The unusual rainbow trout sex determination gene hijacked the canonical vertebrate gonadal differentiation pathway

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Evolutionary novelties require rewiring of transcriptional networks and/or the evolution of new gene functions. Sex determination (SD), one of the most plastic evolutionary processes, requires such novelties. Studies on the evolution of vertebrate SD revealed that new master SD genes are generally recruited from genes involved in the downstream SD regulatory genetic network. Only a single exception to this rule is currently known in vertebrates: the intriguing case of the salmonid master SD gene (sdY), which arose from duplication of an immune-related gene. This exception immediately posed the question of how a gene outside from the classical sex differentiation cascade could acquire its function as a male SD gene. Here we show that sdY became integrated in the classical vertebrate sex differentiation cascade by interacting with the Forkhead box domain of the female-determining transcription factor, Foxl2. In the presence of Foxl2, SdY is translocated to the nucleus where the SdY:Foxl2 complex prevents activation of the aromatase (cyp19a1a) promoter, consequently allowing testicular differentiation to proceed. These results also suggest that the evolution of unusual vertebrate master sex determination genes recruited from outside the classical pathway like sdY is strongly constrained by their ability to interact with the canonical gonadal differentiation pathway.

Significance

Sex determination is one of the most fundamental but also extraordinary plastic processes in nature. Many different master sex-determining genes have been characterized in vertebrates, and most of them are known to fulfill essential functions during sexual development and thus are already tightly linked to the process that they now govern. Only one exception is currently known: the salmonid master sex-determining gene (sdY), which arose from the duplication of an immune-related gene. Here we show that SdY prevents female differentiation by interacting and blocking the action of a key ovarian differentiation factor. These results suggest that the evolution of unusual vertebrate master sex determination genes is strongly constrained by their ability to interact with the canonical gonadal differentiation pathway.


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roles have been described in neurons and liver and heart physiology (19), but so far no evidence implicates it in sex determination or sex differentiation processes. The birth of such a master SD gene recruited from outside of the classical sex differentiation cascade raises the intriguing question of its functional evolution and how this unusual SD gene determines sex. Did SdY evolve a new function to be able to interact directly with the classical gonadal sex differentiation cascade, or does it use part of its ancestral pathway, that is, the IFN immune-related response, for its action?

Results and Discussion

During evolution of SdY, the DNA-binding domain and the nuclear localization signals of Irf9 were lost, while the protein–protein interaction domain (IRF association domain, or IAD) was preserved and underwent some sequence diversification (16). To test the hypothesis that the IAD domain of SdY still functions in protein binding, we first performed molecular modeling and found that the 3D structure of SdY strongly overlaps the IAD domain of IRF proteins (Fig. 1A). As the IAD domain is the only domain of known function predicted from the primary sequence of the sdY gene (16, 17), we hypothesized that SdY could still exert its function based on protein–protein interactions. We thus searched for SdY interacting proteins using a yeast two-hybrid (Y2H) screen with SdY used as bait and with a rainbow trout prey cDNA library prepared from late differentiating testes sampled when sdY expression is still high (16). Among the 46 different putative interacting proteins there were none of the known Irf9 partners like Stat1 or Stat2. Instead, we found a very strong enrichment of many members of the Forkhead box (FOX) family (11 FOX proteins, SI Appendix, Tables S1–S3). The Forkhead box, a highly conserved DNA-binding domain (20) common to all FOX proteins, was identified as the minimum domain needed for an effective interaction with SdY (Fig. 1B and SI Appendix, Table S3). Interestingly, among all of the FOX proteins interacting with SdY in yeast, we found the well-known female sex differentiation protein Fox2 (21, 22). Taking into account the importance of Fox2 in vertebrate sex differentiation, we reasoned that this would be an interesting and biologically relevant SdY partner. We then explored the interaction of SdY with trout Fox2 in a direct yeast interaction assay and confirmed that SdY and Fox2 can interact together (SI Appendix, Fig. S1).

To better characterize this interaction, an in vitro approach was developed using cell transfection assays. In HEK 293T cells transfected only with sdY plasmid, SdY protein was localized predominantly in the cytoplasm (Fig. 2A–A′ and SI Appendix, Fig. S2). However, when cotransfected along with Fox2, SdY was completely translocated into the nucleus (Fig. 2B–B′ and SI Appendix, Fig. S2). Such a complete SdY nuclear translocation was observed only with fish Fox2 proteins (Fig. 2H), including the two rainbow trout paralogous gene products (Fox2b1 and Fox2b2) resulting from the salmonid whole-genome duplication (23) (Fig. 2I–O) and the medaka, Oryzias latipes, Fox2 (SI Appendix, Figs. S3 and S4). No complete nuclear translocation was observed with some other rainbow trout Fox proteins (Fig. 2C–H and SI Appendix, Fig. S2), with some mammalian Fox2, that is, mouse and goat (SI Appendix, Fig. S3), and with the rainbow trout Fox2b2 containing a modified mouselike Forkhead box domain (SI Appendix, Fig. S4). This complete nuclear relocalization of SdY with trout and medaka Fox2s indicated some specific protein–protein interaction and that this interaction required the formation of a fish Forkhead domain. This interaction was also confirmed in vitro by co-immunoprecipitation experiments (Fig. 2P and Q and SI Appendix, Fig. S5A) and in vivo by showing that SdY was also translocated into the nucleus following coinjection with Fox2 in medaka embryos (SI Appendix, Fig. SS B and C).

To obtain further insights into the physiological relevance of the SdY and Fox2 interaction in vivo, a gene expression time course was done (fox2, nr5a1, and sdY gene expression data in differentiating gonads were performed. In agreement with its male-determining role, sdY expression was detected only in male gonads, with a peak of expression around 45 d postfertilization (dpf) (Fig. 3A). In contrast, fox2b1, fox2b2, and nr5a1 were not expressed in a sexually dimorphic fashion before the time point at which sdY peaks in males; after this time point fox2b1 and fox2b2 are markedly up-regulated in females and down-regulated in males (Fig. 3 A and B and SI Appendix, Fig. S6). We also explored expression of gonadal aromatase (cyp19a1a), as this gene is a well-known direct target of Fox2 (24). We found that cyp19a1a expression was only in the differentiating testes and was markedly down-regulated in females (Fig. 3A). We also confirmed in vitro by co-immunoprecipitation experiments (SI Appendix, Figs. S3 and S4), and with the rainbow trout Fox2b2 containing a modified mouselike Forkhead box domain (SI Appendix, Fig. S4). This complete nuclear relocalization of SdY with trout and medaka Fox2s indicated some specific protein–protein interaction and that this interaction required the formation of a fish Forkhead domain. This interaction was also confirmed in vitro by co-immunoprecipitation experiments (Fig. 2P and Q and SI Appendix, Fig. S5A) and in vivo by showing that SdY was also translocated into the nucleus following coinjection with Fox2 in medaka embryos (SI Appendix, Fig. SS B and C).

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Fig. 1. SdY conserves the structure of the IRF protein–protein interaction domain and interacts with the Forkhead box domain of FOX proteins. (A) SdY shares structural homologies with IAD, a protein–protein interaction domain. The structure of SdY (in gray) was modeled using the crystal structure of IRF5 as a template (in green). This SdY structure reveals eight α-helices that are highly conserved with IRF5. (B) SdY interacts in yeast with Fox proteins through their highly conserved DNA-binding domain. The alignments of the SdY-Fox interacting clone sequences (gray lines) delineate the minimum domain or selected interacting domain needed for an effective interaction with SdY in yeast, which is the Forkhead box domain (110 aa, black lines). The 11 Fox proteins characterized in the Y2H screen are represented by open cylinders with numbers of interacting clones indicated on the right side.

The alignments of the SdY-Fox interacting clone sequences (gray lines) delineate the minimum domain or selected interacting domain needed for an effective interaction with SdY in yeast, which is the Forkhead box domain (110 aa, black lines). The 11 Fox proteins characterized in the Y2H screen are represented by open cylinders with numbers of interacting clones indicated on the right side.
Fig. 2. SdY interacts with Foxl2, resulting in its nuclear translocation. (A–H) GFP:SdY alone (A–A’) and GFP:SdY in combination with different trout Fox proteins, Fox2 (Fox2b1) (B–B’), Fox3 (C–C’), Fox1 (D–D’), Foxn2 (E–E’), Foxn3 (F–F’), Foxo3 (G–G’), were cotransfected in HEK 293T cells (delimited by yellow dotted lines) and Foxl2b2 are both able to drive SdY complete nuclear translocation (delimited by yellow dotted lines and stained in blue with Hoechst staining) only in the presence of Fox2 (B–B’). Scale bar, 5 μm. (H) Percentage of transfected cells (mean ± standard deviation on 200 cells) in which SdY is completely translocated in the nucleus after three independent cotransfection experiments with different trout Fox proteins. Significant differences compared with SdY alone were calculated using an unpaired two-tailed Student’s t test. *** P < 0.001; ns, nonsignificant. (I–O) Foxl2b1 and Foxl2b2 were both able to drive SdY complete nuclear translocation (delimited by yellow dotted lines and stained in blue with Hoechst staining). Confocal images of HEK 293T cells (delimited by white dotted lines) transiently transfected with SdY (I–I’), mCherry:Foxl2b1 alone (J–J’), SdY and mCherry:Foxl2b1 (K–K’), mCherry:Foxl2b2 alone (L–L’), SdY and mCherry:Foxl2b2 (M–M’). Scale bar, 10 μm. (N) Quantitative analysis in the presence or absence of Foxl2b1 and Foxl2b2. Percentage of complete SdY nuclear translocation (mean ± standard deviation on 100 cells) after three independent cotransfection experiments. Statistical significances compared with SdY alone were calculated using an unpaired two-tailed Student’s t test. (Q) SdY colocalizes with Foxl2 in the nucleus. SdY, SdY-Foxl2b1, and SdY-Foxl2b2 colocalizations were measured in the nucleus for SdY (n = 5), SdY and Foxl2b1 (n = 5), and SdY and Foxl2b2 (n = 5) with Pearson’s correlation. Statistical significance was calculated using an unpaired two-tailed Student’s t test, *** P < 0.001. (P and Q) SdY binds with Foxl2 in co-immunoprecipitation (IP) experiments. HEK 293T cells were transiently transfected with expression plasmids for SdY fused to a hemagglutinin tag (3xHA:SdY) and for Foxl2 fused to a 3xFlag tag (3xFlag:Foxl2b1 or 3xFlag:Foxl2b2). Whole-cell lysates were used for immunoprecipitation with anti-Flag or anti-Foxl2 (P) or with anti-HA or anti-SdY (Q) followed by immunoblotting with the appropriate antibodies. Input represents 10% whole-cell lysate. IgG mouse antibody was used as the control. In P, 3xFlag:Foxl2b1 or 3xFlag:Foxl2b2 was immunoprecipitated with either Flag (Top) or Foxl2 (Bottom) antibodies followed by immunoblotting with an antibody against the HA tag to reveal the interaction with 3xHA:SdY. In Q, 3xHA:SdY was immunoprecipitated with an HA or SdY antibody, followed by immunoblotting with an antibody against the Flag tag to reveal 3xFlag:Foxl2b1 (Foxl2b1) (Top) or 3xFlag:Foxl2b2 (Foxl2b2) (Bottom).

By doing so very early in the differentiation process of the gonads, i.e., long before implementation of the cyp19a1a loop of regulation in females (Fig. 3A), SdY would completely prevent estrogen production in the differentiating male gonads. The absence of estrogen production subsequently triggers masculinization as it has been demonstrated in many fish species, including rainbow trout, by treating specimens with an aromatase inhibitor (30) or, more recently, by direct inactivation of the cyp19a1a gene (26, 31, 32). Such masculinization following the blockade of estrogen production is even effective in adult females (27), showing that estrogens in fish are needed not only for ovarian differentiation but also for ovarian maintenance. However, the fact that fox2b1 and fox2b2 gene expression is not down-regulated in the male gonad before 45–50 dpf suggests that the inhibition of the positive regulatory loop between cyp19a1a expression, estrogen production, and fox2 gene expression is not active at these early testicular differentiation stages. This absence of inhibition of fox2 expression in the early male differentiating gonad could suggest that there is additional regulation of this positive loop or that expression of fox2 is not sensitive to estrogens at these early developmental stages. Such a mechanism of action through the blockade of cyp19a1a and estrogen production assigns to SdY an activity as an antiovian determining factor directly preventing the ovarian differentiation pathway instead of activating the male pathway. However, it cannot be totally excluded that SdY, besides suppressing the female pathway, may also affect directly the activation of the male pathway. Nevertheless, known important male developmental actors such as Dmrt1, Amh, and Sox9 (1) were not identified in the Y2H screen, and this along with their late expression during rainbow trout male gonad development (33) compared with SdY expression suggests that they are not directly interacting partners of SdY.

In summary, we provide strong evidence that SdY determines sex in rainbow trout not by using part of its ancestral IrF9 pathway but by directly interacting with Foxl2, an important member of the classical gonadal sex differentiation cascade. This suggests that innovation at the top of the vertebrate sex determination cascade may be constrained because novel master SD genes have to cope with the regulation of the conserved vertebrate sex differentiation cascade. The “limited option” hypothesis is mainly based on the idea that only a small subset of genes and chromosomes, because they are better at doing the job, would be independently and repeatedly selected as new vertebrate master SD genes (15). We now propose that the limited option is actually more constrained by solstication of the sex differentiation pathway and that evolution of SdY genes may include some innovations like SdY if these unusual SD genes evolved a means to build an interface with the sex differentiation cascade.
given to each interaction as previously described (35). PBS scores were divided (National Center for Biotechnology
Genomic DNA Extraction. To clone rainbow trout
extraction phenol
fragments of these 202 positive clones were amplified by PCR and sequenced at

Yeast Two-Hybrid Screen. Yeast two-hybrid screening was performed by

Male Female

Plasmids and primers used are listed in the SI Appendix, Tables S4 and S5. The coding sequence of SdY was amplified from the psyd:psy:prcy: cfp plasmid (16) and inserted into pcS2*-HA::mCherry (gift from Manfred Gessler, University of Wuerzburg, Wuerzburg, Germany), pcS2*-emGFP, and pcS2*-3xHA expression plasmids. Rainbow trout foxl2b1 and foxl2b2 and medaka foxl2 (Ol-foxl2) were cloned by PCR amplification on genomic DNA and inserted in pcS2* plasmid, pcS2*-HA::mCherry, and pcS2*-3xFlag between the EcoRI-Xhol restriction sites. The constructs 3xHA-pcS2* and 3xFlag-pcS2* were obtained by concatenating three single HA sequences (3xHA) or three single FLAG sequences (3xFLAG) flanked by HindIII restriction sites. pcS2*-emGFP:SdY was obtained by inserting a PCR-amplified fragment corresponding to emGFP in frame into the EcoRI site. To explore the hypothesis that SdY could be able to interact with all Fox proteins through an interaction with their highly conserved Forkhead domain, we selected different rainbow trout Fox proteins from an EST resource collection in which ESTs were cloned into a CMV expression (pcCMV-Sport6) plasmid (36). Five trout cDNA clones encoding for Foxd3, Foxd3, Foxd1, Foxn3, and Foxn2 were found. Foxd1 and Foxn3 were found in the C-term transcriptional activation domain (34) as a template. The resulting model was obtained by the su-

Materials and Methods

Protein Structure Prediction. Three-dimensional homology modeling of SdY was predicted with the software SWISS-MODEL (https://swissmodel.expasy.org)) using the structure of the dimeric IRF5 (PDB ID 3D5H) transcriptional domain (34) as a template. The resulting model was obtained by the superposition of the template and SdY. The 3D views of SdY were made with PyMOL (molecular graphics system, version 1.7.4; Schrödinger).

Yeast Two-Hybrid Screen. Yeast two-hybrid screening was performed by Hybrigenics Services (https://www.hybrigenics-services.com). The coding sequence for SdY (amino acids 1–215) (GenBank accession number GI:392583258) was PCR amplified and cloned into pB27_A as a C-terminal fusion to LexA (W-LexA-SdY-Q) and into pB66_A as a C-terminal fusion to the Gal4 DNA-binding domain (W-Gal4-SdY-C). These constructs were checked by sequencing and used as baits to screen a random-primed Oncorhynchus mykiss immature male gonad (gonads sampled around 75 dpf) cDNA library; 112 million interactions were

For transfection, RTG2 cells were detached by Trypsin-EDTA (P0781; Sigma-Aldrich) and pelleted by centrifugation at 1,000 × g for 5 min, washed once with medium and once with PBS. The pellet was drained and resuspended in solution V (Amaxa Kit) at a density of 10⁶ cells/mL. 2 µg of plasmid were added to the suspension. After mixing, the suspension was transferred to a cuvette (Kit V; Amaxa). Program D-23 was used to electroporate the cells. After transfection, cells were immediately transferred to six-well plates filled with medium. All experiments were performed 72 h after transfection.

Immunofluorescence. HEK 293T cells were seeded in a 6-well plate containing coverslips. After pcS2*-meGFP:SDY and pcS2*-mCherry-Foxl2b1 (or pcS2*-3xFLAG:Foxl2b2 or pcS2*-3xFLAG-OL-Foxl2) cotransfection for 48 h, cells were fixed in 4% fresh paraformaldehyde for 15 min, extensively washed, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Then cells were blocked with 1% BSA for 20 min. The primary antibody (SI Appendix, Table S6) was incubated overnight at 4 °C. After extensive washing with PBS, cells were incubated with Alexa 488 or Alexa 594 conjugated secondary antibodies in 1% BSA for 1 h, followed by Hoechst 33342 (Invitrogen) staining for 5 min (1 µg/mL final concentration). Cells were mounted using Mowiol 4–88 (Roth). Confocal images were acquired using a Nikon Eclipse C1 laser-scanning microscope (Nikon), fitted with a 60× Nikon objective (PL APO, 1.4 N.A.), and Nikon image software. Images were collected at 1,024 × 1,024 pixel resolution. The stained cells were optically sectioned in the z axis. The step size in the z axis varied from 0.2 to 0.25 µm to obtain 50 slices per imaged file. All experiments were independently repeated at least three times.

Colocalization Analyses. The Nikon NIS-Elements imaging analysis software was used for the colocalization analyses. Confocal images of double-stained sections were first subjected to background correction. SdY nuclear translocation was counted as complete translocation when the majority of the SdY nuclear signal was found in the nucleus. Pearson’s correlation coefficient was calculated and used to obtain the colocalization values as percentages of SdY overlapping with Foxl2b1 or Foxl2b2 for a minimum of five cells (n = 5). The Pearson’s coefficient values were defined as very strong colocalization (24:1) extraction was done. DNA precipitation was performed with an equal volume of isopropanol (1:1). The precipitate was pelleted by centrifugation at 15,000 × g and washed twice in 70% ethanol, dried at room temperature, and dissolved in 2 mL of distilled water.
between 0.85 and 1, strong colocalization between 0.5 and 0.85, and weak or no colocalization between ~1 and 0.5.

**Co-Immunoprecipitation.** HEK-293T cells were transfected with pcS2*-3xHA: SdY and pcS2*-3xFlag-Foxl2b1 or pcS2*-3xFlag-Foxl2b2 or pcS2*-3xFlag-Ol-Foxl2 constructs to be assessed for their ability to co-immunoprecipitate. After 48 h cells were scraped and resuspended in 50 μL lysis buffer [20 mM Hepes (pH 7.8), 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.5% Nonidet-P40, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 200 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 100 mM NaF]. Cells were incubated in lysis buffer for 30 min at 4 °C and then cleared by high-speed centrifugation for 20 min. After Bradford protein concentration measurement, HNTG buffer (20 mM Hepes pH 7.5; 150 mM NaCl; 10% glycerol; 0.1% Triton X-100) was added (1:1 to 250 μg of the whole-cell lysate. After preclearing with IgG antibodies for 1 h at 4°C whole-cell lysates were used for immunoprecipitation with the corresponding antibodies. One microgram of anti-Flag, anti-HA, or IgG antibody was added to 500 μL of cell lysate or 5 μg of anti-SdY or anti-Foxl2 (SI Appendix, Table S6) and then incubated at 4 °C overnight. After the addition of washed protein G agarose beads (Pierce, 20398), incubation in HNTG buffer was continued for another 2 h. Immunoprecipitates were washed (five times with centrifugation in 1,000 x g, supernatant discarded, HNTG lysis buffer added) and eluted with SDS/PAGE loading buffer by boiling for 10 min. Co-immunoprecipitation was detected by standard Western blot analysis procedure.

**Western Blotting.** Cells were lysed in a Hepes-based lysis buffer [20 mM Hepes (pH 7.8), 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 200 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 100 mM NaF] for 3 h. Cells debris was pelleted by centrifugation for 15 min at 16,000 x g. Cell lysate protein concentration was measured with a Bradford assay (Cay Gröf Spectrophotometer; Varian). The protein lysates (30-50 μg) were resolved by SDS/PAGE on 12% Tris-glycine gels followed by transfer to nitrocellulose membranes. Unspecific binding was blocked with 5% BSA in Tris buffered saline with Tween-20 (TBST) [10 mM Tris (pH 7.9), 150 mM NaCl, 0.1% Tween-20] for 1 h at room temperature. Incubation with primary antibodies was performed overnight at 4 °C. After three washes with TBST, HRP conjugated antibodies were incubated with blocking solution for 1 h. Following the washes, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) for 1 min. The signal from the membrane was detected using a Photo Image Station 4000MM (Kodak). At least two independent experiments were performed, and representative protein blot images are shown.

**Quantitative PCR.** Expression levels of sdY, foxl2b1, foxl2b2, nr5a1, and cyp19a1a were measured by qPCR as previously described (37). Gonads (15-20 pairs of gonads per time points) were sampled in triplicate at 33, 35, 37, 40, 44, 50, 61, 85, and 125 dpf in both genetic all-male (XY) and all-female (XX) populations of rainbow trout. Total RNA was extracted using the RNAqueous-Micro Kit (Ambion, Life Technologies) for the 33- to 50-dpf samples and the RNAqueous-Kit (Ambion, Life Technologies) for the 61- to 125-dpf samples. All samples were then treated with the TURBO DNA-free Kit (Ambion, Life Technologies) to remove any leftover genomic DNA. Reverse transcriptions were carried out using 150 ng of total RNA as the starting material with the Ovation RNA Amplification System (NuGEN Technologies), following the manufacturer’s recommendations. Quantitative PCR was performed using the StepOnePlus system (Applied Biosystems) using 4 μL of cDNA (single tube quantification per sample, with three biological replicates for each sex and time point) diluted to 1:90, the Fast SYBR Green Master Mix (Applied Biosystem), and 600 nmol of each primer listed in SI Appendix, Table S7. The enzyme was activated for 20 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and elongation at 60 °C for 30 s.

**Whole-Mount In Situ Hybridization.** In situ hybridization was performed as previously described (38). RNA probes were produced from PCR products obtained by amplification of foxl2b2. Ten nanograms of the PCR product were used as a template for digoxigenin-labeled RNA probe synthesis using digoxigenin-11-UTP (Roche Diagnostics Corp.) and T3 or T7 RNA polymerase (Promega) following standard protocols. Whole-mount in situ hybridization was carried out using an in situ Pro, Intavis AG robotic station. Male and female embryos were fixed overnight in 4% paraformaldehyde at 4 °C, dehydrated in 100% methanol, and stored at ~−20 °C. Before in situ hybridization they were rehydrated, permeabilized by proteinase K treatment (25 μg/mL, 30 min, at room temperature), and postfixed (4% paraformaldehyde and glutaraldehyde 0.2%, for 20 min). Prehybridization and hybridization media contained 50% formamide, 5XSSC, 0.1% Tween 20, 0.005% heparin, 0.1 mg/mL RNA. Hybridization was carried out at 65 °C for 16 h. After post-hybridization washes, embryos were incubated in blocking buffer (PBS/0.1%Tween 20 0.2%, containing 2% serum) for 2 h before the addition of the alkaline phosphatase conjugated digoxigenin antibody (1:2,000; Roche Diagnostics Corp.) for 6 h. After washing, the color reaction was performed in...
the presence of nitro-blue tetrazolium/5-Bromo-4-chloro-3-indoly phosphosphate (NB/T/BCIP) (Roche). Briefly, dehydration and paraffin infiltration were performed for 16 h. A 1000 tissue processor (Shandon). Dehydrated tissues were embedded in plastic molds in paraffin using a HistoEmbedder (TS888; Medite). Each embedded sample was sectioned 5 μm thick on a MICRO HM355 (Thermo Fisher Scientific).

**Colocalization of sdy and fox2b by in Situ hybridization.** Fifty-dpf male rainbow trout were fixed in Bouin’s fixative for 4 h. One hour after fixation and dehydration, the tissues were embedded in paraffin and cut into 4-μm sections. Anti-sdy and foxz2b RNA probes were synthesized using in vitro transcription with a fluorescein RNA labeling mix (Roche) and a DIG RNA labeling mix (Roche). Sections were deparaffinized, rehydrated, and treated with 1% H2O2 in TBST buffer at room temperature for 30 min and 1 μg/mL proteinase K (Roche) at 37 °C for 13 min. After the enzymatic treatment, sections were dehydrated with ethanol and chloroform and then hybridized with sdy and fox2b probes simultaneously at 60 °C for 18 h. Fluorescin was visualized by using an anti-fluorescein-alkaline phosphatase (anti-fluorescein–AP) Fab fragment (Roche) (1:1000) and the HNPP Fluorescent Detection Set (Roche). Digoxigenin (DIG) was visualized by using an anti-digoxigenin–AP Fab fragment (Roche) (1:500) and NB/T/BCIP. Before the DIG visualization, alkaline phosphatase was inactivated in 0.1 M glycine–0.1% Tween 20 at room temperature for 30 min. DAPI staining was performed to visualize nuclei.

**Luciferase Assay.** HEK 293T cells were transfected using PEI with the following luciferase constructs: firefly luciferase readings were used for calibration. Each experiment was performed with a 1.0-μL luciferase reaction mixture containing 0.5 μg of pCS2−“mEGFP-Sdy,” 0.5 μg of pCS2−“HAmyFoxz2b,” and 0.5 μg of pCS2−“Olafoxz2b,” respectively, was transcribed from linearized pCS2+ plasmid using the SP6/T7 T7 MESSMAGE mMachine Kit (Ambion). One nanoliter was injected into the cytoplasm of one-cell stage Medaka embryos.

**Statistical Analysis.** Most of the data were analyzed using an unpaired Student’s t test. A Mann–Whitney U test was then used to compare the median value when the Kolmogorov–Smirnov test was negative. Statistical analysis of multiple groups was performed using an ANOVA followed by a Dunnett or Turkey test for multiple comparison (a control group compared with an experimental group or a control sample compared to an experimental group, respectively). All statistical analyses were performed with GraphPad Prism (version 5; GraphPad Software). Significant differences are symbolized in figures by asterisks if P < 0.001 (**), P < 0.05 (**), or P < 0.01 (*) or indicated by ns if not significant.

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**One-by-one Yeast Two-Hybrid Assay.** Diploid cells containing the same bait construct of the yeast two-hybrid assay (p66_Sdy) and a prey plasmid constructed coding for Foxz2b cloned in frame with the activation domain of GAL4 (p14-N–GAL4-Foxz2b-C) were mated and spotted on selective media. The medium lacking tryptophan and leucine was used as a control for the yeast growth test and to check for the presence of the bait or the prey. The assay is based on the histidine reporter gene. A triple-negative medium (tryptophan, leucine, and histidine) selects yeast growth if interaction occurs. Interaction pairs were tested at decreasing concentrations (10−1, 10−2, 10−3, 10−4) from two independent clones. An inhibitor of the histidine gene product 3-AT was used to increase stringency at four different concentrations (1, 5, 10, 50 mM).

**RNA injections.** For injections capped RNA GFP-sdy, mCherry-Foxz2b, Olafoxz2b from pCS2−“mEGFP-Sdy,” pCS2−“HAmFoxz2b,” and pCS2−“Olafoxz2b,” respectively, was transcribed from linearized pCS2+ plasmid using the SP6/T3/ T7 MESSMAGE mMachine Kit (Ambion). One nanoliter was injected into the cytoplasm of one-cell stage Medaka embryos.