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Supplemental Information

The core circadian component, Bmal1, is maintained in the pineal gland of old killifish brain

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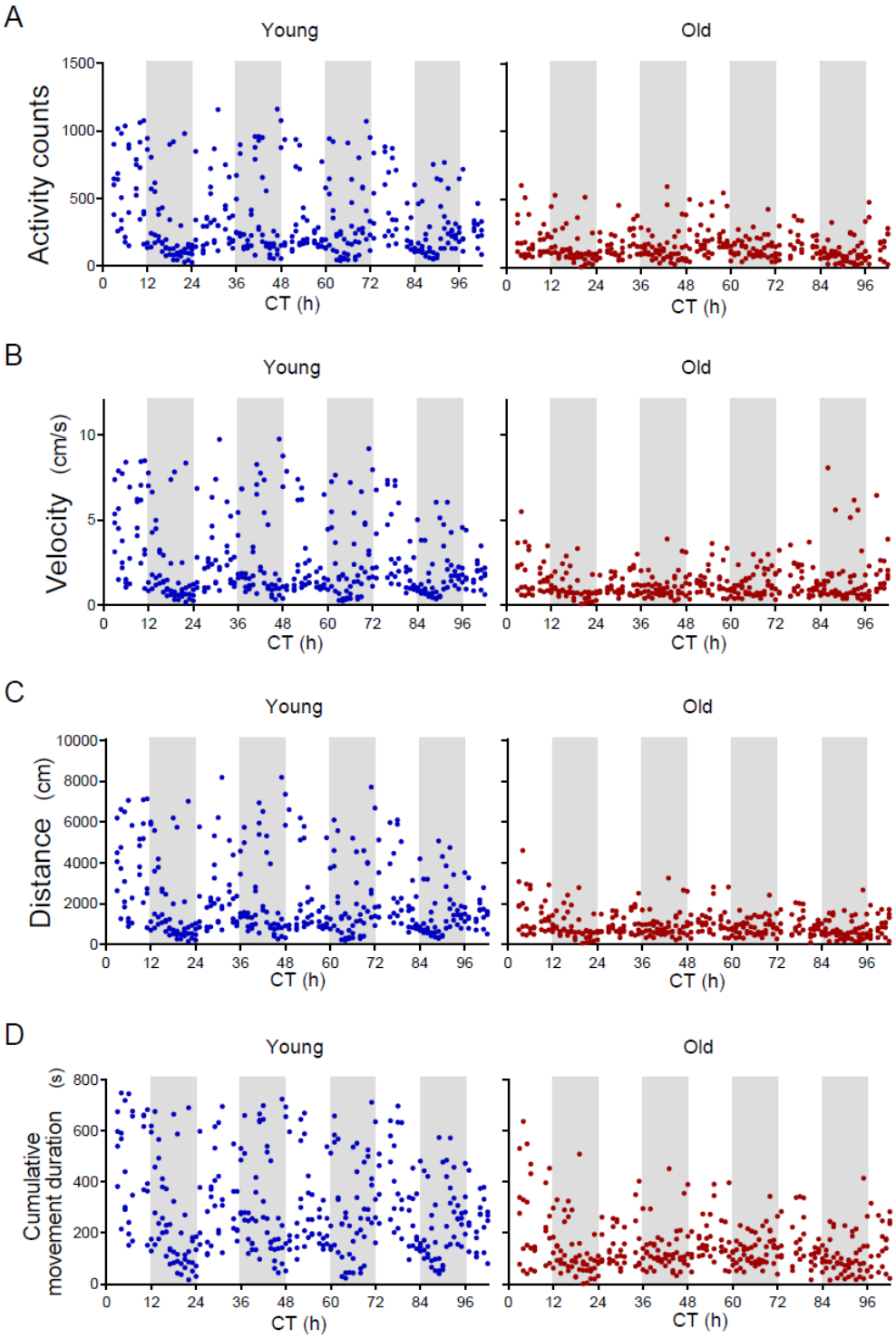

Figure S1. Locomotion of circadian rhythm measurements under the free-running LL condition. Related to Figure 1. Activity counts recapitulating a conventional method for locomotion *(A)*, mean velocity *(B)*, total distance *(C),* and cumulative movement duration *(D)* of young and old fish were measured under the free-running LL condition. CT represent “Circadian time.” Each dot represents a single fish at each measurement (n = 4).

Figure S2. Amino acid alignment of the circadian core components BMAL1, CLOCK, PER3, and CRY1 among *Nothobranchius furzeri*, *Danio rerio*, *Mus musculus*, and *Homo sapiens*. Related to Figure 2. Compared are the translated amino acid sequences of turquoise killifish, zebrafish, mouse, and human BMAL1 [XM_015965280 (*bmal1*), NM_131577 (*bmal1a*), NM_007489, and NM_001030272, respectively], CLOCK [XM_015971720 (*clockb*), BC163244 (*clock1a*), AF000998, and AF011568, respectively], CRY1 [XM_015964931 (*cry1b*), NM_001099297 (*cry1aa*), NM_004075, and NM_004075, respectively], and PER3 (XM_015959552 (*per3*), AF254792, NM_011065, and NM_001289862, respectively). Identical amino acid sequences are highlighted in red characters and a yellow background, weakly similar amino acids are displayed in green characters, a block of similar amino acids is shown in black characters with a green background, conserved sequences are displayed in blue characters and sky-blue background, and non-similar sequences are indicated in black characters. Red box in BMAL1 consensus sequence indicate an epitope region of human BMAL1 for anti-BMAL1 antibody (ab93806, Abcam).

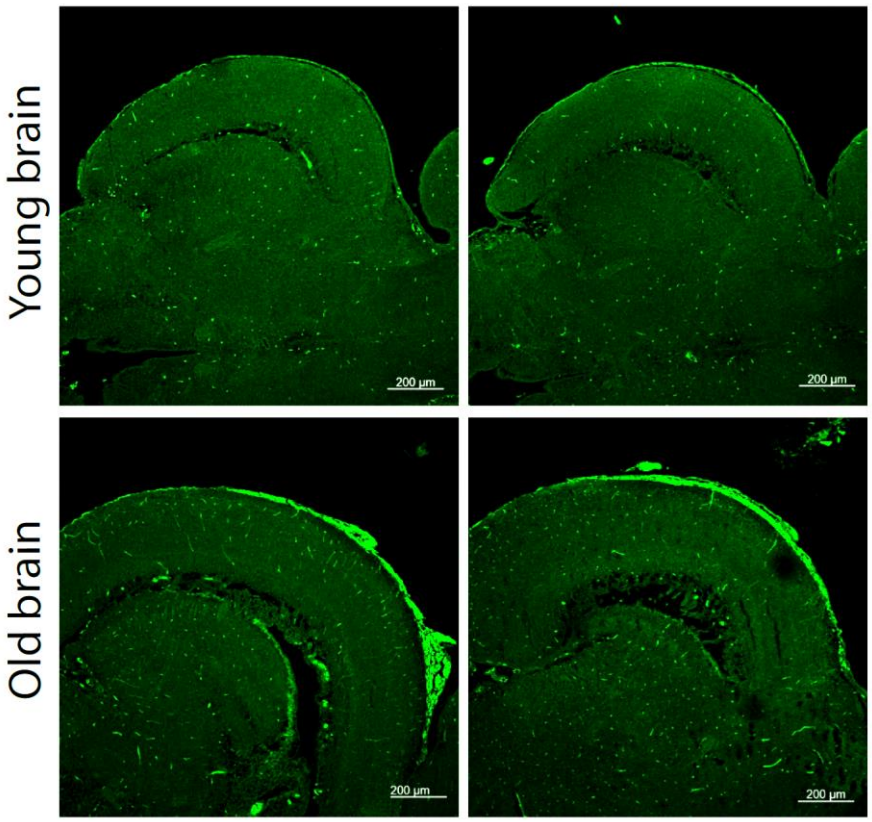

Figure S3. Neurodegeneration in the aged killifish brain. Related to Figure 3. Degenerated neurons were stained with Fluoro Jade B, and image sections from two independent brains are displayed.

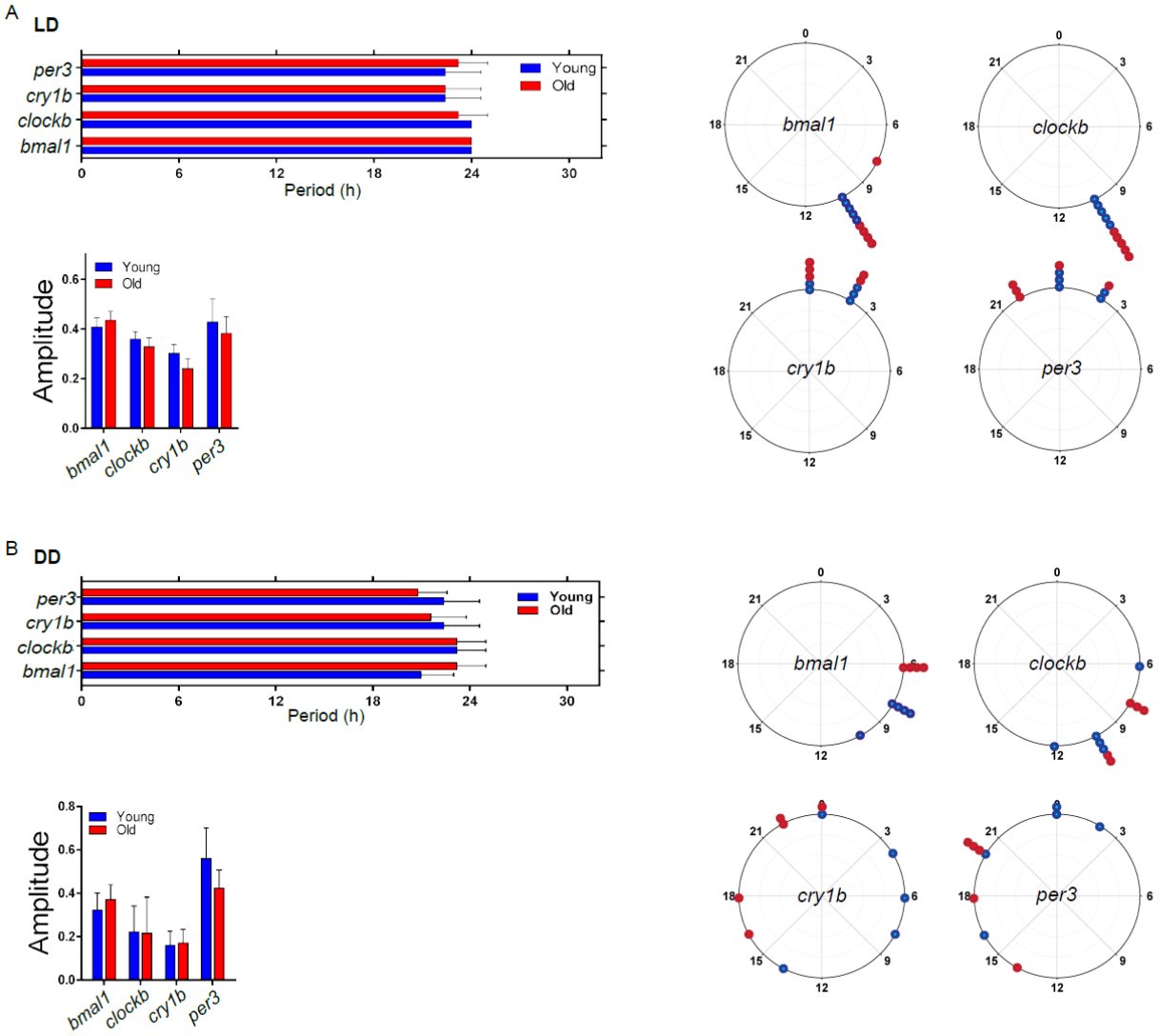

Figure S4. Circadian characteristics of clock gene expression in LD and DD. Related to Figure 3. Period, amplitude, and phase from the cyclic expression under LD *(A)* and DD *(B)* conditions were analyzed using JTK_CYCLE. Data are presented as the mean ± SD for period and amplitude.

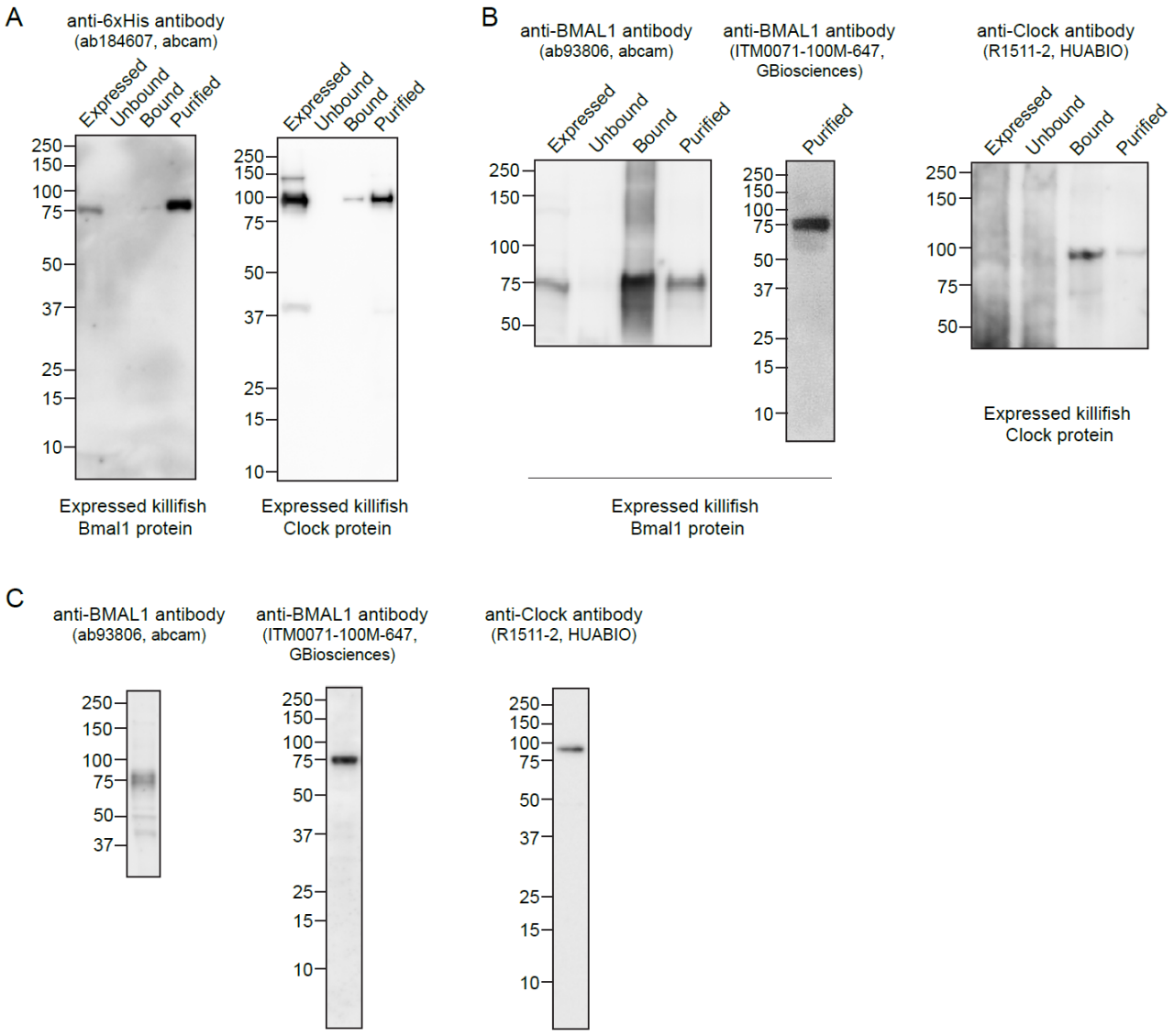

Figure S5. Validation of commercial BMAL1 and CLOCK antibodies. Related to Figure 4. (A) Synthesized Bmal1 and Clock proteins were detected with anti-His antibody (ab184607, Abcam). (B) Synthesized Bmal1 and Clock proteins were detected with commercial BMAL1 (ab93806, Abcam and ITM0071-100M-647, GBiosciences) and CLOCK (R1511-2, HUABIO) antibodies. (C) Specificity of antibodies in this study. Total protein extracts from the turquoise killifish brain were used to test the specificity of commercial BMAL1 and CLOCK antibodies.

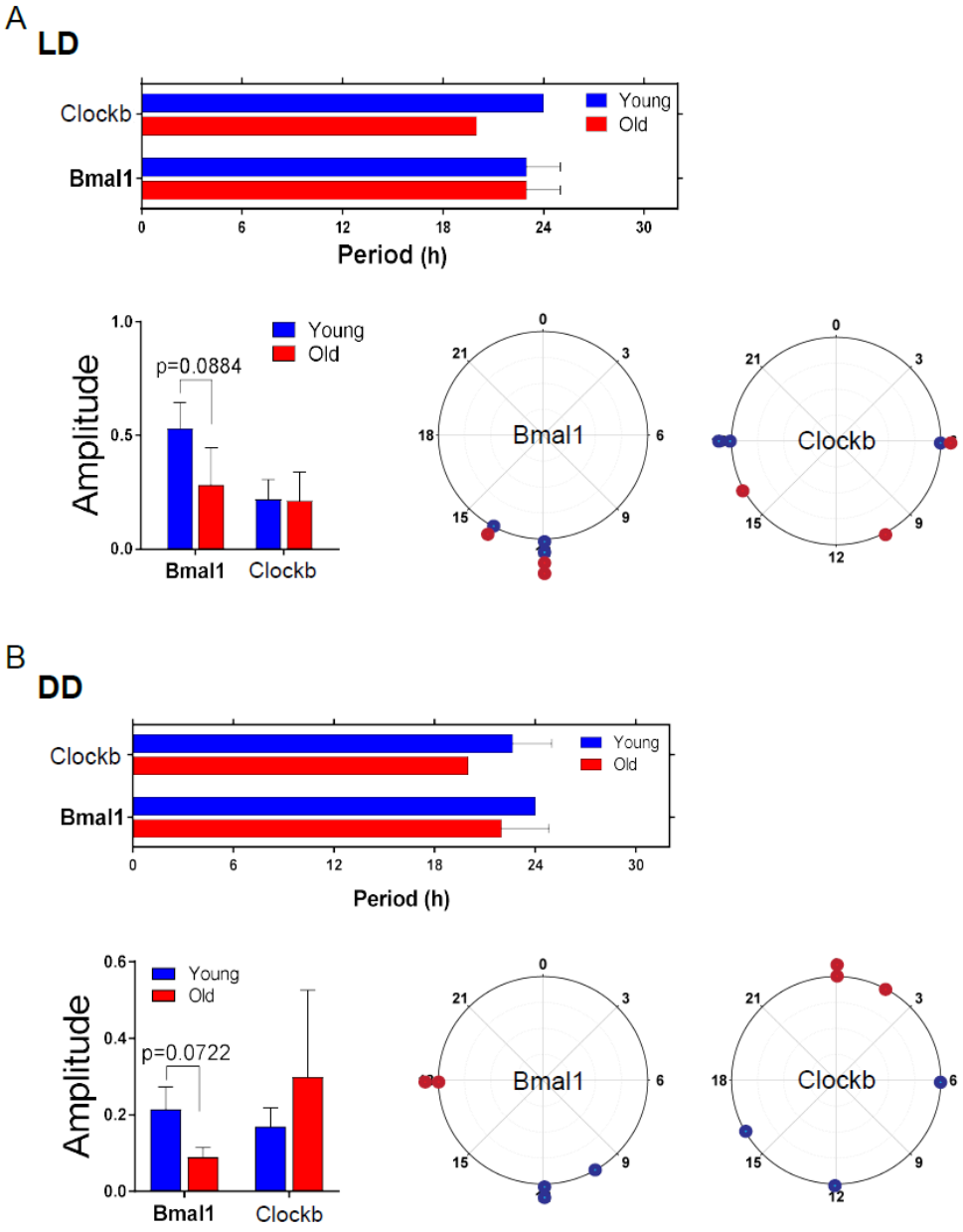

Figure S6. Circadian characteristics of Bmal1 and Clockb protein abundance in LD and DD. Related to Figure 4. Abundance cycling of Bmal1 and Clockb in LD (A) and DD (B) was analyzed using JTK_CYCLE. Data are presented as the mean ± SD for period and amplitude.

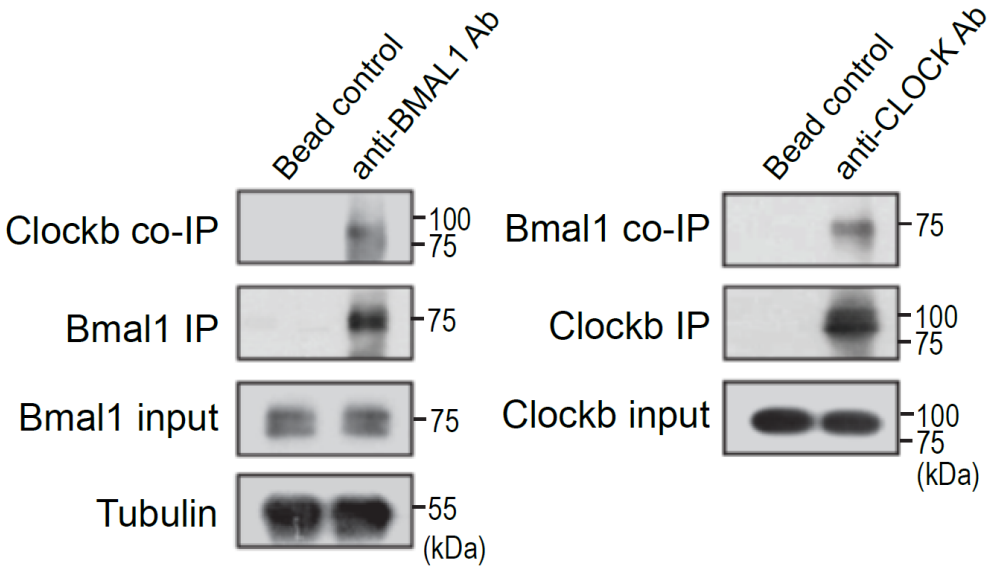

Figure S7. *In vivo* interaction between Bmal1 and Clock. Related to Figure 5. Heterodimer formation between Bmal1 and Clockb in the turquoise killifish brain was detected using BMAL1 or CLOCK antibody.

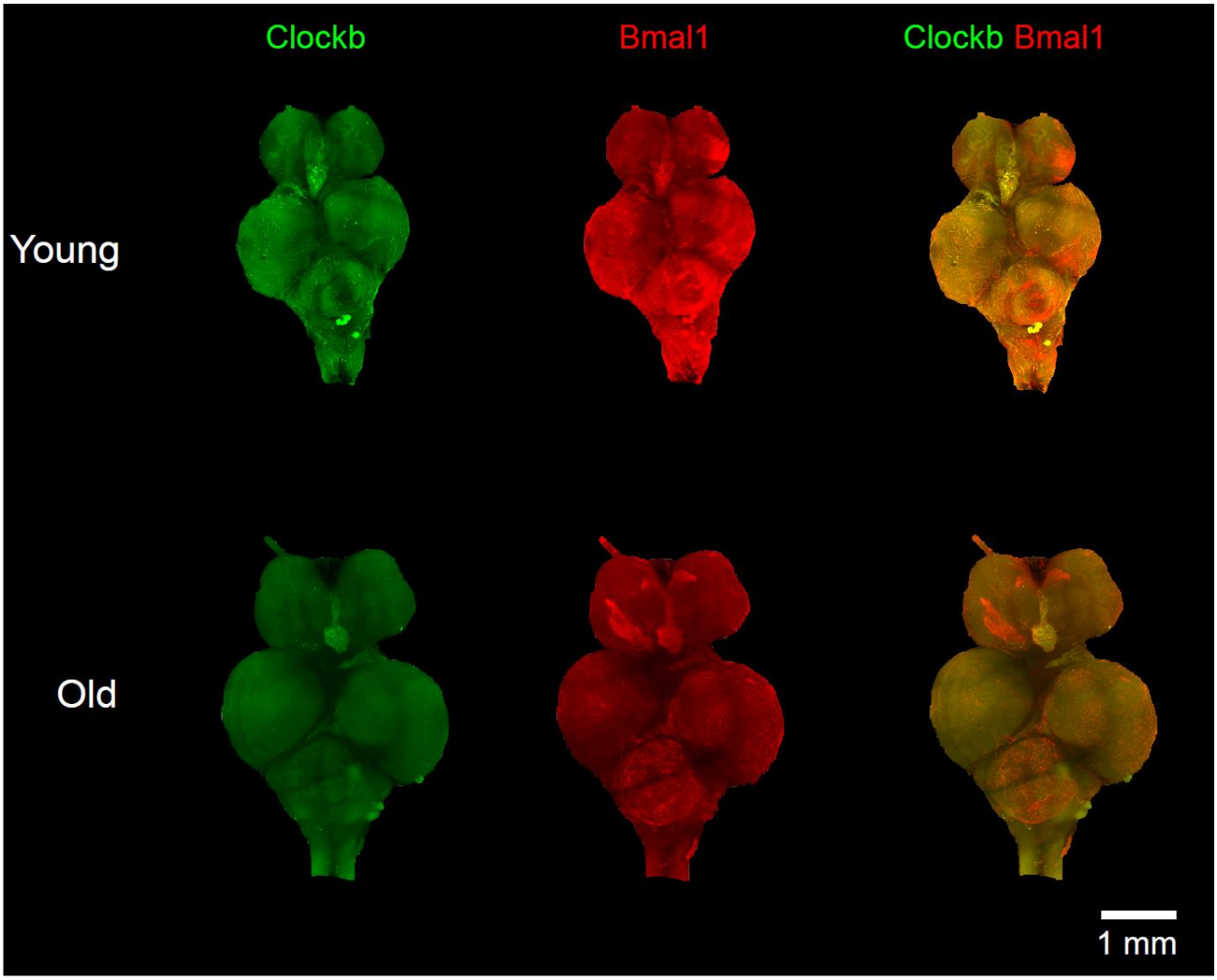

Figure S8. Biological replicates of whole brain staining with BMAL1 and CLOCK antibodies. Related to Figure 6.

Transparent Methods

Fish husbandry

The GRZ-AD strain, which is a short-lived strain of the turquoise killifish, was used and maintained as previously described (Dodzian et al., 2018). The fish were cultivated under 12 h light and 12 h dark cycle. The fish were singly housed in a 1.8 L tank and fed twice a day at 1 h and 8 h after light on. Young and old fish were sacrificed after 6 and 14 weeks after hatching, respectively.

Fish care and experiments were performed in accordance with the animal care and use protocol that is reviewed and approved by the Institutional Animal Care and Use Committee at Daegu Gyeongbuk Institute of Science and Technology, Republic of Korea (Approval number: DGIST-IACUC-17103001-00).

Development of the assay system for measuring the free-running circadian rhythm

A wide tank measured 17 cm (*w*) × 24 cm (*l*) × 18 cm (*h*) was designed to contain relatively shallow water. The water level was maintained at a 3 cm in depth with continuous circulation of system water in the fish culture facility. The tanks were equipped on the LED light panel (LED light pad GB4, GAOMON) for a video recording in LL and 940 nm LED illuminator (custom-built) was installed on top of the tank for a video recording in DD. A web camera [WideCam F100 (Genius) for LL measurement and C920 PRO HD WEBCAM (Logitec) for DD measurement] was installed on top of each tank. One fish per tank was placed and the fish husbandry were consistently carried out following the ordinary fish husbandry scheme. The cameras were connected to a computer, and video images were acquired automatically every 15 min for 4 days using open source software (OBS studio 23.0.1). The raw video images were concatenated into one file per fish, and analyzed and visualized with a behavior analysis program (EthoVision XT, Version 13.0, Noldus Information Technology).

Characterization of circadian rhythms

Only male fish for each age groups were used for measuring circadian rhythmicity as indicated in the main figure and Table S1. The raw data (mean velocity from EthoVision XT outputs of LL and DD free-running rhythm measurements, and normalized circadian clock gene and protein expressions) were used to analyze the circadian rhythmicity with JTK_CYCLE (Hughes et al., 2010). The outputs of every analysis are shown in Supplementary Table 1; period, amplitude, and phase were used for visualization.

Measurement of circadian gene expression

The turquoise killifish brains were dissected from young (6-week-old) and old (14-week-old) fish. Each age group contains three males and two females per time point. Total RNAs were isolated individually from five fish per age group at each time point using QIAzol Lysis Reagent (79306, QIAGEN) following the manufacturer's instructions. cDNAs were synthesized from 2.5 μg of total RNA using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (11766050, Invitrogen). Synthesized cDNAs were diluted 10-fold, and 3 μL of diluted cDNA was used as a template for qPCR (SsoAdvanced Universal SYBR Green Supermix, BioRad). The primers used are listed below. The

core gene expressions were normalized with *insr* expressions which have been known to be well maintained over the fish age (Hartmann et al., 2011).

Gene name	Forward primer	Reverse primer
bmal1	CGATGGAAAGTTTGTCTTCGT	TGGGGCAAATACGCTAGG
clockb	CAGCTTTCAGCCATGCAG	TGGGTAGATTGGTTTCCATGA
per3	TCATGAGGAAATAAAAGATCTACAAGC	CGCTGGAGCCATTGTTGT
cry1b	GGCTCTCATGCAGCTCGT	TGCTGGTAAATAGCGTCTGATG
insr	TGCCTCTTCAAACCCTGAGT	AGGATGGCGATCTTATCACG

Immunoblot analysis and co-immunoprecipitation

Total proteins were isolated from young and old brains using 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 0.05% NP40, 3 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail; approximately 10 μg of total protein was used for protein gel blot analysis. Each age group contains two males and one female per time point. The specificity of BMAL1 and CLOCK antibodies was determined using synthetic Bmal1 and Clockb proteins expressed in the turquoise killifish. XM_015965280 for Bmal1 and XM_015971720 for Clockb were used as a template to synthesize control proteins. The synthetic killifish Bmal1 and Clockb proteins were obtained using a cell-free expression system after conjugation with a 6xHIS tag for purification (Gene to Protein Synthesis service, Bioneer). The synthetic proteins were detected with an anti-6xHis antibody (ab184607, Abcam, RRID:AB_2868537). Tubulin (T5168-2mL, Sigma, RRID:AB_477579) was used as a loading control, and BMAL1 (ab93806, Abcam, RRID:AB_10675117) and CLOCK (R1511-2, HUABIO, RRID:AB_2859538) antibodies were used to detect protein expressions and for immunoprecipitation. Co-immunoprecipitation was performed with BMAL1 or CLOCK antibody conjugated with IgG-conjugated agarose beads (20423, Pierce™ Protein A/G Plus Agarose, Thermo Scientific™).

Chromatin immunoprecipitation

Each biological group contained five brains from three males and two females. Chromatin immunoprecipitation was performed using an antibody against BMAL1 (ab93806, Abcam, RRID:AB_10675117), and GFP (ab290, Abcam, RRID:AB_303395) antibody was used as the control according to a previously described method (Haring et al., 2007). Chromatin shearing was performed using a sonicator (VCX 130, Vibra-Cell™ Ultrasonic Liquid Processors, Sonics & Materials, Inc.) under the following conditions: 2 s of sonication and 2 s of rest for 16 min. Two nanograms of input and ChIPed DNA were used for qPCR. The primers targeting the E-boxes of *cry1* and *per3* promoter regions are listed below.

Name	Forward primer	Reverse primer
cry1bp-amp1	AATAAAGAGGGATGGGGCATG	TGTAATTCAGTAACGTAATGGCC
cry1bp-amp2	ATGCCAAACAATTACTCCTTTCT	AACAGGTGGAGGTGAGACTAAAG
cry1bp-amp3	CTGTGGTTTGTTTACATGCAATC	CCAGTCATATAATGTTGAAACTTGT
cry1bp-amp4	AAATCGTAACTAGGTAAGCTGAC	CTCACGTTAATAGATGTTCGACC
cry1bp-amp5	TATAGAGCTCCGGACGTCA	AGGTCCGGTAAGAAGTCC
per3p-amp1	CTACTGACTCCCCCTCATC	AGAAGTTTCAACGTGAATGAAGC
per3p-amp2	GGTGCACGTGTGTAAACTGG	GCTGAAAAACTTCAAAGGCGTA
per3p-amp3	TTGGCCGATAATGATGCAGA	GTCATTTGCTGTATACCACTTGT
per3p-amp4	TAACATAGCCAAAGTTATCAC	GTGTGTACTGTACATAATAAC
per3p-amp5	CAACGTGATTCCGGCATG	CACTTGTTGCTCCTCTTGT

Whole brain immunostaining

Two young and two old male brains were dissected carefully under a microscope and fixed in 4% PFA overnight. The whole brain was cleared with a tissue immune-staining kit (Binaree Immuno Staining^{TM} Kit for Brain, BINAREE) following the manufacturer's protocol. Cleared brains were stained using anti-BMAL1-Alexa647 (ITM0071-100M-647, GBiosciences, RRID:AB_2868539) or anti-CLOCK (R1511-2, HUABIO, RRID:AB_2868538)/goat anti-rabbit IgG Cross-Adsorbed, Alexa Fluor488 (A11008, Invitrogen, RRID:143165) antibodies. The nucleus was labeled with DAPI. Cleared and stained brains were embedded in 2% of low melting agarose in 2-mm capillaries (inner diameter). The embedded brain was tiled by 9-30 regions to cover the whole brain, and imaged with a 20× objective lens (W Plan-APOCHROMAT 20, Zeiss) under light sheet microscopy (Lightsheet Z.1, Zeiss). Tiled images were converted (Imaris File Converter x64.9.2, Bitplane) and merged (Imaris Stitcher 9.2.1, Bitplane) into one image for visualization.

Statistics

Statistical comparison between young and old of gene and protein expression for circadian core components was performed in Graph Pad PRISM (Prism 7 for Windows, version 7.03) by putting raw data in the data tables. Statistical analysis of circadian rhythmicity was performed JTK_CYCLE (Hughes et al., 2010), and Every raw data and output after JTK_CYCLE analysis were provided in Supplemental Table 1.

Supplemental References

Dodzian, J., Kean, S., Seidel, J., and Valenzano, D.R. (2018). A Protocol for Laboratory Housing of Turquoise Killifish (Nothobranchius furzeri). Journal of visualized experiments : JoVE.

Haring, M., Offermann, S., Danker, T., Horst, I., Peterhansel, C., and Stam, M. (2007). Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. Plant Methods *3*, 11.

Hartmann, N., Reichwald, K., Wittig, I., Drose, S., Schmeisser, S., Luck, C., Hahn, C., Graf, M., Gausmann, U., Terzibasi, E.*, et al.* (2011). Mitochondrial DNA copy number and function decrease with age in the short-lived fish Nothobranchius furzeri. Aging cell *10*, 824-831.

Hughes, M.E., Hogenesch, J.B., and Kornacker, K. (2010). JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. Journal of biological rhythms *25*, 372-380.