression in the \textit{rx2} domain (Fig. S2C), and \textit{rx2}-expressing MG and PRCs were also positive for pAkt. These results show that in the retina of \textit{rx2::caigf1r} transgenic fish, Akt is activated downstream of Igf1r in stem and progenitor cells in the CMZ as well as in MG and PRCs in the differentiated retina.

To address the potential of Igf1r signaling to modulate retinal growth we next examined hatchlings for changes in retinal morphology related to \textit{rx2::caigf1r} expression. Intriguingly, transgenic \textit{rx2::caigf1r} hatchlings displayed an oversized retina compared to wildtype siblings (Fig. 2A-C), while other body parts remained unaffected. Relative eye size (anterior-posterior eye diameter normalized to body length) was increased in \textit{rx2::caigf1r} hatchlings by 15\% (Fig. 2D, Table S1), persisting throughout postembryonic growth until adulthood, where relative eye size was enlarged by 9\% in \textit{rx2::caigf1r} fish (Fig. 2E, Table S2).

These data indicate that CMZ-targeted activation of Igf1r signaling uncouples retinal growth from overall body growth in medaka, disrupting the coordinated, Igf1r-mediated scaling of retina and body size.

Size increase of a tissue can arise due to different mechanisms of tissue expansion, such as increase in cell size or number, or stretching and increase in fluid or pressure (Ritchey et al., 2012; Stujenske et al., 2011; Veth et al., 2011). To understand how constitutive Igf1r signaling impacts on retina size, we examined cryosections of wildtype and \textit{rx2::caigf1r} hatchling retinae. \textit{Rx2::caigf1r} eyes exhibited a noticeable expansion of the neuroretina in all dimensions in contrast to
wildtype controls (Fig. 2F,G). Importantly, nuclear layer morphologies and arrangement indicated that the overall retinal architecture in *rx2::caigf1r* fish remained intact. The stereotypical structure of the neuroretina with the CMZ at the periphery, and three nuclear and two plexiform layers in the differentiated part was undisturbed by retinal expansion.

We further assessed retinal architecture using the expression of *rx2* as a landmark. All cell types expressing *rx2* in wildtype are present in the oversized *rx2::caigf1r* retinae, both in the CMZ as well as centrally in MG and PRCs (Fig. 2F,G). To characterize the apparent neuroretinal expansion, we compared the neuroretinal thickness in the peripheral, fully laminated region in *rx2::caigf1r* and wildtype retinae, along a line perpendicular to the inner plexiform layer (IPL). Retinal column height was increased by 23-24% on average in the dorsal as well as the ventral retina (Fig. 2H). In addition, we measured height of the retinal column in central regions, reflecting the embryonic contribution of *rx2* expression in retinal progenitor cells to the oversized retinae. Column height of the central neuroretina in *rx2::caigf1r* hatchlings was increased by 15% compared to wildtype (Fig. 2H). However, the increase was even more pronounced in peripheral, CMZ-derived regions, arguing for a greater impact of the post-developmental contribution via growth mediated by the CMZ.

To further substantiate our hypothesis that the increased size of the neuroretina is predominantly mediated by CMZ-derived cells, we assessed the relative contribution of the differentiated, central retinal region by proliferation analysis. A 2 h BrdU incubation of wildtype and *rx2::caigf1r* hatchlings yielded very low numbers of BrdU-positive cells per µm² retinal area in both groups, with a slight elevation of proliferative MG and PRCs in oversized retinae reflecting a minor to negligible contribution to the apparent neuroretinal expansion (Fig. S3A-C). Moreover, we also excluded that, reduced apoptosis levels in the retina contributed to the size increase. TUNEL analysis revealed comparable numbers of apoptotic cells in the CMZ of wildtype and *rx2::caigf1r* hatchlings, and increased levels of TUNEL-positive cells in the central region of *rx2::caigf1r* retinae (Fig. S3D-F). This indicates that neither increased central proliferation nor decreased apoptosis are major contributors to cell number expansion in *rx2::caigf1r* retinae.
Taken together, these results show that the activation of Igf1r signaling in the CMZ increases retinal diameter and layer thickness, and the enlargement is achieved by a neuroretinal expansion through the increase in cell number rather than in cell size.

**Neuroretinal cell type composition is shifted toward INL neurons upon constitutive Igf1r activation**

The relative composition of retinal cell types reflects the functionality of the retina and differs considerably between teleost species. To elucidate whether in the oversized retinae cell type numbers were increased proportionally or whether cell type proportions were shifted, we analyzed the neuroretinal cell type composition in rx2::caigf1r and wildtype hatchlings in a 30 µm-wide column spanning the entire neuroretinal column in the fully differentiated region proximal to the CMZ (dashed frame in Fig. 3A-D).

Cell type identity was determined by shape and position or by cell type-specific markers. PRCs are unambiguously characterized by their stereotypic localization in the outermost of the three nuclear layers. Retinal ganglion cells (RGCs) and displaced amacrine cells (ACs) located in the ganglion cell layer (GCL) were classified as one group. In the outer nuclear layer (ONL), cone PRCs were identified as outermost nuclear layer, and rods as the inner layer of the PRC nuclei. Other retinal cell types were distinguished by immunostaining with cell type-specific markers such as GS, labeling MG, and Otx2, labeling bipolar cells (BCs) (Fig. 3A-D). Horizontal (HCs) and ACs in the INL were characterized as being Otx2-negative, together with their specific apicobasal localization and nuclear morphology (Fig. 3C,D). In the oversized retinae, the total cell number within the 30 µm-wide column was increased by 15% on average in rx2::caigf1r retinae, with a more prominent increase in the ventral (21%) compared to the dorsal (10%) retina (Fig. 3E). The most prominent shift in the proportion of cell types was detected for ACs (24%) and BCs (31%) (Fig. 3F), while GCL and PRC numbers were rather similar in rx2::caigf1r and wildtype retinae (Fig. 3F). Some differences in cell type number changes were discernible between dorsal and ventral retina, with most distinctive increases in ACs, MG and BCs dorsally (Fig. S4A) and ACs and BCs ventrally (Fig. S4B).

These results indicate that Igf1r signaling activation in the CMZ shifts the neuroretinal cell type composition toward the INL and specifically to AC and BC fate.
Igf1r signaling activation decreases cell cycle length in the CMZ

Igf1r signaling has been shown to influence cell cycle progression in different in vitro and in vivo models (Hodge et al., 2004; Schlueter et al., 2007). To determine the impact of the stem and progenitor cell-specific activation of Igf1r signaling in rx2::caigf1r on cell cycle duration, we next analyzed cell cycle and S phase length of the proliferating cells in the CMZ of wildtype and rx2::caigf1r hatchlings. To this end, we employed a dual pulse S phase labeling regime using BrdU and EdU (Das et al., 2009; Klimova and Kozmik, 2014). Hatchlings were incubated in BrdU for 2 h, washed, and incubated in EdU for 30 min before analysis. BrdU, EdU and Pcna staining (Fig. 4A,B) allowed to quantify fractions of cells positive for one or more marker, allowing to derive cell cycle and S phase length. In the oversized rx2::caigf1r retinas, the cell cycle length was reduced from 12 h to 10.5 h on average (Fig. 4C), whereas the S phase length remained constant with an average duration of 4.5 h (Fig. 4D). This had a drastic impact on the number of BrdU-positive cells in the CMZ, which was more than doubled in rx2::caigf1r compared to wildtype fish (Fig. 4E), a fact that is also confirmed by the analysis of the EdU- and Pcna-positive cells (Fig. S5A,B). The ratio of BrdU- to Pcna-positive cells increased from 50% in wildtype to 57% in rx2::caigf1r hatchlings in the dorsal CMZ, and from 55% to 60% in the ventral CMZ, indicating shortened G phases in the rx2::caigf1r CMZ.

Taken together, these data show that in rx2::caigf1r hatchlings, more cells in the CMZ progress faster through the cell cycle, thereby expanding retinal cell numbers resulting in oversized retinas with shifted cell type proportions and increased height of the neuroretinal column.

Cndp is a bona fide marker specific for neuroretinal stem cells

Differential responses of retinal stem and progenitor cells to extrinsic stimuli have been previously described (Centanin et al., 2014; Love et al., 2014). Given that an Igf signaling-mediated proliferative trigger in the combined stem and progenitor cell domain impacts on retinal size, we addressed the individual contribution of the stem and progenitor cell populations to the scaling response. This required to establish a retinal stem cell-specific driver since such a marker with the required specificity was not available for any vertebrate system. Exploiting existing resources for gene expression patterns we focused on the cytosolic non-specific dipeptidase zgc:114181 (hereafter cndp), which had been identified in a systematic in situ screen.
of full length medaka cDNAs (Alonso-Barba et al., 2016) and showed a promising expression pattern at the retinal periphery.

To establish specific tools and address the expression of *cndp* relative to *rx2* we established transgenic reporter lines carrying putative regulatory regions driving a membrane-coupled GFP or nuclear mCherry reporter. The 5 kb genomic region upstream of the *cndp* coding region was sufficient to recapitulate the mRNA expression pattern in transgenic reporter lines (*cndp::eGFP-caax, cndp::H2A-mCherry*) in the retina (Fig. 5A, Fig. S6A-D) as well as the choroid plexi in the brain (Fig. S6A-D). In the retina, *cndp*-positive cells were found exclusively in a small, peripheral subset of the *rx2*-positive domain in the CMZ (Fig. 5A), by position likely representing retinal stem cells. To address the potency of those putative retinal stem cells and validate their nature (stem or progenitor cells) we employed a lineage analysis tool established in the lab that allows to determine the potency of cells (Centanin et al., 2014). In brief, activation of Cre recombinase specifically expressed in *cndp*-positive cells was used to trigger a permanent switch in reporter gene expression in those cells and all of their descendants. Stem cells and progenitor cells can be distinguished by their proliferative capacity. In the assay, stem cells are resulting in continuous stripes (ArCoS) of labeled descending cells always connected to the CMZ. In contrast, progenitor cells, due to their limited proliferative capacity, result in footprints of labeled cells that are separated from the CMZ. We used the *cndp* promoter (Fig. 5B) characterized above to drive the expression of a Tamoxifen-inducible Cre<sub>ERT2</sub> and combined it with the switchable GaudíRSG reporter line (Centanin et al., 2014). A permanent switch of reporter expression from mCherry to GFP was triggered by the tamoxifen-induced activation of nuclear Cre activity. We characterized the distribution, continuity and cell type identity of the clonally labeled progeny to conclude the nature of the *cndp*-positive mother cell.

GaudíRSG embryos injected with the *cndp::Cre<sub>ERT2</sub>* plasmid at 1-cell stage were induced with Tamoxifen at hatchling stage. After 2-3 weeks, fish were analyzed for GFP expression by whole-mount immunostaining (Fig. 5C). Retinae displayed GFP-positive clones that all originated in the peripheral CMZ and were continuous to the differentiated retina (Fig. 5D,E). This indicates that *cndp*-positive cells are neuroretinal stem cells. Clones comprised cells of all three nuclear layers (Fig. 5F), demonstrating multipotency of their cells of origin (Centanin et al., 2014). Importantly, we never observed clonal footprints originating from progenitor cells.
This establishes *cndp* as a *bona fide* marker for neuroretinal stem cells and an invaluable tool to dissect stem versus progenitor cell behavior.

**Activation of Igf1r signaling in the CMZ preferentially increases progenitor cell numbers**

To address whether stem or progenitor cell populations are expanded in response to activated Igf1r signaling we took advantage of the stem cell-specific expression of *cndp* and used *rx2* as a marker for stem and progenitor cells (Fig. 6A). Retinal stem cells were identified by expressing the *cndp::H2A-mCherry* reporter in a stable transgenic line and retinal stem and progenitor cells were identified by an antibody specific for Rx2 (Reinhardt et al., 2015). Numbers for retinal stem and progenitor cells were determined in hatchlings of wildtype as well as *rx2::caigf1r* retinae harboring the *cndp::H2A-mCherry* reporter (Fig. 6B,C).

Numbers of *cndp*-positive stem cells were comparable in wildtype and *rx2::caigf1r* retinae, ranging from 3 to 11 in the dorsal and 0 to 8 stem cells per section in the ventral CMZ. The number of *cndp*-positive stem cells was rather stable, with a slight increase in *rx2::caigf1r* retinae (Fig. 6D). In contrast to that, the Rx2 domain was prominently expanded in *rx2::caigf1r* hatchlings (Fig. 6E,F), with Rx2-positive stem and progenitor cells in the CMZ more than doubled in *rx2::caigf1r* versus wildtype retinae (Fig. 6G), arguing that the progenitor, but not the stem cell population is expanded by Igf1r signaling activation. In line with this observation, plgf1r staining indicative for the activation of the Igf signaling cascade was only detected in progenitor cells, but not in *cndp*-positive stem cells (Fig. S6E,F).

Since retinal stem cell numbers in *rx2::caigf1r* hatchlings were rather comparable with wildtype, we next wanted to understand if retinal stem cells respond to the modulation of Igf1r signaling. We generated a transgenic line in which activation of Igf1r signaling specifically targeted to *cndp*-positive retinal stem cells (*cndp::caigf1r*). Functionality of the construct was evident through enlarged choroid plexi in *cndp::caigf1r* fish (Fig. S7A,B). However, the GFP expression domain in the retina was unaltered (Fig. S7C,D). We examined relative eye size in hatchling and adult *cndp::caigf1r* fish, and neither stage displayed an alteration in relative eye size compared to wildtype siblings (Fig. S7E,F). These results demonstrate that the retinal stem cell population expressing *cndp* does not expand upon the activation of Igf1r signaling, also not by accumulation over time in adult stages. This further
confirms and refines our findings that the progenitor but not stem cell population is expanded in *rx2::caigf1r* retinae. Taken together, we demonstrate that retinal growth can be uncoupled from overall body growth through the activation of Igf1r signaling targeted to the CMZ. This intrinsic modulation elicits differential responses in stem and progenitor cell populations in the medaka retina, leading to a shortened cell cycle and consequential increase of retinal progenitor but not stem cell number (Fig. 6H, I).

**Discussion**

In this study, we investigated how organ growth is coordinated with overall body growth in medaka by uncoupling growth control in the retina versus body. We focused on the retina and dissected the role of Igf1r signaling in regulating proliferation of stem cells versus progenitor cells in the retinal stem cell niche and its impact on retina size and composition at postembryonic stages. We show that proliferation in the retinal stem cell niche is dependent on and triggered by Igf1r-mediated mitogenic signaling. Combining expression analysis, inhibitor assays and gain-of-function approaches, we show that the Igf pathway is functioning in the postembryonic CMZ where it actively controls proliferation. Our analyses indicate a localized paracrine signaling hub, with Igf1r activity in single progenitors in the CMZ. The inhibition of Igf1r signaling results in a decreased proliferation of CMZ cells. Conversely, the targeted constitutive activation of Igf signaling in the CMZ results in a clear increase of retina size and a shift in relative composition of the retinal layers. Strikingly, this is due to a specific response of the progenitor cells, while the proliferation of stem cells is not affected. Progenitor cells speed up their cell cycle without affecting the subsequent differentiation potential in response to Igf1r signaling activation (Fig. 6I). The specific activation of Igf signaling results in an increased proliferation rate in the progenitor pool that in turn causes a shift in retinal layer composition with the potential to impact on functional optical parameters.

Upon activation of Igf1r signaling in the CMZ, the thickness of the neuroretina increases by a quarter. Intriguingly, the enlarged retinae are structurally intact displaying correct lamination and differentiation. While retinal enlargement due to enhanced progenitor proliferation might be an expected phenotype, the resulting
intact retinal lamination with shifted cell type composition indicates a high degree of self-organization depending on the number of progenitor cells. The specific action of the Igf/insulin pathway as signal integrating nutritional status (Boulan et al., 2015) opens the field for interesting evolutionary hypotheses. In response to an Igf1r-mediated proliferative trigger in the progenitor cell domain higher retinal columns and thus an extended light path are generated. Coordinated changes in relevant optical parameters such as light path length, lens size and photoreceptor density might therefore increase visual acuity. Thus, a signal representing nutrient availability can prompt an immediate selective advantage when preferentially acting on the retina and accessory tissues.

In the teleost retina, several populations of progenitor cells exist that differ in their proliferative and lineage capacities. Early progenitor cells in the CMZ are known to undergo self-renewing divisions generating two progenitor cells (PP), while old progenitor cells favor differentiating divisions (Wan et al., 2016). Among these are different lineage-specified progenitors which have been shown to produce different sets of cell type ratios upon modification of their transcriptional signatures (Pérez Saturnino et al., 2018). The activation of Igf1r signaling elicits two major changes in progenitor cells: first, increased numbers of Pcna- and rx2-positive cells indicates an increase in self-renewing PP divisions. Second, a pronounced increase in ACs and BCs suggests that Igf1r activation might either convey certain cell fates or favor expansion of a specific progenitor population lineage-committed to generate INL cells.

We showed that the uncoupling of Igf signaling in the retina by CMZ-specific constitutive activation of Igf1r signaling preferentially expands the rx2-positive progenitor but not the stem cell population. Targeting stem cells by specific Igf1r activation in cndp-expressing cells does not impact on retinal size, indicating that retinal stem cells do not respond to Igf1r signaling with an immediate and long-lasting increase in proliferation, nor could a cumulative effect be observed in adult fish. Conversely, progenitor cell numbers are more than doubled in response to constitutive activation of Igf1r signaling, rendering the rx2-positive progenitor population receptive for this mitogenic stimulus. Differential responses of stem and progenitor cells have been observed in the opposite direction after nutrient deprivation in the Xenopus retina. While stem cells are resistant to nutrient
deprivation and mTOR inhibition, retinal progenitors respond with changes in proliferation and differentiation in an mTOR-mediated manner (Love et al., 2014). These differences between retinal stem and progenitor cells are complementary to our observations where stem cells appear resistant to the Igf1r-mediated mitogenic stimulus, while progenitor proliferation is increased. Interestingly, outside of the retina, divergence at the level of stem and progenitor cell behavior has been proposed as the basis for varying brain sizes in different amniote species (Nomura et al., 2013). Studies in geckos have proposed that progenitor cells are key for the evolutionary scaling of brain size in amniotes. The lower cell division rates of progenitor cells, their longer cell cycle and the predominance of self-renewing progenitor cell divisions are likely responsible for the comparatively smaller size of the gecko brain (Nomura et al., 2013).

Enlargement of the eye had been reported in few experimental and mutant conditions. While the overexpression of Yap by mRNA injection could increase eye size in *Xenopus* embryos (Caboche et al., 2015), retinal morphology and lamination were disturbed in those Yap overexpressing tadpoles (Caboche et al., 2015). Increase in retinal size has also been observed in a *patched2* mutant in zebrafish where retinal patterning and morphology are largely intact, but the number of MG cells is reduced (Bibliowicz and Gross, 2009). In the CMZ of *patched2* mutant embryos, the number of retinal progenitor cells is increased, but their cell cycle length is unaffected (Bibliowicz and Gross, 2009). We observed that in response to active Igf1r signaling, the overall cell cycle was shortened without affecting S phase length, in agreement with results from various systems where Igf signaling influences G1 or G2 phase length (Hodge et al., 2004; Schlueter et al., 2007; Wang et al., 2015). The observed shortened cell cycle length of retinal progenitor cells of the oversized retinas is likely causal for the expansion of the CMZ and ultimately the entire retina in the *rx2::caigf1r* fish.

In contrast to eye size expansion resulting from increased retinal cell numbers, zebrafish and goldfish *bugeye* mutants exhibit enlarged eyes due to increased intraocular pressure (Kon et al., 2020; Stujenske et al., 2011; Veth et al., 2011). The *bugeye* phenotype is adult-onset and shows retinal cell density decrease and thinning of all nuclear layers. Moreover, *bugeye* mutants display relative refractive errors concordant with myopia due to vitreous chamber expansion between lens and
retina (Veth et al., 2011), which displaces the retina behind the point at which the lens focuses light. Interestingly, the expansion of the eye in adult fish did not impact on the size of the lens in those mutants. We also did not observe an increase in lens size in rx2::caigf1r fish compared to wildtype siblings, indicating that there is no paracrine signal emanating from the neuroretinal niche in response to elevated Igf signaling to coordinate the size of the lens. Those data suggest an independent size control of retina and lens, as opposed to our previous results on neuroretina and retinal pigmented epithelium coordination, where behavior of neuroretinal stem cells in the CMZ drives retinal shape and impacts on retinal pigmented epithelium stem cells that passively follow (Tsingos et al., 2019). Uncoupling neuroretinal Igf signaling from the rest of the body as described here provides indication toward independent size control of retina and lens already during development and postembryonic growth. Expression of insulin receptor and IGF1 receptor at high levels at the lens periphery in embryonic and P0 mice (Xie et al., 2007) as well as proliferation of lens epithelial cells upon IGF1 treatment in rat lens epithelial explants (Iyengar et al., 2006) indicates however that lens growth and size control could be modulated by Igf signaling as well. In the future, it will be important to address whether Igf signaling impacts on lens germinal cell proliferation in the medaka retina and whether Igf signaling contributes to the regulation of lens and retina sizes to achieve a continuous functional consistency during the life-long growth of the eye.

The targeted, isolated size increase of the retina and the concomitant change in cell type composition opens a wide area for discussing the evolutionary and ecological significance of coordinating retinal size and architecture. Throughout the teleost clade, adaptations to specific habitats and niches are evident in the retinal architecture. Surface-dwelling fish such as medaka have two layers of PRCs, one light-sensitive rod and one cone layer responsible for color vision, while zebrafish possess one layer of cones as well as three to four layers of rods for enhanced light perception, as they live in deeper waters (Lust and Wittbrodt, 2018). One particularly interesting example of retinal architecture changes is the „four-eyed“ fish Anableps anableps, which displays structural differences within the dorsal and ventral retina to accommodate its specific optic requirements. The ventral retina features an INL that is twice as thick as the dorsal INL, concordant with increased proliferation in the ventral compared to the dorsal CMZ during the development of the larval eye (Perez...
et al., 2017). Since we observe a preferential increase in the INL neuron population in \textit{rx2::caigf1r} it is tempting to speculate that an altered proliferation of progenitor cells (initiated by altered Igf signaling) in the ventral CMZ contributes to manifesting the structural differences in the \textit{Anableps anableps} retina.

We speculate that Igf signaling can act as an evolutionary handle, through which retinal size, morphology, cell type composition and consequently function can be adapted by modifying signaling activity in distinct populations of progenitor cells in the CMZ and beyond.

\textbf{Materials and Methods}

Animals and transgenic lines

Medaka (\textit{Oryzias latipes}) used in this study were kept as closed stocks at Heidelberg University. All experimental procedures and husbandry were performed in accordance with the German animal welfare law and approved by the local government (Tierschutzgesetz §11, Abs. 1, Nr. 1, husbandry permit AZ 35–9185.64/BH and line generation permit AZ 35–9185.81/G-145-15). Fish were maintained in a constant recirculating system at 28°C on a 14 h light/10 h dark cycle. The following stocks and transgenic lines were used: wildtype Cabs, \textit{Heino} mutants (Loosli et al., 2000), \textit{rx2::caigf1r rx2::lifeact-eGFP, cndp::H2A-mCherry, cndp::eGFP-caax, cndp::H2B-eGFP, cndp::caigf1r cndp::H2B-eGFP, cndp::Cre\textsuperscript{ERT2}, GaudiRSG} (Centanin et al., 2014). All transgenic lines were created by microinjection with Meganuclease (I-SceI) in medaka embryos at the one-cell stage, as previously described (Thermes et al., 2002).

The constitutively active Igf1r variant (Cd8a:Igf1ra, calgf1r) was generated by an in-frame fusion of the codon-optimized extracellular and transmembrane domain of olCd8a (synthesized by Geneart) and the intracellular domain of the medaka Igf1ra, as previously described (Carboni et al., 2005). To generate the \textit{rx2::caigf1r rx2::lifeact-eGFP} line, two F0 fish were crossed, and each subsequent generation was derived from one transgenic male outcrossed to wildtype Cabs. All experiments were done in the F2 to F4 generations.
The *cndp::Cre\(^{ERT2}\)* plasmid (*cndp* Ensembl ID: ENSORLG00000003701) was generated by cloning the 5 kb *cndp* regulatory region in a pBS/I-SceI-vector containing a tamoxifen-inducible Cre recombinase. The plasmid contains *cm1c2::eCFP* as insertional reporter.

**BrdU/EdU incorporation**
For BrdU incorporation, hatchlings (stage 40 (Iwamatsu, 2004)) were incubated in 2.5 mM BrdU (Sigma-Aldrich) diluted in 1x embryo rearing medium (ERM, 17 mM NaCl, 40 mM KCl, 0.27 mM CaCl\(_2\), 0.66 mM MgSO\(_4\), 17 mM Hepes) for 2 h. For EdU incorporation, hatchlings were incubated in 250 μM EdU (ThermoFisher) diluted in 1x ERM for 30 min. Quantification of BrdU-positive cells was performed in four retinae from individual hatchlings. Cell counts were performed in z = 6 μm of two to three central sections per retina.

**Igf1r inhibition**
For inhibition of Igf1r, hatchling fish were incubated in 10 μM NVP-AEW541 (Selleckchem, 10 mM stock solution solved in DMSO) diluted in 1x ERM at 28°C for 24 h. In a parallel control group, hatchling fish were incubated in 0.1% DMSO/1x ERM at 28°C for 24 h. Directly afterwards fish were euthanised and fixed for analysis.

**Induction of Cre/lox system**
For Cre\(^{ERT2}\) induction, hatchlings were treated with a 5 μM tamoxifen solution (Sigma-Aldrich) in 1x ERM overnight.

**Immunohistochemistry on cryosections**
Fish were euthanised using 20x Tricaine and fixed overnight in 4% PFA, 1x PTW at 4°C. After fixation samples were washed with 1x PTW and cryoprotected in 30% sucrose in 1x PTW at 4°C. To improve section quality, the sections were incubated in a half/half mixture of 30% sucrose and Tissue Freezing Medium for at least 3 days at 4°C. 16 μm thick serial sections were obtained on a cryostat. Sections were rehydrated in 1x PTW for 30 min at room temperature. Blocking was performed for 1-2 h with 10% NGS (normal goat serum) in 1x PTW at room temperature. The
respective primary antibodies were applied diluted in 1% NGS o/n at 4°C. The secondary antibody was applied in 1% NGS together with DAPI (Sigma-Aldrich, D9564; 1:500 dilution in 1x PTW of 5 mg/ml stock) for 2-3 h at 37°C. Slides were mounted with 60% glycerol and kept at 4°C until imaging.

BrdU and Pcna immunohistochemistry on cryosections
BrdU and Pcna antibody staining was performed with an antigen retrieval step. After all antibody stainings and DAPI staining, except for BrdU/Pcna, were complete, a fixation for 30 min was performed with 4% PFA. Slides were incubated for 1.5 h at 37°C in 2 N HCl solution, and pH was recovered by washing with a 40% Borax solution in 1x PTW before incubation with the primary BrdU or Pcna antibody.

EdU staining on cryosections
EdU staining reaction was performed after all other antibody stainings were completed using the Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit according to manufacturer’s protocol (Thermo Fisher).

TUNEL staining on cryosections
TUNEL staining was performed after all other antibody stainings were completed using the In Situ Cell Death Detection Kit Fluorescein from Roche. Staining was performed according to manufacturer’s protocol with the following modification: washes were performed with 1x PTW instead of PBS.

Immunohistochemistry on whole-mount retinae
Fish were euthanised using 20x Tricaine and fixed overnight in 4% PFA in 1x PTW at 4°C. After fixation, samples were washed with 1x PTW. Fish were bleached with 3% H₂O₂, 0.5% KOH in 1x PTW for 2-3 h in the dark. Retinae were enucleated and permeabilised with acetone for 15 min at −20°C. Blocking was performed in 1% bovine serum albumin (Sigma-Aldrich), 1% DMSO (Roth/Merck), 4% sheep serum (Sigma-Aldrich) in 1x PTW for 2 h. Samples were incubated with primary antibody in blocking buffer overnight at 4°C. The secondary antibody was applied together with DAPI in blocking buffer overnight at 4°C. Primary antibodies were used at 1:200, secondary antibodies at 1:250 and DAPI at 1:500.
Antibodies

The following primary antibodies were used: anti-BrdU (rat; Abcam, ab6326; 1:200), anti-DsRed (rabbit; Clontech, 632496; 1:500), anti-eGFP (chicken; Life Technologies, A10262; 1:500), anti-GS (mouse; Chemicon, MAB302; 1:500), anti-Otx2 (goat; R&D systems, AF1979; 1:200), anti-pAkt (rabbit; Cell Signaling, 4060; 1:200), anti-Pcna (mouse; Millipore, CBL407; 1:100), anti-plgf1r (rabbit; Abcam, ab39398; 1:100), anti-Rx2 (rabbit, (Reinhardt et al., 2015); 1:500), anti-Zpr-1 (mouse; ZIRC, ZDB-ATB-081002-43; 1:200). The following secondary antibodies were used (at 1:750): anti-chicken Alexa Fluor 488 (donkey; Jackson, 703-485-155), anti-goat Alexa 633 (donkey; Life Technologies, A-21082), anti-mouse Alexa 546 (goat; Life Technologies, A-11030), anti-mouse Alexa 647 (donkey; Jackson, 715-605-151), anti-rabbit Alexa Fluor 488 (goat; Life Technologies, A-11034), anti-rabbit DyLight549 (goat; Jackson, 112-505-144), anti-rabbit Alexa Fluor 647 (goat; Life Technologies, A-21245), anti-rat DyLight488 (goat; Jackson, 112-485-143). DAPI (Sigma-Aldrich, D9564) nuclear counterstaining was performed as described by (Inoue and Wittbrodt, 2011).

Measurement of cell cycle and S phase length

To determine cell cycle length of retinal progenitor cells, dual pulse S phase labeling with BrdU and EdU were used as previously described (Das et al., 2009; Klimova and Kozmik, 2014). This approach allows an estimation of S phase length by consecutive BrdU and EdU incubation, and subsequent extrapolation of total cell cycle length in the Pcna-positive progenitor population. Hatchlings were incubated for 2 h in BrdU, then 30 min in EdU before fixation. Pcna antibody staining was used to label all cycling retinal progenitor cells. Pcna-, EdU- and BrdU-positive cells as well as cells positive for only BrdU were quantified.

Cell cycle length and S phase length were determined with the following formulas:

\[ T_{\text{cell cycle}} = 2 \text{ h} \times \left( \frac{\text{Pcna}^+ \text{ cells/BrdU}^+ \text{ EdU}^-}{\text{BrdU}^+ \text{ EdU}^-} \right) \]

\[ T_{S \text{ phase}} = 2 \text{ h} \times \left( \frac{\text{EdU}^+ \text{ cells/BrdU}^+ \text{ EdU}^-}{\text{BrdU}^+ \text{ EdU}^-} \right) \]

Four retinae from individual hatchlings were used for analysis. Cell counts were performed in \( z = 6 \mu m \) of two to three central sections per retina. \( T_{\text{cell cycle}} \) and \( T_{S \text{ phase}} \) were determined for individual sections.
Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridizations using NBT/BCIP detection were carried out as previously described (Loosli et al., 1998). Afterwards, samples were cryoprotected in 30% sucrose in 1x PTW overnight at 4°C and 20 µm thick serial sections were obtained on a Leica cryostat. Sections were rehydrated in 1x PTW for 30 min at room temperature and washed several times with 1x PTW. Slides were mounted with 60% glycerol and kept at 4°C until imaging.

Image acquisition

All immunohistochemistry images were acquired by confocal microscopy at a Leica TCS SP8 with 20x or 63x glycerol objective. Sections of whole-mount *in situ* hybridizations were imaged at a Zeiss Axio Imager M1 microscope. Images of whole hatchlings were acquired with a Nikon SMZ18 Stereomicroscope equipped with the camera Nikon DS-Ri1.

Image processing and statistical analysis

Images were processed via Fiji image processing software. Statistical analysis and graphical representation of the data were performed using the Prism software package (GraphPad). Box plots show median, 25th and 75th percentiles with whiskers from minimum to maximum data points. All data points, represented by black dots, are superimposed on box plots. Normal distribution was tested with the Shapiro-Wilk normality test, and unpaired two-tailed t-tests or Mann-Whitney tests were performed to determine statistical significance. The P value P < 0.05 was considered significant, P values are given in the figure legends. Sample size (n) is mentioned in every figure legend, all sections were treated as independent samples. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally used in the field. The experimental groups were allocated randomly, and no blinding was done during allocation.
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Author Contributions
C.B., K.L. and J.W. conceived the study and designed the experiments. C.B. performed the experiments. C.B., K.L. and J.W. wrote the manuscript.

Competing interests
Authors declare no competing interests.

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Data and materials availability
All data are available in the main text or the supplementary materials.
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Fig. 1: Igf1r signaling regulates proliferation in the CMZ. (A) Schematic representation of a transverse retinal section. Dashed squares represent dorsal (d) and ventral (v) CMZ. All CMZ sections in this paper depict the dorsal CMZ, with separate quantifications for dorsal and ventral CMZ, unless stated otherwise. (B) Cryosection of wildtype (wt) hatchling with anti-pIgf1r (green) staining shows Igf1r activity in single CMZ cells (arrowhead, n = 35 cells in 15 sections from 5 fish) and in MG (arrow) in the INL. Scale bar is 10 μm. (C,D) Wt hatchlings were incubated for 24 h in BrdU and 10 μM Igf1r inhibitor NVP-AEW541 or DMSO. Cryosections of DMSO- (C) and Igf1r-inhibitor-treated (D) retinae with BrdU staining (green) display decreased BrdU incorporation upon Igf1r inhibition. Scale bars are 10 μm. (E) Quantification of BrdU-positive cell number in one optical section per central section shows a decrease in Igf1r-inhibitor-treated retinae (n = 26 (dorsal)/23 (ventral) sections from 10 retinae in 6 fish) compared to DMSO (n = 28 (dorsal)/27 (ventral) sections from 10 retinae in 6 fish) (data obtained from two independent experiments; t-test: ****Pd < 0.0001, Mann-Whitney test: ****Pv < 0.0001). Box plots depict median + quartiles with min-max whiskers.
Fig. 2: Constitutive activation of Igf1r in retinal stem and progenitor cells results in increased neuroretina size. (A-C) Eye size of rx2::caigf1r hatchlings (B) is increased compared to wt siblings (A). Scale bars are 100 μm. (D-E) Quantification of relative eye size (eye diameter normalized to body length) of wt (D: n = 12; E: n = 11) and rx2::caigf1r (D: n = 38; E: n = 15) hatchlings (D) and 3-month-old adults (E) (t-test, ****P< 0.0001, ***P = 0.0009). (F,G) Cryosections of wt (F) and rx2::caigf1r (G) hatchling retinas with staining against Rx2 (magenta) display neuroretinal expansion. Wt section corresponds to fish 5 and rx2::caigf1r section to fish 16 in Table S1. Scale bars are 50 μm. (H) Neuroretinal thickness was measured perpendicular to the IPL in the fully laminated, CMZ-proximal region in wt and rx2::caigf1r retinas. Quantification of retinal column height in the central (wt: n =
11 sections from 8 retinae in 5 fish, \textit{rx2::caigf1r}: n = 23 sections from 10 retinae in 8 fish), dorsal and ventral (wt: n = 18 sections from 12 retinae in 6 fish, \textit{rx2::caigf1r}: n = 24 sections from 14 retinae in 8 fish) retina shows increase in \textit{rx2::caigf1r} compared to wt fish and CMZ-derived to embryonic retina (t-test, ****P < 0.0001, *P_{\text{wt c-v}} = 0.0130). Box plots depict median + quartiles with min-max whiskers.
Fig. 3: Cell type composition of *rx2::caigf1r* retinae is shifted toward INL neurons. (A-D) Cell type numbers were quantified in a 30 μm wide region (dashed rectangles) in the differentiated peripheral neuroretina in cryosections of wt (A,C) and *rx2::caigf1r* (B,D) hatchlings: cone and rod PRCs and cells in the retinal ganglion layer (GCL) are identified by location, MG are GS-positive (A,B, magenta), bipolar cells (BCs) are Otx2-positive (C,D, magenta), horizontal (HCs) and amacrine cells (ACs) are Otx2-negative (C,D). Scale bars are 20 μm. (E) Quantification of total cell number shows increase in *rx2::caigf1r* (n = 36 (total)/18 (dorsal/ventral) sections from 6 retinae in 3 fish) compared to wt (n = 36 (total)/18 (dorsal/ventral) sections from 6 retinae in 3 fish) retinae (t-test, ****P < 0.0001). Percentage change of cell number means from wt to *rx2::caigf1r* shows significant changes in cell number. (F) Quantification of cell type number shows increase in GCL, AC and BC number in *rx2::caigf1r* (n ≥ 35 sections from 6 retinae in 3 fish) compared to wt (n ≥ 36 sections from 6 retinae in 3 fish) retinae (t-test: ****P<0.0001, Mann-Whitney test: **P_{GCL} = 0.0076, nsP_{MG} = 0.0724, ****P_{BC} < 0.0001, nsP_{HC} = 0.3444, nsP_{rod} = 0.1771, nsP_{cone} =
0.3114). Percentage change of the cell number means of wt and *rx2::caigf1r* shows substantial changes in AC and BC number. ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. 4: Constitutive activation of Igf1r signaling decreases cell cycle length in the CMZ. (A,B) Cryosections of wt (A) and rx2::caigf1r (B) hatchling retinae incubated for 2 h in BrdU and 30 min in EdU to determine cell cycle length. BrdU (green), EdU (magenta) and Pcna (cyan) stainings partially overlap in the CMZ. Scale bars are 10 μm. (C) Quantification of cell cycle length shows a reduction of 1-2 h in rx2::caigf1r (n = 11 sections from 4 retinae in 4 fish) compared to wt (n = 11 sections from 4 retinae in 4 fish) retinae (data obtained from two independent experiments; Mann-Whitney test: **P_d = 0.0018, t-test: *P_v = 0.0188). (D) Quantification of S phase length in rx2::caigf1r (n = 11 sections from 4 retinae in 4 fish) compared to wt (n = 11 sections from 4 retinae in 4 fish) retinae (data obtained from two independent experiments; t-test: nsP_d = 0.6764, nsP_v = 0.8223). S phase length is not altered in rx2::caigf1r retinae. (E) Quantification of BrdU-positive cell number in the CMZ per 6 μm central section shows that numbers have more than doubled in rx2::caigf1r (n = 11 sections from 4 retinae in 4 fish) compared to wt (n = 11 sections from 4 retinae in 4 fish) retinae (data obtained from two independent experiments; t-test: ****P_d/v < 0.0001). ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. 5: *Cndp* is expressed in multipotent neuroretinal stem cells. (A) Cryosection of a *cndp::eGFP-caax* hatchling retina with GFP staining (green) in a peripheral subset of the Rx2 (magenta) domain in the CMZ. Scale bar is 10 μm. (B) Schematic representation of the constructs used for lineage tracing. Upon tamoxifen induction, *mCherry* is floxed out and *H2B-eGFP* is expressed in GaudíRSG fish. (C) Experimental outline: *cndp::CreERT2* is injected in 1-cell stage GaudíRSG embryos. At hatch, fish are incubated in tamoxifen overnight and grown for 2 - 3 weeks before analysis. (D) Schematic representation of a whole-mount neuroretina containing stem cell clones (arrowheads) and progenitor clones (asterisks). Progenitor clones are not connected to the CMZ. (E,F) Whole-mount immunostainings of *cndp::CreERT2*, GaudíRSG retinae against GFP (green) with neuroretinal clones (E, arrowheads) labeling the whole retinal column (F), originating from multipotent neuroretinal stem cells (*n* = 9 clones in 4 retinae from 4 fish, data obtained from two independent experiments). Scale bars are 100 μm (E) and 20 μm (F).
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**A**

Stem cells:
- *cnnp* expression
- Stem & progenitor cells:
  - *rx2* expression

**B**

C

D

E

F

H

Proliferating cells in the CMZ
- BrdU-positive cells

I

Cell populations and cell cycle
- *cnnp*: stem cells
- *rx2*: stem & progenitor cells

**G**

Number of mCherry cells in CMZ per 16 μm central section

**D**

Number of mCherry cells in CMZ per 16 μm central section

**G**

Number of R2b stem and progenitor cells in CMZ per 16 μm central section

**H**

30%

Igf1r inhibition

100%

Igf1r activation

**I**

12 h

**Development • Accepted manuscript**
**Fig. 6: Constitutive activation of IGF1R signaling expands retinal progenitor cell numbers.** (A) Schematic representation of the dorsal CMZ of a retinal section with cndp (magenta) and rx2 (green) expression in stem and progenitor cells. (B,C) Cryosections of wt (B) and rx2::caigf1r (C) cndp::H2A-mCherry reporter hatchling retinae. mCherry (magenta) is visible in peripheral-most cells in the CMZ (arrowheads). (D) Quantification of H2A-mCherry-positive cell number in the CMZ per 16 µm central section does not indicate an expansion of cndp-positive stem cells in rx2::caigf1r (n = 18 sections from 6 retinae in 3 fish) compared to wt (n = 18 sections from 6 retinae in 3 fish) retinae (Mann-Whitney test: *P_{d} = 0.0402, t-test: nsP_{v} = 0.2177). (E,F) Cryosections of wt (E) and rx2::caigf1r (F) hatchling retinae. Rx2 staining (green) marks peripheral cells in the CMZ. (G) Quantification of Rx2-positive cell number in the CMZ per 16 µm central section demonstrates that Rx2-positive stem and progenitor cells are more than doubled in rx2::caigf1r (n = 9 sections from 6 retinae in 3 fish) compared to wt (n = 9 sections from 6 retinae in 3 fish) retinae (t-test: ****P_{d/v} < 0.0001). (H) IGF1R inhibition decreases proliferating cells (green) in the CMZ by 30%, while IGF1R activation increases proliferation by ≥ 100%. (I) IGF1R activation in the CMZ expands progenitor numbers (rx2-positive, green) in rx2::caigf1r fish, but does not enlarge the stem cell population (cndp-positive, magenta). Cell cycle duration in the CMZ is shortened from 12 h in wt fish to 10 h upon IGF1R activation in rx2::caigf1r fish. ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. S1: Igf signaling pathway components are expressed in the hatchling CMZ.

(A-C) Cryosections of whole-mount in situ hybridizations of hatchling retinae (n = 3 fish each). Expression of *igf1ra* (A) is visible in CMZ, outer (ONL) and inner (INL) nuclear layer (asterisks). *Igf2* (B) is expressed in CMZ, ONL, INL and ganglion cell layer (GCL). CMZ and INL show expression of *insrb* (C).
**Fig. S2: Caigf1r** expression results in increased downstream signaling activation in the CMZ. (A) Quantification of relative eye size (eye diameter normalized to body length) of wt hatchlings incubated for 24 h in DMSO (n = 8) and 10 μM Igf1r inhibitor NVP-AEW541 (n = 8) (t-test: ns P = 0.4799). The percentage change of relative eye size means of DMSO- and inhibitor-treated hatchlings is 1.01%. (B,C) Cryosections of wt (B) and rx2::caigf1r (C) retinas at hatching stage. The pAkt-positive domain (magenta, arrowheads) is enlarged in rx2::caigf1r (C) compared to wt (B) retinas, co-localizing with GFP signal (green) also in PRCs (C, arrow) (n = 3 fish each). Scale bars are 10 μm. ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. S3: Neuroretinal expansion in \textit{rx2::caigf1r} retinas is driven neither by central retina proliferation nor by decreased apoptosis. (A,B) Cryosections of \textit{wt} (A) and \textit{rx2::caigf1r} (B) retinas at hatching stage fixed after 2h BrdU incubation. BrdU-positive cells (magenta, arrowheads) are present in the central region of \textit{wt} (A) and \textit{rx2::caigf1r} (B) retinas. Asterisks mark BrdU-positive cells in the lens epithelium and the optic nerve. Scale bars are 50 μm. (C) Quantification of BrdU-positive cell number in the central retina per 16 μm section normalized to retinal area [μm$^2$] shows slightly elevated, very low numbers in \textit{rx2::caigf1r} (n = 32 sections from 8 retinas in 4 fish) compared to \textit{wt} (n = 32 sections from 8 retinas in 4 fish) retinas (Mann-Whitney test: ****\text{P} > 0.0001). (D,E) Cryosections of \textit{wt} (D) and \textit{rx2::caigf1r} (E) retinas at hatching stage. TUNEL-positive nuclei (magenta, arrowheads) are present in \textit{rx2::caigf1r} (D) and \textit{wt} (E) retinas. Scale bars are 50 μm. (F) Quantification of TUNEL-positive cell number per 16 μm section shows increased apoptosis in the central but not CMZ region of \textit{rx2::caigf1r} (n = 11 sections from 8 retinas in 4 fish) compared to \textit{wt} (n = 10 sections from 8 retinas in 4 fish) retinas (t-test: ns\text{P}_{CMZ} = 0.6142, ****\text{P}_{central} > 0.0001). ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. S4: Cell type composition of the dorsal and ventral neuroretina is changed in rx2::caigf1r fish. (A) Quantification of cell type number in a 30 μm wide region in the dorsal differentiated peripheral neuroretina shows increased AC and BC number in rx2::caigf1r (n ≥ 35 sections from 6 retinae in 3 fish) compared to wt (n ≥ 36 sections from 6 retinae in 3 fish) retinae (t-test: ***P_{AC} = 0.0001, ***P_{BC} < 0.0001, Mann-Whitney test: nsP_{GCL} = 0.4193, *P_{MG} = 0.0127, nsP_{HC} = 0.2326, nsP_{rod} = 0.6162, **P_{cone} = 0.0031). Percentage change of the cell number means of wt and rx2::caigf1r shows large changes (≥ 10%) in AC, MG and BC number. (B) Quantification of cell type number in...
a 30 μm wide region in the ventral differentiated peripheral neuroretina shows increased GCL, AC and BC number in rx2::caigf1r (n ≥ 35 sections from 6 retinae in 3 fish) compared to wt (n ≥ 36 sections from 6 retinae in 3 fish) retinae (t-test: **P_{GCL} = 0.0023, ****P_{AC} < 0.0001, ****P_{BC} < 0.0001, Mann-Whitney test: nsp_{MG} = 0.8407, nsp_{HC} = 0.6608, **P_{rod} = 0.0017, *P_{cone} = 0.0150). Percentage change of the cell number means of wt and rx2::caigf1r shows large changes (≥ 10%) in GCL, AC and BC number. ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Fig. S5: EdU- and Pcna-positive cell number is increased in *rx2::caigf1r* retinae. (A,B) Quantification of EdU- (A) and Pcna-positive (B) cell number in the CMZ per 6 μm central section shows increased numbers in *rx2::caigf1r* (*n* = 11 sections from 4 retinae in 4 fish) compared to wt (*n* = 11 sections from 4 retinae in 4 fish) retinae (data obtained from two independent experiments; t-test: ****$P_{d/v} < 0.0001$). Box plots depict median + quartiles with min-max whiskers.
**Fig. S6: Cndp is expressed in the choroid plexi.** (A) Cndp::eGFP-caax hatchling shows GFP expression in the choroid plexi in the brain (arrowheads). Retinal GFP expression is masked by the retinal pigmented epithelium. (B) Whole-mount in situ hybridization for cndp in a stage 32 medaka embryo (dorsal view). Expression of cndp is visible in the peripheral retina (arrows) and the choroid plexi (arrowheads). Adapted from MEPD/(Alonso-Barba et al., 2016). (C) Whole-mount immunostaining against GFP (green) in a stage 32 cndp::eGFP-caax embryo. GFP expression (green) is visible in the peripheral retina (arrows) and the choroid plexi (arrowheads). Asterisk indicates unspecific background staining. (D) Cryosection of a cndp::eGFP-caax hatchling brain. The diencephalic choroid plexus is positive for GFP (green). (E) Cryosection of cndp::eGFP-caax hatchling with anti-plgf1r (magenta) staining shows Igf1r activity in single progenitors (arrow) but not in cndp-positive stem cells (green,
arrowhead). Scale bars are 20 μm. (F) Quantification of plgf1r-positive/cndp-negative and plgf1r/cndp-double positive cell numbers in the CMZ per 16 μm section show no GFP-positive cells co-labeled with plgf1r (n = 157 cells in 40 sections from 5 fish). Box plots depict median + quartiles with min-max whiskers.
Fig. S7: Expression of caigf1r in retinal stem cells does not result in increased eye size. (A-D) Cryosections of wt (A,C) and cndp::caigf1r (B,D) cndp::H2B-eGFP reporter hatchlings. The GFP-positive (green) choroid plexi are enlarged in cndp::caigf1r (B) compared to wt (A) brains. Cndp-driven GFP expression (green, arrowheads) in the CMZ of cndp::caigf1r hatchlings (C) is not expanded compared to wt (D) (n = 3 fish each). Scale bars are 20 μm. (E) Quantification of relative eye size (eye diameter normalized to body length) of wt (n = 28) and cndp::caigf1r (n = 20) hatchlings (Mann-Whitney test: nsP = 0.7023). (F) Quantification of relative eye size (eye diameter normalized to body length) of wt (n = 8) and cndp::caigf1r (n = 13) 3-month-old adult fish (t-test: nsP = 0.7894). The percentage change of relative eye size means of wt and cndp::caigf1r is > 1% in hatchlings (E) and adults (F). ns, not significant. Box plots depict median + quartiles with min-max whiskers.
Table S2: Raw data and calculated values for relative eye size quantification at hatchling stage, with eye diameter measured in the anterior-posterior axis.

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