Sox2, Tlx, Gli3, and Her9 converge on Rx2 to define retinal stem cells in vivo

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Abstract

Transcriptional networks defining stemness in adult neural stem cells (NSCs) are largely unknown. We used the proximal cis-regulatory element (pCRE) of the retina-specific homeobox gene 2 (rx2) to address such a network. Lineage analysis in the fish retina identified rx2 as marker for multipotent NSCs. rx2-positive cells located in the peripheral ciliary marginal zone behave as stem cells for the neuroretina, or the retinal pigmented epithelium. We identified upstream regulators of rx2 interrogating the rx2 pCRE in a transcriptional screen and focused on four TFs (Sox2, Tlx, Gli3, and Her9) activating or repressing rx2 expression. We demonstrated direct interaction of the rx2 pCRE with the four factors in vitro and in vivo. By conditional mosaic gain- and loss-of-function analyses, we validated the activity of those factors on regulating rx2 transcription and consequently modulating neuroretinal and RPE stem cell features. This becomes obvious by the rx2-mutant phenotypes that together with the data presented above identify rx2 as a transcriptional hub balancing stemness of neuroretinal and RPE stem cells in the adult fish retina.

Keywords de-differentiation; gene regulation; neural stem cells; retinal stem cells; transcriptional network

Subject Categories Development & Differentiation; Stem Cells; Transcription

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Introduction

Post-embryonic neurogenesis relies on the activity of neural stem cells (NSCs) (Adolf et al., 2006; Zhao et al., 2008). The fish neural retina (NR) constitutes an ideal model to study embryonic and post-embryonic NSCs in their physiological environment. It consists of seven main cell types distributed in three nuclear layers, and all these cell types are added lifelong from retinal stem cells (RSCs) that reside in the peripheral ciliary marginal zone (CMZ) (Johns, 1977; Lamba et al., 2008; Centanin et al., 2011). The CMZ also contributes to the retinal pigmented epithelium (RPE), a monolayer of pigmented cells surrounding and synchronously growing with the NR (Johns, 1977; Amato et al., 2004a; Moshiri et al., 2004; Centanin et al., 2011). As an attractive model for life-long neurogenesis as well as growth of the RPE, the CMZ has been extensively studied in fish, frog, and chicken (Amato et al., 2004b; Raymond et al., 2006; Lamba et al., 2008).

The lack of genetic tools to follow lineages in these species, however, was a long-lasting limitation to validate putative RSC-specific markers, which in turn prevented understanding the regulatory framework that generates and maintains this stem cell fate. In medaka, retinal multipotent NSCs were identified by their virtue to form arched continuous stripes (ArCoS) via cell transplantation at early embryonic stages (Centanin et al., 2011) and by inducible recombination in late embryonic and post-embryonic stages (Centanin et al., 2014). These experiments demonstrated that the maintenance of the NR and the RPE is achieved by independent RSCs located in the CMZ.

How the function of NSCs is maintained in the CNS remains largely unknown. Two of the best-studied factors expressed by post-embryonic NSCs in other niches are Sox2 and Tlx, which have been shown to be crucial for the self-renewal and differentiation of NSCs (Monaghan et al., 1995; Graham et al., 2003; Shi et al., 2004). Furthermore, fate-mapping studies showed that sox2 (Suh et al., 2007) and tlx (Liu et al., 2008) are markers for multipotent stem cells in the mammalian brain. Identifying target genes regulated by NSC-determining TFs is crucial for the understanding of the molecular and signaling pathways underlying adult neurogenesis. Previously, Sox2 has been demonstrated to bind and regulate the gfla regulatory element (Cavallaro et al., 2008). Target genes regulated by Tlx include cell cycle regulators p21 and pten (Sun et al., 2007). The function of Tlx is context dependent; inhibition of target genes relies on the interaction with HDACs (Sun et al., 2007), while Tlx can also act as activator of transcription (Iwahara et al., 2009).

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Characterizing a NSC regulatory network crucially depends on the identification of a reliable molecular marker and the main cis-acting regulators controlling its proper expression pattern. Here, we followed a long-term lineage analysis to identify the transcription factor (TF) retina-specific homeobox gene-2 (rx2) as a bona fide marker for multipotent adult RSCs in the post-embryonic medaka CMZ. We show that individual rx2+ RSCs give rise to progeny contributing either to NR or to RPE. Next, we identified TFs acting upstream of rx2 in a trans-regulation assay using the rx2 proximal cis-regulatory element (pCRE) and characterize sox2, tlx, gli3, and her9 as transcriptional modulators of stem cell features in the post-embryonic fish retina. Clonal expression of sox2 or tlx induces RSC-specific characteristics, including ectopic rx2 expression in differentiated neurons of the central retina. Conversely, conditional clonal gain of Gli3 or Her9 function leads to rx2 repression and inhibits stem cell proliferation in the post-embryonic CMZ. We also identify and validate the cis-regulatory motifs within the proximal rx2 pCRE that activate expression in the peripheral most CMZ, and repress expression in the adjacent RPE. Additionally, we show that the rx2 expression levels resulting from this transcriptional network establish the balance between NR and RPE stem cells. Our in vitro and in vivo results provide evidence for the importance of direct TF-DNA binding for proper spatial rx2 expression in RSCs. Taken together, we present a regulatory framework of TFs that establish, expand, and restrict RSC features in the post-embryonic fish retina and reveal that rx2+ cells locate to the peripheral most domain of the CMZ (Fig 1C), suggesting that Rx2 constitutes a marker for RSCs. On the central side of the CMZ, differentiating Atoh7+ cells demarcate the boundary between the central most domain of the CMZ and the differentiated, layered retina (Fig 1C) (Del Bene et al., 2007; Cerveny et al., 2010). As expected for stem cells, rx2+ cells in the post-embryonic CMZ barely incorporate the thymidine analog bromodeoxyuridine (BrdU) when applied in short pulses (Supplementary Fig S2A) and similarly, there is only partial overlap with proliferation markers like phospho-histone H3 (pHH3) and proliferating cell nuclear antigen (PCNA) (Supplementary Fig S2B and C). Most of the BrdU+, PCNA+, and/or pH3+ cells of the CMZ map to the progenitor domain, between the rx2+ cells and the Atoh7+ cells.

In medaka, transplantation of EGFP+ cells from the Wimbledon line (a transgenic line that expresses EGFP ubiquitously during the entire life of the fish) (Centanin et al., 2011) into an unlabeled blastula results in fish with mosaic retinae. In these chimeric fish, RSCs were addressed by their property to form ArCoSs, which are continuous clonal strings of EGFP+ cells consisting of differentiated neurons and glia at central positions, and undifferentiated cells in the peripheral domain. We reasoned that if rx2+ cells were indeed RSCs, all the ArCoSs should contain rx2+ cells located at the most peripheral position of the clone. We therefore transplanted Wimbledon rx2+/−, rx2#: H2B-mRFP donor cells into wild-type hosts and consistently found rx2+ cells at the peripheral tip in all resulting ArCoSs (Fig 1D and E). Thus, the expression domain of rx2+ and the relative position of rx2+ cells within an ArCoS match the expected location of RSCs and suggest that rx2+ is a RSC marker in the mature medaka retina.

**Results**

Rx2 labels the most peripheral cells in the ciliary marginal zone of the medaka retina

To specifically target RSCs in the CMZ, we followed a candidate gene approach and systematically searched for genes and their regulatory regions with expression confined to the CMZ. Both in amphibians and fish, the retina-specific homeobox gene-1 and retina-specific homeobox gene-2 (rx1 and rx2 respectively) are expressed in the peripheral CMZ at embryonic and post-embryonic stages (Locker et al., 2006; Raymond et al., 2006; Borday et al., 2012). In medaka, rx2 is first expressed in the undifferentiated retinal progenitor cells (RPCs) that form the optic vesicle (Loosli et al., 1999). At later stages, when the fish retina already contains all the main cell types and is functionally active, rx2 expression is confined to photoreceptors (cones and rods in the outer nuclear layer, ONL), to the Müller glia cells, and to the peripheral most part of the CMZ, as revealed by in situ hybridization and immunostaining (Fig 1A and B) (Sinn et al., 2014). Medaka transgenic lines in which 2.4 kb of the proximal rx2 pCRE control the expression of a reporter fluorescent protein (FP) (rx2#:H2B-mRFP and rx2#:Tub-GFP) (Martinez-Morales et al., 2009; Inoue & Witthbrodt, 2011) (Fig 1B and C; Supplementary Fig S1) exhibit the same expression pattern, indicating that the 2.4-kb rx2 pCRE contains the regulatory cues driving rx2 expression to those cell types.

The post-embryonic medaka retina grows outwards, with the RSCs located in the most peripheral domain of the CMZ (Centanin et al., 2011). A close analysis at rx2#:H2B-mRFP transgenic juveniles revealed that rx2+ cells locate to the peripheral most domain of the CMZ (Fig 1C), suggesting that Rx2 constitutes a marker for RSCs. On the central side of the CMZ, differentiating Atoh7+ cells demarcate the boundary between the central most domain of the CMZ and the differentiated, layered retina (Fig 1C) (Del Bene et al., 2007; Cerveny et al., 2010). As expected for stem cells, rx2+ cells in the post-embryonic CMZ barely incorporate the thymidine analog bromodeoxyuridine (BrdU) when applied in short pulses (Supplementary Fig S2A) and similarly, there is only partial overlap with proliferation markers like phospho-histone H3 (pHH3) and proliferating cell nuclear antigen (PCNA) (Supplementary Fig S2B and C). Most of the BrdU+, PCNA+, and/or pH3+ cells of the CMZ map to the progenitor domain, between the rx2+ cells and the Atoh7+ cells.

Rx2 is a molecular marker of adult RSCs

The ultimate validation of rx2+ as a stem cell marker is to follow the lineage of an individual rx2+ cell in the medaka CMZ during several months of post-embryonic life. The Gaudi toolkit allows single-cell labeling and lineage analysis in medaka (Centanin et al., 2014), based on Cre/LoxP-mediated recombination. When fish bearing a ubiquitous Cre driver and a Gaudi reporter are induced for stochastic recombination, single RSCs generate induced ArCoSs (iArCoSs) of the same characteristics than the ArCoSs generated by transplantation (Centanin et al., 2014).

To address if rx2 marks RSCs and if the progeny of an rx2+ cell in the CMZ forms an iArCoS, we generated a transgenic line expressing a tamoxifen-inducible Cre recombinase under the control of the well-characterized 2.4-kb rx2 pCRE (rx2::ERT2Cre). We induced Cre recombinase within the rx2 expression domain to trigger recombination in the ubiquitously expressed four-color reporter cassette (Gaudi4) (Centanin et al., 2014). Tamoxifen induction of rx2::ERT2Cre, Gaudi4 at 10 dpf resulted in the specific labeling of individual rx2+ cells, as shown by stochastic expression of FPs labeling single photoreceptors, Müller glia, and peripheral cells in the CMZ (Fig 2A).

Long-term lineage experiments showed that rx2+ cells formed iArCoSs and thus indeed represent RSCs (n = 162 red-colored iArCoSs distributed over 7 retinae, ranging from 15 to 31 iArCoSs per retina, average 23.1 iArCoSs/retina) (Fig 2B). Lineage analyses indicate as well that every single rx2+ post-embryonic NR stem cell analyzed is multipotent, equivalent to ArCoSs-forming RSCs in transplantation experiments. Each iArCoS contains the full repertoire of differentiated retinal cell types of the NR (n = 125 NR iArCoSs) (Fig 2C).
Remarkably, rx2+ RSCs as a population give rise to iArCoSs in both the NR and the RPE (Fig 2B). An individual rx2+ RSC, however, can be a stem cell either for the NR or for the RPE (n = 25 NR iArCoSs and 136 RPE iArCoS distributed over 8 retinae, 98.5% of independent iArCoSs).

Identification of Sox2, Tlx, Gli3, and Her9 as transcriptional regulators in control of retinal stemness

To identify genes controlling RSC features, we followed a high-throughput trans-regulation screen (Souren et al, 2009) to systematically detect factors operating on the rx2 pCRE. The trans-regulation screen is based on two nested screens. In a first step, it employs a high-throughput luciferase assay based upon the co-expression of an rx2 pCRE reporter construct driving firefly luciferase together with individual full-length candidate TFs (Fig 3A). This cell culture-based assay allows transcriptome scale analyses and has been used reliably to identify so far unknown upstream regulators (Souren et al, 2009). We took advantage of the relatively short 2.4-kb proximal rx2 CRE sufficient to recapitulate the rx2 expression pattern and assayed more than one thousand individual full-length cDNA clones, which represented a large complement of all putative medaka TFs. We controlled for transfection efficiencies in a dual luciferase-based screen in cultured cells through co-transfection of a

Figure 1. Rx2 functions as retinal stem cell (RSC) marker.

A rx2 mRNA is strongly detected in the peripheral CMZ of juvenile medaka (black box).
B Expression analysis of boxed area in (A). Transgenic reporter (rx2::Tub-GFP in green) overlaps with Rx2 immunostaining (in red).
C Cross-section of a transgenic rx2::H2B-mRFP juvenile medaka. rx2+ cells are located in the most peripheral domain of the CMZ (bracket), limited centrally by Atoh7::mYFP. rx2+ cells in the central retina are Müller glia (arrowhead) and photoreceptors (arrow).
D, E Transplantation of rx2::H2B-mRFP, Wimbledon cells into wild-type medaka demonstrates that rx2+ cells represent the most peripheral cells in an ArCoS.

Data information: Scale bars represent 25 µm in (B), 50 µm in (C), and 100 µm in (D).
control plasmid encoding Renilla luciferase (Fig 3A). To exclude potential false positives, we performed a secondary, nested, whole-mount in situ screen to analyze the expression pattern of putative candidate TFs relative to rx2 by a semi-automated whole-mount in situ hybridization approach (Quiring et al, 2004). We eventually selected activating or repressing candidates based on their co-expression with or adjacent to rx2 in the juvenile CMZ.

This nested screening pipeline delivered clear candidates from the more than one thousand TFs analyzed: sox2 was the top activator, while gli3 and her9 (a medaka Hes1 ortholog) showed the strongest repressive activities. tlx—not initially present in the full-length TF library—showed a strong activation of the rx2 pCRE (Fig 3C) and was assayed in a parallel candidate screen because of its role in mouse NSCs (Yu et al, 1994; Monaghan et al, 1995; Shi et al, 2004). To test whether Sox2, Tlx, Her9, and Gli3 regulate rx2 transcription in a concentration-dependent manner, we performed dual luciferase assays with increasing amounts of the respective TF cDNA. For Sox2 (Fig 3B), we observed the activation of relative luciferase activity in a dose-dependent manner. Likewise, for Tlx (Fig 3C) activation of transcription peaked with the highest cDNA concentration (160 ng), implicating tlx as an activator of rx2 expression. Conversely, stepwise increase of Her9 resulted in the gradual reduction of reporter expression (Fig 3D). Interestingly, Gli3-mediated repression of rx2 pCRE activity was strongest at the lowest Gli3 concentration (Fig 3E), while increasing cDNA amounts led to a gradual reduction of its repressive potential.

Next, we addressed the expression patterns of sox2, tlx, gli3, and her9 with respect to their putative target gene rx2 in the juvenile CMZ by two-color fluorescent whole-mount in situ hybridization (WISH). All four regulators are expressed in nested domains that partially overlap with the rx2 expression domain in the CMZ. We detected transcripts of the pan-neural determinant sox2 throughout the CMZ overlapping with the Rx2 expression domain (Fig 3F–H). tlx and her9 were both expressed in the central CMZ where they partially overlapped with the rx2 expression domain (Fig 3I–N). gli3...
Figure 3.
transcripts were found in the peripheral CMZ overlapping with rx2 expression and were also found in the adjacent RPE (Fig 3O–Q). Out of all the rx2 regulators identified in the trans-regulation screen, gli3 was the only factor expressed in the peripheral RPE adjacent to the CMZ.

**Gli3 and Her9 antagonize stem cell features in vivo**

To test whether these candidate factors regulate rx2 expression in vivo, we employed a conditional clonal analysis in the post-embryonic retina. For this purpose, we adopted a hormone-inducible binary gene expression system, which consists of a TF (LexPR) that upon hormone induction will dimerize and bind to the corresponding promoter element (LexOP) to activate the expression of the gene of interest with high efficiency (up to 90%). Consequently, the analyses based on the expression of the fluorescent reporter are conservative and always underestimate the effects of the gene of interest.

To assess the repressive potential of gli3 and her9, respectively, on the expression of rx2 in vivo, we targeted their clonal expression to the peripheral rx2+ CMZ. Gain-of-function clones were highlighted by the co-expression of nuclear FPs (Fig 4F and I). The expression of Rx2 protein was determined by antibody staining and analyzed by the co-expression of nuclear FPs (Fig 4F and I). The expression of Sox2 and Tlx co-expression on the proliferative capacity of RSCs in the CMZ.

PCR staining, which labels S-phase cells within the CMZ, was low only in very few cells in the CMZ of GFP control retinas (n = 3/38). Conversely, PCNA expression was affected in the gli3 and her9 gain-of-function clones at rates comparable to those observed for the repression of Rx2. PCNA was severely affected in gli3 gain-of-function clones (n = 39/106) (compare Fig 4L–N to O–Q) or her9 gain-of-function clones (n = 21/64) (compare Fig 4L–N to R–T). Taken together, these results indicate that ectopic clonal gli3 and her9 expression in the CMZ represses rx2 expression and impacts negatively on the proliferation of RSCs.

**Sox2 and Tlx promote rx2 expression**

Since our in vitro characterization and the overlapping expression pattern of sox2 and tlx with rx2 consistently argued for an activating function of sox2 and tlx, we tested the consequences of acute clonal activation of sox2 (cska::LexPR LexOP::sox2) and tlx (cska::LexPR LexOP::tlx) gain-of-function (Fig 5A). Gain-of-function clones were marked by the expression of red FPs (LexOP::cherry), encoded by co-injected reporter plasmids. In combination with the ubiquitous cska promoter (Grabher et al, 2003), this approach allowed clonal, mosaic expression throughout all three nuclear layers of the differentiated retina (Fig 5B).

We first examined the consequences of Sox2 and Tlx co-expression on rx2 promoter activity in vivo. The transgenic reporter line expressed FP under the rx2 pCRE (rx2::Tub-GFP). The combined expression of sox2 and tlx resulted in strong rx2 reporter activation (n = 40/48) in all three nuclear layers (Fig 5C and D). Individual clonal mis-expression of sox2 (n = 32/48) (Supplementary Fig S3A–D) or tlx (n = 142/173) (Supplementary Fig S3F–I) also resulted in ectopic rx2 reporter activation with high efficiency. To corroborate that sox2 and tlx activate endogenous rx2 expression in vivo, we combined WISH and immunohistochemistry in whole-mount preparations. Clones expressing sox2 (n = 53/62) (Supplementary Fig S3E–F) or tlx (n = 34/56) (Supplementary Fig S3J–K) efficiently triggered the ectopic expression of endogenous rx2 mRNA, which was never detected in controls.

We next asked whether the clonal activation of sox2 or tlx was sufficient to trigger the ectopic induction of RSC features. While control clones in central retinal cell types never showed proliferating activity, the expression of Sox2 (n = 7/11) (Fig 5E–I) or Tlx (n = 3/23) (Fig 5J–N) resulted in the re-acquisition of proliferative features as indicated by PCNA staining. PCNA+ clones were observed in the INL and OPL, indicating that de-differentiation and re-initiation of proliferation were not restricted to one particular type of retinal neurons. Together, these results revealed that both sox2 and tlx induce the endogenous expression of rx2 in vivo, and re-activate the proliferative potential of post-mitotic cells.
Sox2 and Gli3 regulate rx2 expression through direct protein–DNA interaction with the rx2 pCRE

Our analysis showed that sox2 and tlx trigger some RSC features in differentiated retinal cells, while gli3 and her9 constrain rx2 expression and stem cell proliferation in the CMZ. To investigate whether these effects are mediated by direct trans-regulation on the rx2 pCRE, we carried out a combination of in vitro and in vivo analyses.

We started by performing an evolutionary footprinting analysis on the cis-regulatory elements present in the rx2 pCRE. We identified evolutionarily conserved binding sites for Gli (Sasaki et al., 1997) and for Sox (Danno et al., 2008), the strongest repressor and activator in the in vitro assay, respectively (Fig 6A). Direct interaction between Sox2 and the Rix promoter has been demonstrated in Xenopus (Danno et al., 2008). To test whether Sox2 binds to the predicted, putative Sox-binding site in the medaka rx2 pCRE, we next performed electromobility shift assays (EMSA). Sox2 showed sequence-specific binding to the rx2 pCRE, which was weakened upon mutation of the predicted Sox TFBS (Fig 6B).

Similarly, the specific affinity of Gli3 protein to the rx2 pCRE was abolished when we tested the rx2 pCRE lacking the Gli-binding motif (Fig 6B).

Next, we investigated whether the transcriptional regulation of rx2 expression is impaired upon mutation of the identified Sox or Gli TFBSs. For this, we employed the luciferase trans-regulation assay and compared the transcriptional activity of rx2 pCREs with mutated Sox or Gli motifs (rx2 pCRE mtSox and rx2 pCRE delGli) to the corresponding wild-type activity (Fig 6C). The transcriptional activation of the rx2 pCRE through the predicted Sox2-binding site was severely attenuated by the introduction of mutations into the
Figure 5. Expression of sox2 or tlx promotes stem cell features in terminally differentiated neurons.

A Hormone-inducible expression plasmids for co-injection into transgenic rx2::Tub-GFP or wild-type embryos.

B Positive clones were traced by the expression of FPs (LexOP::Cherry), encoded by co-injected reporter plasmids. In combination with the ubiquitous cskα promoter, this approach facilitated mosaic expression throughout all three nuclear layers in transgenic rx2::Tub-GFP embryos. Expression of the candidate factors was hormonally induced (4 dpf) when the majority of cells in the central retina (CR) had exited the cell cycle and already differentiated into the neuronal and glial cell types.

C, D Compared to control (C), combined expression of tlx and sox2 (D) triggered ectopic rx2::pCRE activation.

E–N In addition to ectopic rx2 expression, PCNA protein was detected upon sox2 or tlx expression at 7 dpf.

Data information: White arrowheads point to representative co-expressing cells. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RSCs, retinal stem cells. Scale bar represents 10 μm.
Figure 6. The rx2 pCRE contains conserved Sox- and Gli-binding sites, which interact with Sox2 and Gli3 proteins.

A Result of BLAT search for the 2.4-kb medaka rx2 pCRE using the UCSC Genome Browser. The region, which contains the predicted Sox- and Gli-binding sites, is conserved. Blue peaks indicate conservation of coding and non-coding DNA.

B Sox2 and Gli3 proteins bind to predicted sites within the rx2 pCRE. The DNA–protein interaction is reduced/abolished through mutations introduced in the binding motifs.

C, D Reporter vectors carrying mutations (indicated by pink bars) in Sox (rx2 pCRE mtSox) or Gli (rx2 pCRE delGli) motifs in rx2 pCRE followed by firefly luciferase were co-transfected with 20 ng of sox2 or gli3 cDNA. Results of dual luciferase assays show that relative luciferase activity is reduced with rx2 pCRE mtSox compared to rx2 pCRE (C). Repression mediated by Gli3 is attenuated with rx2 pCRE delGli (D).

E, F Fold enrichments of two independent ChIP-PCR assays for Sox2 (E) and Gli3 (F) on rx2 pCRE. Enrichments for Sox2 and Gli3 TFBSs were normalized against control luciferase fragments (summarized in Supplementary Table S1).
Sox-binding site (from 50.99 ± 2.19 to 9.93 ± 1.41) (Fig 6D). Conversely, absence of the Gli TFBS lifted the strong Gli3-mediated repression of the rx2 pCRE (0.37 ± 0.01 to 0.56 ± 0.11) (Fig 6D). These results of the trans-regulation assays underscore the significance of both identified motifs for the regulation of rx2 expression through Sox2 and Gli3.

To gain further insight into the molecular mechanism underlying the regulation of rx2, we performed chromatin immunoprecipitation (ChIP)-PCR assays. To this end, rx2 pCRE luciferase plasmids co-expressed with GFP-tagged Sox2 or Gli3 fusion proteins were analyzed by immunoprecipitation of chromatin with antibodies directed against GFP. Subsequent amplification of co-precipitated DNA by qPCR using oligonucleotides flanking the specific TFBSs directed against GFP. Subsequent amplification of co-precipitated DNA by qPCR using oligonucleotides flanking the specific TFBSs directed against GFP. Subsequent amplification of co-precipitated DNA by qPCR using oligonucleotides flanking the specific TFBSs directed against GFP.

Conversely, absence of the Gli TFBS lifted the strong Gli3-mediated repression of the rx2 pCRE (0.37 ± 0.01 to 0.56 ± 0.11) (Fig 6D). Interestingly, the mutation of the Sox-binding site also affected rx2 reporter expression in Müller glia cells, the only other retinal cell type that co-expresses sox2 and rx2. In contrast, rx2 mSox reporter expression was unaffected in post-mitotic photoreceptor cells, which express rx2, but not sox2 (compare Supplementary Fig S6A–A” to Supplementary Fig S6B–B”).

These results indicate a critical in vivo role of the Sox-binding site and its interaction with the TF Sox2 for the onset and maintenance of rx2 expression in RSCs in the CMZ and in Müller glia cells. Our analyses support a dual role for the TF Gli3 as a direct context-dependent mediator of rx2 activation and repression, respectively.

Rx2 activity is required for the balance between RPE and neuroretinal stem cells

To ultimately address the role of Rx2 in the retinal stem cells in the peripheral CMZ, we established mutant alleles targeting the rx2 locus with transcription activator-like effector nucleases (TALENs). Customized TALEN pairs were designed to bind the coding sequence in the homeodomain of the rx2 gene. Mutations were introduced by injection of mRNA (Ansai et al, 2013) encoding the Rx2-specific TALEN pair at the one-cell stage. Successful targeting of the rx2 locus was validated by PCR and followed by sequence analysis in the injected and subsequent generation. Embryos carrying a homozygous mutation in rx2 (RNA null) are transiently delayed in retinal development but ultimately develop morphologically normal eyes with slightly reduced eye size (Fig 8A). They are viable and can be maintained as homozygous stock (Fig 8A). The spatiotemporal redundancy of rx2 expression in comparison to other highly related paralogous genes (rx3 in RCPs, rx1 in RSCs) presents a likely argument for the lack of a strong phenotype in mutant fish.

To challenge the capacity of rx2-mutant cells to contribute to retinal stem cells, we generated chimeric retinai by transplanting labeled wild-type cells into rx2-mutant embryos. When wild-type blastula cells are transplanted into a wild-type blastula host, they contribute to both stem cell populations, either to neuroretinal stem cells or to RPE stem cells as indicated by the formation of NR ArCoS and RPE ArCoS, respectively (34 NR ArCoSs and 13 RPE ArCoSs distributed in eight retinae; NR/RPE ratio = 2.6) (Fig 8B and C). Conversely, in the context of a rx2-mutant background, wild-type blastula cells transplanted into a mutant host preferentially give rise to neuroretinal stem cell ArCoSs, and contribute only occasionally to the RPE (24 NR ArCoSs and three RPE ArCoSs distributed in eight retinae; NR/RPE ratio = 8) (Fig 8D). Thus, our mosaic analysis revealed a requirement for Rx2 activity in balancing the contribution to the stem cell pools of neuroretinal stem cells and RPE stem cells. In the absence of Rx2 activity, RPE is preferentially formed, resulting in the exclusion of wild-type contribution in a mosaic situation. These data corroborate the findings presented above and, altogether, highlight an intricate connection between fate determinations of stem cell populations via the activity of Rx2, which ensures the proper balance of the two stem cell populations.
Discussion

Here, we identify and characterize the TF *rx2* as a proxy for retinal stem cells with a key function in balancing retinal stem cell populations and establish a scaffold network of directly interacting TFs that control and likely confine *rx2* expression to RSCs *in vivo*. Strikingly, an individual *rx2*+ cell is a stem cell either for the NR or for the RPE. Each individual *rx2*+ NSC is multipotent and gives rise to the full complement of retinal cell types. By identifying upstream regulators of *rx2*, we have gained mechanistic insight into the transcriptional control of retinal stemness. The bifunctional *rx2*+ stem cell domain within the CMZ is defined by both transcriptional activators (Sox2, Tlx) and transcriptional repressors (Gli3, Her9) (Fig 9A). We hypothesize that the combined activities of those factors confine *Rx2* expression and facilitate the balanced establishment of stem cells for the NR and the RPE within the CMZ.

We followed an unbiased approach (Souren et al, 2009) to initially identify proteins interacting with the *rx2* CRE *in vitro*. This approach has clear limitations due to the composition of the initially screened gene set and the fact that we only assayed for the activity of individual genes. Nevertheless, we successfully identified key transcriptional regulators of *rx2* that directly interact with the *rx2*
CRE in vitro and in vivo and exert key functions in confining the rx2+ RSCs to the periphery of the CMZ.

Conditional mosaic expression of individual rx2 activators in the central retina resulted in the de-differentiation of post-mitotic retinal cells and the induction of stem cell features therein. These terminally differentiated cells are efficiently reprogrammed by the combination of Tlx and Sox2, consistent with reports in cultured NSCs, where a direct interaction between Sox2 and Tlx alleviates a negative feedback loop acting on the tlx promoter (Shimozaki et al., 2012). Additionally, both factors have been shown to be crucial for the maintenance of mammalian NSCs (Bylund et al., 2003; Graham et al., 2003; Shi et al., 2004; Taranova et al., 2006; Liu et al., 2008). We hypothesize that both factors synergize in the central CMZ to establish the niche for neuroretinal stem cells (Fig 9A).

Our data connect Sox2, Tlx, Gli3, and Her9 forming a core scaffold, which converges on the rx2 pCRE and modulates rx2 expression in adult RSCs (Fig 9B). Because these TFs are known to participate in non-exclusive protein–protein interactions (Kageyama et al., 2007; Qu & Shi, 2009; Kondoh & Kamachi, 2010), it is possible that a combinatorial code with additional cofactors modulates the spatiotemporal function and activity of the core components of the network. Previous studies in Xenopus reported the interdependence of Sox2 and Otx2 for rx activation (Danno et al., 2008; Martinez-De Luna et al., 2010). Even though we failed to detect Otx2 transcripts in the CMZ (data not shown), we cannot entirely exclude the presence of Otx2 at basal levels in the trans-regulation assays. However, the graded response to increasing concentrations of Sox2 strongly argues for a Sox2-mediated rx2 regulation independent of Otx2. Since fish and amphibia possess the highest regenerative capacity amongst vertebrates and show lifelong, post-embryonic retinogenesis, the difference in rx regulation is unexpected. Addressing how the regulatory scaffold presented here has evolved from fish over frogs to mammals will present one aspect toward our understanding of the gradual loss of proliferative and regenerative capacity in the retina of higher vertebrates.

One of the striking findings of this work relates to the fact that a single retinal stem cell expressing rx2 can either be a multipotential stem cell for the neuroretina or be a stem cell for the RPE. Here, distally adjacent to the CMZ, Gli3 (Fig 9A, blue) acts as repressor, confining rx2 expression (Fig 9A, red) to the CMZ. The shift of rx2 reporter expression from the CMZ to the RPE upon deletion of the Gli-binding site underlines a dual function of Gli3: It mediates rx2 repression in the RPE as well as rx2 activation in the directly adjacent CMZ. These data together with the fact that rx2-mutant cells preferentially contribute to the RPE RSC pool (discussed below) indicate that Rx repression (mediated by Gli 3 in the wild-type retina) is a prerequisite for commitment toward RPE fate.

Figure 8. Rx2 balances fate decisions of retinal stem cells.

A Adult wild-type (top) and rx2 homozygous mutant fish (bottom).
B Transplantation of permanently labeled wild-type cells at the blastula stage into a wild-type host results in the formation of both NR (asterisk, green) and RPE (arrowhead, blue) ArCoSs.
C Control transplantations (wild-type to wild-type).
D Transplantation of wild-type cells into rx2-mutant host blastulae results in the preferential formation of NR ArCoSs.
In the absence of Rx2 (by repression or mutation), RPE fate is strongly favored. Intriguingly, rx2-mutant cells display a striking phenotype when challenged with wild-type cells within the same niche. In a mosaic retina, the absence of Rx2 activity favors the formation of RPE and consequently prevents wild-type cells to take RPE stem cell fate. Therefore, our data not only highlight an intricate connection between fate determinations of both stem cell domains via the activity of Rx2. They furthermore also imply a cell non-autonomous feedback activity ensuring a balance of both stem cell populations.

The transcriptional confinement of rx2 to the CMZ by the activity of the regulatory scaffold presented here connects the stem cells of the NR and those of the RPE and thus sheds light on the mechanism specifying this composite stem cell niche.

Materials and Methods

Medaka stocks

Medaka (Oryzias latipes) stocks were maintained as previously described (Koster et al, 1997). All fish are maintained in the closed stocks of COS at Heidelberg University. Fish husbandry and experiments were performed according to local animal welfare standards (Tierschutzgesetz 111, Abs. 1, Nr. 1, Haltungserlaubnis) and in accordance with European Union animal welfare guidelines. The fish facility is under the supervision of the local representative of the animal welfare agency. Embryos were staged according to Iwamatsu (2004).

Transgenesis

Injections were done as previously described (Rembold et al, 2006b). For transient expression, driver and effector plasmids were co-injected (30 ng/ul final concentration) in two-cell stage medaka embryos (Rembold et al, 2006a).

Molecular cloning

LexPR and LexOP cassettes were derived from pDs[krt8:LP–LPR::G4] and pDs[cska::Cy-LO–LEXOP::Ch] to generate driver, effector, or driver–effector constructs (Emelyanov & Parinov, 2008). A cassette containing the LexOP operator upstream of the Cherry coding sequence was extracted from pDs[cska::Cy-LO–LEXOP::Ch]. The Cherry coding sequence was replaced with H2B-EGFP and H2A-Cherry. Effectors: LexOP::H2A-Cherry; LexOP::H2B-EGFP; LexOP::Cherry. A cassette containing the coding sequence for the LexPR trans-activator followed by the LexOP operator was released from pDs[krt8:LP–LO–LPR::G4] and inserted downstream of the rx2 pCRE (Inoue & Wittbrodt, 2011). Coding sequences for gli3 and her9 were inserted downstream of the LexOP operator. A second LexOP operator followed by H2B-EGFP coding sequence was added (released from LexOP::H2B-EGFP). Driver–Effectors: rx2::LexPR LexOP::Gli3 LexOP::H2B-EGFP; rx2::LexPR LexOP::Her9 LexOP::H2B-EGFP; rx2::LexPR LexOP::H2B-EGFP.

A cassette containing the coding sequence for the LexPR trans-activator followed by the LexOP operator was introduced downstream of the cska promoter (Grabher et al, 2003). Coding sequences for sox2 and tlx were inserted downstream of the LexOP operator. Drivers: cska::LexPR LexOP::Sox2; cska::LexPR LexOP::Tlx; cska::LexPR LexOP.

Figure 9. A proposed model summarizing spatial regulation of rx2 expression and RSC-specific features through sox2, tlx, her9, and gli3.

A Schematic illustration outlining the spatial distribution of rx2 (red), sox2 (black dashed line), tlx (green), her9 (green), and gli3 (blue) transcripts in the CMZ and RPE. Purple indicates overlap between rx2 and gli3; yellow indicates overlap between rx2 and tlx/her9.

B RSC-specific gene regulatory network operating in vivo. sox2 (black) and tlx (green) directly interact with the rx2 pCRE to activate rx2 expression in the CMZ as well as other stem cell features. Her9 (green) restricts rx2 expression centrally, and gli3 prevents Rx2 from being expressed in the RPE. Proximity to the hedgehog morphogen (gray) secreted from the ganglion cell layer could dictate the activating function (purple) in the peripheral CMZ and repressive action (blue) in the RPE of Gli3 protein isoforms.
The coding sequence of the nuclear FPs in the \(rx2::H_2B\)-mRFP vector (Inoue & Wittbrodt, 2011) was replaced by tubulin-GFP fusion. The 2.4-kb \(rx2\) pCRE was released through restriction digest and cloned upstream of the luciferase gene into the pGL4.1 luciferase reporter vector (Promega).

Coding sequences for \(sox2\), \(gli3\), and \(her9\) were derived from a full-length cDNA library based on the pCMV-Sport6.1 vector (Souren et al., 2009); \(tlx\) cDNA was derived from a Lambda ZAP cloning vector. Full-length \(Olrx2\) (NP_001098373.1) for antibody generation was cloned by PCR from medaka stage 32 cDNA using the following primers: forward primer: 5’-GAATTCTCATATGATGCATTGTCTAATGGATAC-3’; reverse primer: 5’-GGGATCCCTTTACATGCTTGGCAACGGGTG and the antisense FL was generated by annealing with the sense oligo: GATCCTCCCTTTACATGCTTGGCAACGGGTG and the antisense oligonucleotides (25 fmol) were incubated with 5 \(\mu\)l protein translation reaction for 1 h at 4°C in binding buffer (for \(Gli3\) and \(Tlx\): 10 mM Tris, 1 mM DTT, 5 mM MgCl\(_2\), 5% glycerol, 1 mM EDTA, 0.05% NP-40, and 50 ng/\(\mu\)l poly(dI-dC); for \(Sox2\) and \(Her9\): 10 mM Tris, 1 mM DTT, 2.5 mM MgCl\(_2\), 100 mM KCl, 5% glycerol, 1 mM EDTA, 0.05% NP-40, and 50 ng/\(\mu\)l poly(dI-dC)) in 20 \(\mu\)l total volume.

The binding reactions were subjected to electrophoresis on a pre-run 6% polyacrylamide gel in 0.5× TBE. Signal was detected using the LightShift Chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer’s instructions. Oligonucleotide sequences used are summarized in Supplementary Table S3.

**Dose–response assay**

pGL4.1 vector containing the \(rx2\) pCRE with different mutations (40 ng) and pRL-CMV vector (5 ng) were co-transfected with 20, 40, 80 or 160 ng of medaka cDNA (pSport6.1-cDNA) per well. A total amount of 205 ng DNA was transfected in each well through addition of empty pCS2+ vector. The assays were carried out in quadruplicate.

**Chromatin immunoprecipitation**

ChIPs were performed with the SimpleChIP Enzymatic Chromatin IP kit (New England Biolabs) according to the manufacturer’s instructions. BHK cells were co-transfected with 6 mg pGL4.1-rx2 and 3 mg pSport6.1-mGFP-FL-cDNA. Chromatin was incubated with 20 \(\mu\)l of agarose beads attached to anti-GFP antibodies (Chromotek) at 4°C overnight. Quantification of DNA by PCR was carried out in a thermocycler (Bio-Rad) using Absolute qPCR SYBR Green Mix (Thermoscientific). Primers used to amplify regions containing putative binding sites within the \(rx2\) pCRE and control primers for firefly luciferase coding sequence are summarized in Supplementary Table S4. Two independent biological replicates were carried out for each genomic region of interest. Each qPCR was performed as duplicates.

**Whole-mount in situ hybridization**

For anti-sense riboprobe synthesis, linear templates were produced from full-length cDNA clones either through standard PCR (\(sox2\), \(gli3\) and \(her9\)) or restriction enzyme digestion 5’ of the start codon (\(rx2\) and \(tlx\)). T7 RNA polymerase-based transcription was carried out as previously described (Loosli et al., 1998). Fluorescent whole-mount in situ hybridizations were performed as previously described (Schuhmacher et al., 2011). Signals were detected using TSA-Plus Fluorescein and Cyanine 5 Systems (Perkin Elmer). For combined single-color fluorescent whole-mount in situ hybridization and immunostaining, embryos were incubated for 2 days with anti-fluorescein antibody conjugated to horseradish peroxidase (Roche).
and anti-GFP antibody (Invitrogen) at 4°C. After riboprobe detection using TSA-Plus Cyanine 3 System (Perkin Elmer), the embryos were incubated with fluorescent-conjugated secondary antibody and 4',6-diamidino-2-phenylindole (DAPI, 1:500, Sigma) for 2 days at 4°C. Whole-mount in situ hybridizations using NBT/BCIP transcriptions were performed using the mMessage machine (SP6, Ambion) according to the manufacturer’s protocol. The mRNA was purified using the RNeasy RNA purification kit (Qiagen) according to the manufacturer’s protocol and stored at −80°C.

mRNAs encoding TALENs directed against the rx2 coding sequence were injected at the one-cell stage as described for medaka (Ansei et al., 2013). To identify mutations in the locus, fin clip DNA of F1 fish was analyzed. The regions of interest for the rx2 TALEN pair were amplified from the isolated genomic DNA with the following primers: rx2 forward primer: 5’-AACAGTGTAGCCGCGTCTGT-3’; reverse primer: 5’-TCTGAGGATGAAATTCTGC-3’. The PCR amplification was performed with a proofreading polymerase: 30 s 98°C; (20 s 98°C, 45 s 67°C, 45 s 72°C) repeated for 29 cycles, 5 min followed by 72°C; and followed by 15 min at 72°C with Taq polymerase. The resulting PCR product (930 bp) was enzymatically digested with HpaII. Uncleaved fragments resulting from TALEN-induced mutations in the HpaII recognition site were purified, cloned, and validated by sequencing. RNA null alleles were closed to homozygosity and maintained as homozygous stock. Stocks are validated each generation by allele-specific PCR on fin clip DNA. Wild-type rx2 alleles were identified with the following primers: forward primer: 5’-GGGGATTGATGGAGATGGAGT-3’; reverse primer: 5’-CGGCTGTJAGACCTTGAGA-3’.

Mutant rx2 alleles were identified with the following primers: forward primer: 5’-GGGGATTGAGATGAGATGGAGT-3’; reverse primer: 5’-CCTCCTCGTCTGAGATGAG-3’.

Transplantation experiments

Transplantations were performed as previously described (Ho & Kane, 1990; Rembold et al., 2006a). For experiments in Fig 1, homozygous Wimbledon males (Centanin et al., 2011) were crossed to heterozygous rx2::H2B-mRFP females; 10–15 blastulae cells from the progeny (100% Wimbledon+/−, 50% rx2::H2B-mRFP+/−) were transplanted to the central part of wild-type (Cab strain) blastulae. Transplanted embryos were kept in agar-coated dishes in 1×EWR supplemented with penicillin–streptomycin (1:200, Sigma) and selected for GFP+, RFP+ cells in the retina.

For experiments shown in Fig 8, heterozygous Wimbledon males were crossed to wild-type females; 20–30 blastulae cells from the progeny were transplanted to the central part of either rx2-mutant or a wild-type blastulae. Transplanted embryos were raised in agar-coated dishes in 1×EWR supplemented with penicillin–streptomycin (1:200, Sigma), selected for GFP+ clones in the retina 5 days post-transplantation and grown to the desired age for ArCoS analysis.

Imaging

Samples were imaged using an Olympus MVX10 binocular coupled to a Leica DFC500 camera, a Nikon AZ100 coupled to a Nikon C1 (entire retinae), or a Leica TCS SPE (sections). Images of NBT/BCIP stainings were taken using a Leica DM5000 scope equipped with a Leica DFC500 camera. For cell counting, an automated segmentation tool (maximum entropy threshold) for ImageJ (Version 1.41o; http://rsbweb.nih.gov/ij/) was used.
Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions
RR (in vivo, in vitro and cell culture assays, generation of rx2 mutants) and LC (lineage-tracing analysis, transplantation assays) performed the experiments with contributions from TT (transplantation assays), DI (Rx2 antibody and GFP fusion proteins), J-PC (design of rx2 talens) BW (rx2 mutants), and JRM-M (rx2: Tub-GFP transgenic line), RR, LC, TT and JW designed the experiments and analyzed the data. RR, LC and JW wrote the manuscript with contributions from DI, TT and JRM-M.

Conflict of interest
The authors declare that they have no conflict of interest.

References

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