The Zebrafish Period2 Protein Positively Regulates the Circadian Clock through Mediation of Retinoic Acid Receptor (RAR)-related Orphan Receptor α (Rora)*

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Background: Null mutants for zebrafish period2 were generated to elucidate its functions.

Results: Both locomotor activity and expression of key circadian clock genes are disrupted in the period2 mutant zebrafish.

Conclusion: Period2 is essential for zebrafish circadian regulation.

Significance: Period2 plays a positive role in the zebrafish circadian clock by enhancing bmal1b expression through binding to nuclear receptor Rora.

We report the characterization of a null mutant for zebrafish period2 (per2) generated by transcription activator-like effector nuclease and a positive role of PER2 in vertebrate circadian regulation. Locomotor experiments showed that per2 mutant zebrafish display reduced activities under light-dark and 2-h phase delay under constant darkness, and quantitative real time PCR analyses showed up-regulation of cry1aa, cry1ba, cry1bb, and aanat2 but down-regulation of per1b, per3, and bmal1b in per2 mutant zebrafish, suggesting that Per2 is essential for the zebrafish circadian clock. Luciferase reporter assays demonstrated that Per2 represses aanat2 expression through E-box and enhances bmal1b expression through the Ror/Rev-erb response element, implicating that Per2 plays dual roles in the zebrafish circadian clock. Cell transfection and co-immunoprecipitation assays revealed that Per2 enhances bmal1b expression through binding to orphan nuclear receptor Rora. The enhancing effect of mouse PER2 on Bmal1 transcription is also mediated by RORα even though it binds to REV-ERβ. Moreover, zebrafish Per2 also appears to have tissue-specific regulatory roles in numerous peripheral organs. These findings help define the essential functions of Per2 in the zebrafish circadian clock and in particular provide strong evidence for a positive role of PER2 in the vertebrate circadian system.

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Significance: Period2 plays a positive role in the zebrafish circadian clock by enhancing bmal1b expression through binding to nuclear receptor Rora.

Circadian clocks, which evolved from long and enduring adaptation of organisms to the light/dark cycle on Earth (1–3), can be reset and synchronized by local environmental cues such as light and even food (4, 5) and regulate behavior and physiology with a period of ~24 h (6–8). The molecular genetic mechanisms underlying circadian regulation have been established by a series of genetic and biochemical studies in several organisms including Cyanobacteria (9), Neurospora crassa (10, 11), Drosophila melanogaster (12, 13), and mice (14–16). In the circadian system, the oscillating pacemaker is regulated by the transcription/translation-based negative feedback loop (17, 18). In flies, CLOCK and CYCLE as positive factors form a heterodimer to activate transcription of period (per)2 and timeless (tim) and PER and TIM as negative factors form a heterodimer to turn off their own transcription (19). Likewise, in mammals, CLOCK and BMAL1 (vertebrate ortholog of fly CYCLE) form a heterodimer to activate transcription of Period and Cryptochrome (Cry) by binding to the E-box elements in the promoter regions of these genes (20–22), and PER and CRY form a heterodimer (PER-CRY) to repress their own transcription (2, 23, 24). Mammals have another loop wherein RORα activates Bmal1 transcription by binding to the ROR/REV-ERβ response element (RORE) in the Bmal1 promoter, whereas REV-ERβ competes to bind to RORE to repress Bmal1 transcription (25–27).

The zebrafish (Danio rerio) is an excellent model for circadian studies and is equipped with circadian regulatory components similar to those of flies and mammals (28–31). Light and temperature are crucial circadian entrainment factors in zebrafish (32–34), and in particular, zebrafish peripheral tissues can directly respond to light signals to generate rhythmicity (35, 36). Two light receptors (Tmt opsin and Opsin4.1) were shown to be crucial for light entrainment in zebrafish peripheral cir-

2 The abbreviations used are: per, period; tim, timeless; Cry, cryptochrome; TALEN, transcription activator-like effector nucleases; LD, light/dark; DD, constant darkness; Rora, retinoic acid receptor (RAR)-related orphan receptor a/Nr1f1, nuclear receptor subfamily 1, group F, member 1; Rev-erba/Nr1d1, nuclear receptor subfamily 1, group D, member 1; RORE, Rev-erb response element; aanat, aryalkylamine N-acetyltransferase; co-IP, co-immunoprecipitation; qRT-PCR, quantitative real time PCR; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.
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circadian clocks (37). In addition, light can entrain the circadian clock to impact the cell cycle and DNA damage repairs in zebrafish (33, 38). Although the transcription/translation-based loops are also thought to operate in zebrafish, there are notable differences in circadian regulation between zebrafish and mammals; for instance, at the transcription level, clock and rora oscillate in numerous tissues in zebrafish but not in mice (29), and csnk1b, which encodes for casein kinase 6, also oscillates in zebrafish pineal gland but not in mice (39), emphasizing the necessity and importance to investigate the zebrafish circadian clock to obtain a full understanding of the vertebrate circadian mechanisms.

Mammalian Period2 as a canonical component of the circadian clock plays important roles in the circadian clock (21, 40), sleeping (41), metabolism (42), and carcinogenesis (43). In humans, a PER2 missense mutation abolishes phosphorylation by CK1δ (44) and results in familial advanced sleep phase syndrome (45). Mouse Per2 is a light-responded gene, and its circadian phase and amplitude of expression in the suprachiasmatic nuclei can be altered by different light/dark cycles (46). Zebrafish per2 is also a light-regulated gene, and its expression is significantly damped under constant darkness (32). Using transgenic fish and stably transfected cell line-based assays, a light-responsive module composed of D-box and E-box motifs within the per2 promoter was identified (32). In addition, zebrafish per2 is required for expression of the clock-controlled arylalkylamine N-acetyltransferase 2 (aanat2) (47, 48) encoding the rate-limiting enzyme for melatonin synthesis (48).

Despite all this important progress concerning zebrafish per2, there have been no stable genetic mutants for zebrafish per2, which are critical for determining its roles in zebrafish circadian regulation as well as other life processes. Recently, genome-editing tools, zinc finger nucleases, transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 have been developed to generate site-specific DNA double strand breaks that trigger the endogenous nonhomologous end joining DNA repair pathway to induce indel mutations in targeted genes in numerous species including zebrafish (49–51). Here we have successfully generated two lines of per2-null mutants with TALEN. Characterization of per2 mutant zebrafish showed that Per2 is essential for maintaining rhythmicity of zebrafish locomotor activities and expression of circadian clock genes as well as a circadian clock-controlled gene. We also determined that Per2 plays dual roles in the zebrafish circadian clock, not only repressing expression of E-box-containing genes but also promoting expression of RORE-containing genes. The positive role of PER2 in Bmal1 expression is conserved from zebrafish to mice with the difference that zebrafish Per2 enhances bmal1b expression through its binding of Rora rather than Rev-erba, whereas the enhancement of Bmal1 expression by mouse PER2 still requires RORα mediation even though it binds to REV-ERBα rather than RORα. Zebrafish Per2 seems to have distinct regulatory functions in the different peripheral organs. These results should help elucidate the essential functions of Per2 in the zebrafish circadian clock and provide critical evidence for a positive role of PER2 in the vertebrate circadian system.

EXPERIMENTAL PROCEDURES

Zebrafish Maintenance and Embryo Production—Zebrafish wild-type AB strain and per2 mutant lines were raised on a 14-h/10-h light/dark (LD) cycle at 28 °C in the Soochow University Zebrafish Facility according to standard protocols (52). Embryos were produced by pair mating, maintained in culture dishes, and used for experiments at specified stages.

TALEN Construction and Microinjection—TALEN target sites were designed using the web tool TALE-NT (53). TALEN expression vectors were constructed using the “unit assembly” method with Sharkey-AS and Sharkey-R forms of FokI cleavage domains as described previously (54). Briefly, TALEN expression vectors were linearized by NotI and used as templates for capped TALEN mRNA synthesis with the SP6 mMESSAGE mMACHINE kit (Ambion). Capped TALEN mRNAs encoding each monomer were simultaneously microinjected into one-cell zebrafish embryos.

Analysis of Mutagenesis Frequencies and Identification of per2 Mutants—The injected embryos were maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28.5 °C. The genomic DNAs of five groups (five embryos each) were extracted at 2 days postfertilization. A 259-bp DNA fragment containing the per2 target site was amplified by PCR (primers used are listed in Table 1). 7 μL of the PCR product was digested using BslI (New England Biolabs) at 55 °C for 3 h. The intensities of cleaved and uncleaved bands were quantified with NIH ImageJ and used for estimating mutagenesis frequencies. The uncleaved bands were recovered and cloned into pMD-19T (Takara) for sequencing analysis. To identify germ line-transmitted mutations, the microinjected founder (F0) embryos were raised to adulthood. The F0 fish were then outcrossed with wild-type zebrafish to produce F1. From each cross, 10 F1 embryos were collected for genomic DNA extraction and enzymatic digestion (data not shown). Siblings of the F1 embryos that carry heritable mutations were raised to adulthood, and an individual F1 fish was reidentified via PCR amplification and sequencing with fin clipped DNAs. Homozygous per2 mutant fish were generated by crossing of the male and female fish carrying the same mutation. Two per2-null mutant lines were established (see Fig. 1D).

Behavioral Analysis for Zebrafish—Locomotor activity analysis was performed as described previously with some modifications (39). On the 4th day postfertilization, larvae were singly placed into each well of the 48-well plate. Locomotor activities of larvae were monitored and recorded for 4 consecutive days using an automated video tracking system (Videotrack, ViewPoint Life Sciences, Montreal, Canada) and analyzed with Zebralab3.10 software (ViewPoint Life Sciences). Locomotor activities were measured from day 5 to day 8 postfertilization as the total distance moved by one larva during 10-min time windows. The data are presented as a moving distance average for each group (n = 24). The period length of each larval locomotor trace was retrieved by a fit to a damped cosine curve using non-linear least square fitting with the CellulaRhythm R script (55). Statistical analysis of period length differences between the treatment groups was performed with one-way analysis of
TABLE 1

All primers used in the study

| Gene      | Primer sequence | Accession no. | Note                  |
|-----------|-----------------|---------------|-----------------------|
| per2      | AATTCACCAGGAAATTGACTTC | ENSDART0000148788 | TALEN target fragment |
| per2      | ATCTAGCGATCATACGTCAC | ENSDART0000148788 | cDNA cloning          |
| per2      | CGCGGTCGGACCGGGAGACATC | ENSDART0000148788 | cDNA cloning          |
| rev-erha  | TGTTGCTGTTGCTGAGTCTG | ENSDART0000126282 | cDNA cloning          |
| roraa     | GCACTGCTGTTGCTGAGTCTG | ENSDART0000148537 | cDNA cloning          |
| rorab     | AGCGTTGCTGTTGCTGAGTCTG | ENSDART000019140 | cDNA cloning          |
| anat2 promoter | AGCGTTGCTGTTGCTGAGTCTG | ENSDART000018205 | Promoter cloning      |
| bmaltb promoter | AGCGTTGCTGTTGCTGAGTCTG | ENSDART0000098259 | Promoter cloning      |
| per1b     | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| per2      | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| per3      | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| cry1aa    | GACGCACAGACATGAAAGAGCG | ENSDART0000034401 | qRT-PCR              |
| cry1ba    | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| cry1bb    | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| bmaltb    | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| anat2     | AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| β-Actin   | AAGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |
| Luciferase| AGAAAGCTCATGAATCAAGATG | ENSDART0000011082 | qRT-PCR              |

variance followed by Dunnett’s posttest comparing each sample group with the control group (55).

RNA Extraction and Quantitative Real Time PCR (qRT-PCR)—Total RNAs were extracted from ~30 larvae of homozygous per2 or wild type at 4-h intervals from 120 to 148 h postfertilization under LD or DD conditions and from adult organs including the brain, muscle, heart, and liver under LD using TRIzol (Invitrogen) reagent, respectively. qRT-PCR was performed in an ABI StepOnePlus instrument with the SYBR Green detection system (Invitrogen). PCR thermal profiles were 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Experiments were performed in triplicates, each with at least two different biological samples for corresponding genotypes and developmental stages. All results were normalized to the expression level of the housekeeping gene β-actin. qRT-PCR results are shown as a relative expression level calculated using the 2−ΔΔCT method, where values were analyzed with one-way analysis of variance test or Student’s t test. All primers used are listed in Table 1.

DNA Site-directed Mutagenesis—The mutated DNA vectors were constructed by PCR-based site-directed mutagenesis. PCR was performed with KOD Plus DNA polymerase (Toyobo). DpnI (New England Biolabs) restriction enzyme-treated PCR products were transformed into Escherichia coli. Positive clones were selected and verified by sequencing.

Cell Transfection and Luciferase Reporter Assays—Human embryonic kidney (HEK) 293T cells were used for cell transfection assays. HEK 293T cells were cultured in DMEM containing 10% serum and penicillin-streptomycin in 24-well plates. Transfection was done with Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. Reporter gene assays were performed with the Dual-Luciferase reporter assay system (Promega) using 100 ng each for bmal1b-luc, roraa, rorab, and rev-erba and 200 ng for per2. 2 ng of pRL-TK vector was added for control, and pCDNA3.1-Myc/His was added to bring to the same amount. Each experiment was conducted in triplicate.

Co-immunoprecipitation Assay and Western Blotting—One day before transfection, HEK 293T cells were seeded in 10-cm Petri dishes. Thirty-four hours following transfection, cells were lysed in radioimmune precipitation assay buffer with protease inhibitor (Sigma). Lysates were released with protein G-Sepharose beads (GE Healthcare) and then incubated with
rabbit polyclonal anti-HA antibodies (Protech) or anti-His antibodies (Protech). After washing five times, the precipitates were resuspended in SDS-PAGE sample buffer, boiled for 3 min, and resolved by 8% SDS-PAGE followed by Western blot analysis using mouse monoclonal anti-His antibody or anti-HA antibody (Protech). Immuno reactive bands were detected by ECL reagents (Biological Industries).

Generation of bmal1b-luc Transgenic Fish and in Vivo Measurement of Bioluminescence Rhythms—The vector bmal1b-luc was linearized by KpnI digestion and microinjected into one- to two-cell zebrafish embryos. Injected embryos were raised to adulthood and individually bred to wild-type fish or pairwise bred to each other. Transgenic progeny were identified by PCR using a pair of primers (Table 1). Transgenic embryos or larval fish were placed individually in a well of a 96-well with 200 μl of Holtfreter solution (7.0 g of NaCl, 0.4 g of sodium bicarbonate, 0.2 g of CaCl2, and 0.1 g of KCl (pH 7.0) in 2 liters of double distilled H2O) aerated overnight and containing 0.5 mM D-luciferin potassium salt (BBI). The monitoring of bioluminescence was performed with a Luminoskan Ascent microplate luminometer (Thermo), and data analysis was performed according to the protocol described previously (55).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed according to the manufacturer’s protocol (Milipore’s ChIP assay kit). Briefly, a group of 200 capped per2 mRNA-injected larvae and control injected larvae at 5 days postfertilization was collected and cross-linked in 2% formaldehyde at room temperature for 30 min, and then a 1/10 volume of 1.25 M glycine was added to stop cross-linking followed by PBS washes (three times, each for 10 min). We used purified rabbit or mouse IgG (Invitrogen) as a negative control. ChIP PCRs were performed using primers flanking the E-boxes or RORE sites as well as primers not flanking the E-boxes or the RORE sites in the promoter regions of annat2 or bmal1b as controls. Primers used for the ChIP PCR are listed in Table 1.

Statistical Analysis—Groups of data are presented as mean ± S.E. We performed statistical analyses with analysis of variance or the unpaired two-tailed Student’s t test. All statistical analyses were performed using SPSS 16.0 software, and p < 0.05 was regarded as a statistically significant difference.

RESULTS

Generation of Zebrafish per2 Mutants—Using the software TALE-NT (53), we selected a TALEN pair targeting the second exon (containing the start codon ATG) of zebrafish per2, and within the targeted 57-bp fragment, there is a BssI restriction site for evaluating mutagenesis efficiency and subsequent mutant identification (Fig. 1A). We then used the “unit assembly” method (54) to construct the two arms of the per2 TALEN. The capped mRNAs of the two TALEN arms were microinjected into one-cell embryos at a concentration of 250 pg. To evaluate the mutagenesis efficiency, a 259-bp genomic DNA fragment containing the target site was PCR-amplified from five groups of injected embryos and a control group of embryos (five embryos each). Enzymatic digestion of the PCR-amplified fragments with BssI showed that the efficiencies of the five groups are 10.5, 18.9, 20, 11.2, and 10.2%, respectively, with an average of 14.2% (Fig. 1B). We cloned the uncleaved PCR fragments into the sequencing vector pMD-19T. Single clone sequencing revealed six different types of indel mutations in the per2 TALEN target site in F0 (Fig. 1C). The siblings of these microinjected F0 embryos were raised to adulthood. Out-crossing F0 fish with wild-type fish produced F1 embryos. Two of 15 fish examined with PCR amplification and BssI digestion of DNAs extracted from their F1 embryos were found to carry heritable mutations (Fig. 1D). DNA sequencing showed that one fish carried a 11-bp deletion and the other carried a 13-bp insertion, which both result in frameshift mutations: the 11-bp deletion mutation might encode a truncated protein with only 160 amino acids, and the 13-bp insertion mutation might encode a truncated protein with only 167 amino acids (Fig. 1E). The homozygous mutant fish with the 11-bp deletion were used primarily for all subsequent experiments.

Disrupted Rhythmicity of Locomotor Activities and Altered Expression of Key Circadian Clock Genes and a Circadian Clock-controlled Gene in per2 Mutant Zebrafish—Locomotor activities of zebrafish larvae exhibit robust circadian rhythmicity and peak during the subjective day (57). To determine whether Per2 affects locomotor activity rhythms, behavior analyses were performed for per2 mutant and wild-type larvae starting at 96 h postfertilization under LD and DD conditions. Under the LD condition, the locomotor activities of per2 mutant larvae were significantly reduced compared with wild types (Fig. 2A); specifically there was an ~30% reduction in the total moving distance during the 3 days examined (Fig. 2B). Under the DD condition, per2 mutant larvae displayed an approximately 2-h phase delay (Fig. 2C) and an ~1.1-h lengthened periodicity (Fig. 2D). These results indicate that the locomotor activity rhythms are disrupted in per2 knock-out fish.

We also examined expression of key circadian clock genes and a circadian clock-controlled gene in per2 mutant fish. Zebrafish possess four per genes, per1a and per1b (co-orthologs of mammalian Per1) and per2 and per3 (single orthologs of mammalian Per2 and Per3) (58). Under the LD condition, per2 exhibited robust oscillation in wild types but still oscillates with much damped amplitude in per2 mutant zebrafish, whereas under the DD condition, per2 became arrhythmic in both wild-type and per2 mutant zebrafish (Fig. 2, E and F). Compared with wild types, per1b and per3 were significantly down-regulated in per2 mutant fish under both LD and DD conditions (Fig. 2, E and F), suggesting that Per2 plays a positive role in regulation of these two zebrafish per genes. Similar to per genes, cry genes are negative regulators in the transcription/translation feedback loop (59). Although mice have two Cry genes (59), zebrafish have six cry genes (31, 56, 60, 61). Under both LD and DD, crylaa, crylba, and cry1bb were primarily up-regulated in per2 mutant fish (Fig. 2, E and F), suggesting that Per2 plays a repressive role in regulation of these cry genes. Intriguingly, bmalb gene, one of the two co-orthologs of mouse Bmal1 (62), was down-regulated in per2 mutant under both LD and DD conditions (Fig. 2, E and F), suggesting that Per2 plays a positive role in bmalb expression. The other two bmal genes (bmal1a and bmal2) also had disrupted expression patterns in per2 mutant under both LD and DD conditions (Fig. 2, E and F).

Melatonin plays an important role in the endogenous circadian clock system in vertebrates (63). Melatonin rhythms are
generated by the oscillating rate-limiting enzyme aralkylamine N-acetyltransferase (AANAT) in the pineal gland (47, 64). Mammals have only a single Aanat gene that is expressed in both the pineal gland and the retina (65). In zebrafish, there are two copies of aanat genes, aanat1 and aanat2. Although aanat1 is expressed only in the retina, aanat2 is expressed in the pineal gland and in the retina at relatively lower levels (66). aanat2 was significantly up-regulated under both LD and DD conditions in per2 mutant fish (Fig. 2, E and F), suggesting that Per2 plays a repressive role in regulation of aanat2.

We also characterized another null per2 mutant line carrying the 13-bp insertion and found that it displays a similar behavioral phenotype (data not shown) and altered gene expression (Fig. 2, E and F) like the 11-bp deletion mutant line. These results demonstrate that like mammalian Per2 zebrafish per2 is also essential for the zebrafish circadian clock.
Our qRT-PCR analysis showed that *aanat2* is up-regulated but *bmal1b* is down-regulated in *per2* mutant fish (Fig. 2, E and F), indicating that Per2 represses *aanat2* expression but enhances *bmal1b* expression in zebrafish. To investigate the role of Per2 in zebrafish *aanat2* expression, a 405-bp *aanat2* promoter containing one E-box was isolated and cloned into the pGL4.17 vector (Fig. 3A). The full-length cDNAs of
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FIGURE 3. Dual roles of zebrafish Per2 are mediated by E-boxes and RORE boxes. A, schematic of the DNA construct aanat2-luc wherein the aanat2 promoter contains one E-box. B, schematic of the DNA construct bmal1b-luc wherein the bmal1b promoter harbors the two RORE motifs (in red), which are identical in human, mouse, and zebrafish. C, Per2 inhibits the Clock1a-Bmal1b heterodimer-mediated aanat2 transcription through E-box. The activities of the E-box containing aanat2 promoter are activated by co-transfection of Clock1a and Bmal1b but repressed by Per2. The effects of both activation and repression are lost without the E-box. D, Per2 enhances Rorαa- or Rorαb-mediated bmal1b expression. Both Rorαa and Rorαb can activate bmal1b transcription. The activation efficacy of Rorαa is ~2-fold that of Rorαb. Rev-erbα outcompetes Rorαa or Rorαb to repress bmal1b expression. Per2 reverses the inhibitory effects of Rev-erbα to enhance bmal1b expression. All effects are lost without the two RORE motifs. E, zebrafish Rev-erbα outcompetes Rorαa to repress bmal1b in a dose-dependent manner. F, zebrafish enhances bmal1b expression in a dose-dependent manner. G, the inhibitory effect of Rev-erbα was not significant when RORE1 was mutated. H, the inhibitory effect of Rev-erbα still persisted when RORE2 was mutated. The Dual-Luciferase reporter system was used in the experiments. ***, p < 0.001, unpaired two-tailed Student’s t test. Error bars represent S.E.
per2, bmal1b, and clock1a were cloned into the pcDNA3.1-Myc/His vector, respectively. In vitro cell transfection showed that aanat2 is significantly up-regulated by a combination of Clock1a and Bmal1b but repressed by Per2, and Per2 could no longer inhibit aanat2 when the E-box in the aanat2 promoter was mutated (Fig. 3C). In addition, ChIP assays showed that Per2 can bind to the E-box in the aanat2 promoter (Fig. 4E). These results demonstrate that zebrafish Per2 can repress the gene expression through the E-box elements, which is consistent with mouse PER2 function (67).

Similar to the two RORE boxes in the mouse Bmal1 promoter (68), we also identified the two RORE motifs in the zebrafish bmal1b promoter region (Fig. 3B). To determine the role of Per2 in zebrafish bmal1b expression, a 1.7-kb promoter of bmal1b harboring the two RORE motifs was isolated and cloned into the pGL4.17 vector. Due to the third round of teleost genome duplication (58, 62, 69), zebrafish have two copies of rora genes, roraa and rorab (70). The full-length cDNAs of rev-erba, roraa, and rorab were PCR-amplified and cloned into the pcDNA3.1-Myc/His vector, respectively. Cell transfection assays showed that these two rora genes can significantly activate bmal1b expression, and in particular, the activation activity of roraa is ~2-fold higher than that of rorab (Fig. 3D). In addition, Rev-erba can outcompete Roraa or Rorab to inhibit bmal1b expression in a dosage-dependent manner (Fig. 4E). These results demonstrate that like what happens in mammals Rora activates bmal1b expression, whereas Rev-erba represses it in zebrafish.

To determine how Per2 regulates bmal1b expression, we also conducted co-transfection experiments with per2, roraa, rorab, or rev-erba. Results showed that Per2 can enhance bmal1b expression via RORE motifs (Fig. 3D), and the enhancing effects of Per2 on bmal1b expression are also dosage-dependent (Fig. 3F). To examine the roles of the two RORE motifs in bmal1b expression, we mutagenized them in the bmal1b promoter. Luciferase assays showed that the inhibitory effect of Rev-erba, the activating effect of Roraa, and the enhancing effect of Per2 on bmal1b expression are all abolished (Fig. 3D). These results indicated that the two RORE motifs are required for Per2 to enhance bmal1b expression in zebrafish (Fig. 3D).

To further distinguish the roles of these two RORE motifs in bmal1b expression, we mutagenized them individually. Results showed that although both the individually mutated RORE motifs can result in the reduction of Rora-mediated bmal1b expression, whereas Rore2 is necessary for enhancement of the level of activation (Fig. 3, G and F).

Per2 Enhances bmal1b Expression in Vivo—To examine the positive role of Per2 in regulating bmal1b in vivo, we generated four stable transgenic zebrafish lines including 1) wild-type Tg(1.7bmal1b-luc) AB wherein luciferase is driven by the normal 1.7-kb bmal1b promoter in wild-type fish, 2) wild-type Tg(1.7bmal1b-ROREmut-luc) AB wherein luciferase is driven by the 1.7-kb bmal1b promoter with the two mutated RORE motifs in wild-type fish, 3) per2 mutant Tg(1.7bmal1b-luc) per2−/− wherein luciferase is driven by the normal 1.7-kb bmal1b promoter in per2 mutant fish, and 4) per2 mutant Tg(1.7bmal1b-ROREmut-luc) per2−/− wherein luciferase is driven by the 1.7-kb bmal1b promoter with the mutated RORE motifs in per2 mutant fish. Bioluminescence assays showed that although in wild-type Tg(1.7bmal1b-luc) AB transgenic fish...
bmal1b displays robust oscillation and in per2 mutant Tg (1.7bmal1b-luc) per2−/− transgenic fish bmal1b exhibits much dampened oscillation (Fig. 4, A and B) in both wild-type Tg (1.7bmal1b-ROREmut-luc) AB and per2 mutant Tg (1.7bmal1b-ROREmut:luc) per2−/− transgenic fish bmal1b shows no oscillation (Fig. 4, C and D). These results indicate that Per2 and RORE motifs are crucial for bmal1b rhythmic expression in vivo.

To determine whether Per2 binds to the RORE motifs in the bmal1b promoter region, capped mRNAs of per2 were microinjected into one-cell embryos for ChIP assays. The results showed that Per2 can bind to RORE motifs in the bmal1b promoter at ZT0 and ZT12 in vivo (Fig. 4E), demonstrating that the enhancing effect of Per2 on bmal1b expression depends upon the RORE motifs in the bmal1b promoter.

We also performed rescue experiments by microinjecting capped wild-type per2 mRNAs into per2 mutant embryos. qRT-PCR results showed that wild-type per2 mRNAs indeed can down-regulate aanat2 but up-regulate bmal1b in per2 mutant fish (Fig. 4, F and G), indicating that the TALEN-induced mutation is responsible for its phenotypes.

Per2 Enhances bmal1b Expression through Rora Rather than Rev-erba—To determine whether Per2 enhances bmal1b expression through Rora, Rorab, or Rev-erba in zebrafish, we conducted cell transfection experiments. The results showed that Per2 alone cannot reverse the inhibitory effect of Rev-erba on bmal1b expression (Fig. 5A) and that Per2 can enhance Rora- or Rorab-mediated bmal1b expression (Fig. 5B). We also performed co-immunoprecipitation (co-IP) assays. The results showed that Per2 can directly bind to Rora rather than to Rev-erba (Fig. 4, C and D). Taken together, these results showed that Per2 can enhance bmal1b expression through Rora or Rorab rather than through Rev-erba in zebrafish.

In mice, the N-terminal LXXLL motif of PER2 was shown to be able to bind to REV-ERBα (24). We observed that zebrafish Per2 also possesses the same LXXLL motif at its N terminus (Fig. 5E). Does zebrafish Per2 bind to Rora through its N-ter-
minal LXLL motif? To address this question, we mutagenized the Per2 LXLL motif and performed co-IP and transfection assays. The results showed that Per2 with the mutated LXLL motif neither binds to Rora (Fig. 5F) nor enhances Rora-mediated bmal1b expression (Fig. 5G), further supporting the notion that the positive role of zebrafish Per2 in bmal1b expression is fulfilled through its binding to Rora rather than to Rev-erbα.

Enhancement of Bmal1 Expression by PER2 Is Evolutionally Conserved—Our results showed that Per2 enhances bmal1b expression through binding to Rora in zebrafish. Although previous studies have shown that Bmal1 expression also was down-regulated in the Per2 knock-out mouse (21, 40, 43), PER2 directly interacts with the REV-ERBα in mice, and overexpression of Per2 can increase Bmal1 expression in cultured cells (24), the mechanisms underlying how mouse PER2 regulates BMAL1 have not been carefully examined. To delineate how PER2 enhances Bmal1 expression in mice, we performed cell transfection experiments. Indeed, the results showed that mouse RORα can activate Bmal1 expression, whereas mouse REV-ERBα can repress RORα-mediated Bmal1 expression, and PER2 can enhance RORα-mediated Bmal1 expression (Fig. 6A). In addition, in the case of the mutated RORE motifs, all these effects were abolished (Fig. 6A). Moreover, PER2 can significantly enhance Bmal1 expression with the presence of RORα (Fig. 6B); however, PER2 cannot reverse the inhibitory effect of REV-ERBα on Bmal1 expression without RORα (Fig. 6C), suggesting that although PER2 interacts with REV-ERBα the enhancing activity of PER2 on Bmal1 expression is still mediated by RORα in mice, which is different from the notion that mouse PER2 binds to REV-ERBα to enhance Bmal1 expression as implicated previously (24).

To elucidate whether the roles of zebrafish Per2/mouse PER2 in regulation of bmal1b or Bmal1 are conserved between mice and zebrafish, zebrafish Per2 was co-transfected with mouse Bmal1luc and RORα. The results showed that mouse PER2 can enhance zebrafish Rora- or Rorab-mediated bmal1b expression (Fig. 6D). Similarly, zebrafish Per2 can enhance mouse RORα-mediates Bmal1 expression (Fig. 6F). These results supported the conservative function of the Per2/PER2 proteins in enhancing expression of bmal1b/Bmal1 in zebrafish and mice.

We also examined the role of Rev-erbα in this system by co-transfection experiments. The results showed that neither can zebrafish Per2 enhance mouse RORα-mediated mouse Bmal1 expression nor can mouse PER2 enhance zebrafish Rora- or Rorab-mediated bmal1b expression (Fig. 6D). The cell transfection experiments also showed that even though zebrafish Rev-erbα can significantly inhibit mouse Bmal1 expression (Fig. 6H) without the presence of RORα and mouse REV-ERBα also can significantly inhibit zebrafish bmal1b expression without the presence of Rora (data not show) zebrafish Rev-erbα cannot outcompete mouse RORα to repress Bmal1 expression. However, the mouse REV-ERBα can outcompete zebrafish Roraα or Rorab to repress bmal1b expression (Fig. 6F), suggesting that mouse REV-ERBα has evolved stronger inhibitory abilities than zebrafish Rev-erbα.

Tissue-specific Circadian Regulation by Zebrafish Per2—Zebrafish per2 is expressed extensively in numerous tissues/organs (32, 71). To investigate the per2 functions in different tissues/organs, we compared expression patterns of several canonical circadian clock genes including per2 itself in different organs of wild-type and per2 mutant zebrafish including the
brain, muscle, heart, and liver. Results showed marked down-regulation of \(\text{per1b}\) and \(\text{per2}\) in all four organs of the \(\text{per2}\) mutant fish (Fig. 7). In contrast, the expression of \(\text{cry1ba}\) was significantly up-regulated in the brain but down-regulated in the muscle, heart, and liver of \(\text{per2}\) mutant fish (Fig. 7). The expression of \(\text{bmal1b}\) exhibited a significant phase delay in the muscle and was down-regulated in the brain, heart, and liver of \(\text{per2}\) mutant fish. Intriguingly, the four circadian clock genes \(\text{per1b}, \text{per2}, \text{cry1ba}, \text{and bmal1b}\) were all down-regulated in the heart and liver of \(\text{per2}\) mutant fish (Fig. 7) implicating that \(\text{Per2}\) is critical for maintaining circadian regulation in the heart and liver and in turn impacts functions of these two important peripheral organs. These results showed that although different circadian clock genes display distinct rhythmic expression patterns in the same organ, the four genes were all significantly disrupted in the \(\text{per2}\) mutant fish (Fig. 7), thereby implicating that Per2 may play distinct regulatory roles in different zebrafish peripheral organs/tissues.

**DISCUSSION**

**Per2 Is Essential for the Zebrafish Circadian Clock—**
Zebrafish have recently figured as an excellent circadian model and contributed to our understanding of vertebrate circadian rhythmicity (30–32). Genetic analysis of the zebrafish circadian clock, however, has lagged behind largely due to difficulties in obtaining stable genetic circadian mutants (14, 31, 72). Here we used TALEN, a fast and convenient genome-editing tool, to generate null mutants for zebrafish \(\text{per2}\). Analysis of \(\text{per2}\) mutant zebrafish helps us to ascertain the roles of Per2 in the zebrafish circadian clock. \(\text{Per2}^{-/-}\) knock-out mice display a short circadian period and arrhythmicity under DD (21), and a human \(\text{PER2}\) mutation results in \(-4\)-h phase advance under LD.
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(45). Our per2 mutant zebrafish displayed reduced activities under LD (Fig. 2, A and B) and a 2-h phase delay and a 1.1-h prolonged period under DD (Fig. 2, C and D), which are completely different from the phenotypes of the zebrafish per1b insertional null mutant that displays hyperactivities under LD and a 2-h phase advance under DD (73). Hence, like mammalian Per2, zebrafish per2 also is critical for maintaining fish locomotor rhythmicity. The fact that both zebrafish per2 and per1b mutant fish still are not completely arrhythmic under DD strongly suggests that zebrafish per genes resemble mouse Per genes in that they are partially redundant and can compensate for the other’s loss for maintaining locomotor rhythmicity (74). However, the intricate roles of Per2 in regulating activity rhythms differ between fish and mammals and likely represent different stages of Per2 functions during evolution from fish to mammals.

We also found that rhythmic expression patterns as well as phases of key circadian genes are disrupted in per2 mutant fish (Fig. 2, E and F). For instance, both per1b and per3 were significantly down-regulated in per2 mutant fish under both LD and DD, suggesting that Per2 plays a positive role in regulating these two per genes (Fig. 2, E and F), which diametrically differs from the repressive role of Per1b in regulating other per genes; i.e. all three other per genes are significantly up-regulated in the per1b-null mutant fish (73). Although Per1 knock-out mice have no effects on rhythmic expression of Per1 or Per2, both Per1 and Per2 are significantly down-regulated in Per2 knock-out mice (21, 40). Thus both zebrafish Per2 and mouse PER2 share the conservative function of positively regulating per genes. Together, the disrupted locomotor behaviors and altered expression of circadian clock genes in per2 mutant indicate that per2 is an essential component in the zebrafish circadian clock.

Dual Roles of Per2 in the Zebrafish Circadian Clock—In the Drosophila negative feedback loop, per and tim act as negative regulators (75). Similarly, it has long been thought that mammalian Per1, Per2, Cry1, and Cry2 proteins as negative factors form heterodimers to repress CLOCK-BMAL1-mediated transcription (2, 59, 76). However, there is evidence that PER2 might also play a positive role in mammalian circadian regulation (21, 40, 77). Although PER2 is not a transcriptional factor, it can directly bind to numerous nuclear receptors to control mammalian physiological processes (24, 78). Our results showed that zebrafish Per2 not only represses aanat expression through E-box but also enhances bmal1b expression through RORE. The repressive role of Per2 is in line with the traditional notion that Per2 and Cry form a heterodimer that represses Clock-Bmal heterodimer-mediated transcription (Fig. 3C) (2, 59), whereas the enhancing role of Per2 is fulfilled by its binding to Rora nuclear receptor (Fig. 3D).

The Positive Role of Zebrafish Per2 and Mouse PER2 in Circadian Regulation Is Fulfilled through Mediation of RORα or Rora—Even though previous studies have shown that mouse PER2 can increase Bmal1 expression (21, 24, 43), the exact mechanisms are not clear. Our detailed cell transfection assays showed that although mouse PER2 binds to REV-ERBα (24) it still requires RORα mediation to enhance BMAL1 expression (Fig. 6, A–C). Our studies also showed down-regulation of RORE-containing bmal1b in per2 mutant zebrafish (Fig. 2, E and F), and zebrafish Per2 positively regulated bmal1b expression through RORE (Fig. 3D). In particular, co-IP experiments showed that Per2 directly interacts with Rora through the LXXLL motif conserved between zebrafish Per2 and mouse PER2 (Fig. 5E).

The roles of RORA, REV-ERBα, and PER2 in regulation of Bmal1 are highly conserved between zebrafish and mice but with a difference. Although zebrafish Rora/Rorob or mouse RORα can bind RORE to activate bmal1b or Bmal1 expression (Fig. 8, A, panel 1, and B, panel 1), zebrafish Rev-erba or mouse REV-ERBα can outcompete Rora or RORα to bind to RORE to repress bmal1b or Bmal1 expression (Fig. 8, A, panel 2, and B, panel 2), and zebrafish Per2 or mouse PER2 alone cannot reverse the repressive effects of Rev-erbo or REV-ERBα on bmal1b or Bmal1 expression without the presence of zebrafish Rora or mouse RORα (Fig. 8, A, panel 3, and B, panel 3), zebrafish Per2 or mouse PER2 alone can significantly enhance Rora-mediated bmal1b expression or RORα-mediated Bmal1 expression without the presence of Rev-erbo or REV-ERBα (Fig. 8, A, panel 4, and B, panel 4), which are all conserved in zebrafish and mice. The difference is that zebrafish Per2 binds to Rora rather than Rev-erbo to enhance bmal1b expression (Fig. 8A, panel 4), whereas mouse PER2 enhances Bmal1 expression still through RORα mediation, but it binds to REV-ERBα rather than RORα (Fig. 8B, panel 5). These results revealed that zebrafish Per2 or mouse PER2 enhances bmal1b or Bmal1 expression through mediation of Rora or RORα. A recent study showed that mouse PER1 and PER2 but not PER3 can inhibit CRY-mediated transcriptional repression by preventing CRY from being recruited to the CLOCK-BMAL1 complex, providing evidence that PER2 and PER1 have positive roles in the mammalian circadian clock (79), which complements our promoter analysis of zebrafish per2 and mouse Per2 in support of PER2 as a positive factor in vertebrate circadian regulation. Hence, the positive role of PER2 in regulation of Bmal1 expression is conserved from zebrafish to mice, which is at odds with the prevailing notion of PER2 as a negative circadian factor (1, 2, 59).

Alignment of the amino acid sequence revealed that numerous functional motifs are highly conserved in zebrafish Per2 and mouse PER2 (data not shown), thereby supporting the notion that their roles in regulation of bmal1b or Bmal1 are conserved. We also examined the possible roles of zebrafish Per2 and Rev-erbo in mice and vice versa and found that although mouse PER2 can enhance zebrafish Rora-mediated bmal1b expression (Fig. 8C, panel 1) so can zebrafish Per2 enhance mouse RORα-mediated Bmal1 transcription (Fig. 8D, panel 1); mouse PER2 cannot reverse the inhibitory effect of zebrafish Rev-erbo on bmal1b expression in the presence of Rora, nor can zebrafish Per2 reverse the inhibitory effect of mouse REV-ERBα on Bmal1 expression in the presence of RORα (Fig. 8, C, panel 2, and D, panel 2). The difference is that although mouse REV-ERBα can outcompete zebrafish Rora to bind to RORE to repress zebrafish bmal1b expression (Fig. 8C, panel 3) zebrafish Rev-erbo cannot (Fig. 8D, panel 3).

Because zebrafish and tetrapods including mammals shared a common ancestor more than 300 million years ago (58, 80), it
is fascinating that most aspects of the functions of zebrafish Per2 and mouse PER2 are still highly conserved (for instance, both can positively regulate expression of *bmal1b* or *Bmal1*). However, it is not unexpected that zebrafish Per2 and mouse PER2 have evolved divergent functions; i.e. zebrafish Per2 binds to Rorα to exert its enhancing effects on *bmal1b* expression, whereas mouse PER2 has evolved to bind to REV-ERBα instead but still requires RORα mediation to exert its enhancing effects.
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on Bmal1 expression (Fig. 8). The functions of zebrafish Rev-erba and mouse REV-ERBα are also mostly conserved but show divergence in that mouse REV-ERBα has evolved a stronger ability to outcompete zebrafish Rora to repress zebrafish bmal1b expression (Fig. 8). Therefore, it is necessary and important to study the functions of zebrafish circadian clock genes because it provides comparative perspectives for how the functions of these key circadian clock genes have evolved.

In conclusion, our analysis of the per2-null mutants generated by TALEN showed that the rhythms of locomotor behaviors and expression of core circadian genes and one circadian clock controlled-gene expression are disrupted in per2 mutant fish, indicating that per2 is essential for the zebrafish circadian clock. We determined that zebrafish per2 plays both positive and negative roles in circadian regulation, and in particular, Per2 positively regulates bmal1b expression by directly binding to Rora. Mouse PER2 enhances Bmal1 expression still through RORα mediation even though it has evolved to bind to REV-ERBα instead. Moreover, zebrafish Per2 appears to have tissuespecific functions in the peripheral circadian clocks. These results help define the Per2 functions in the zebrafish circadian clock and provide invaluable evidence for a positive role of PER2 in the vertebrate circadian system.

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