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Disrupted-in-schizophrenia 1 enhances the quality of circadian rhythm by stabilizing BMAL1

Su Been Lee¹, Jihyun Park², Yongdo Kwak^{1,3}, Young-Un Park^{1,4}, Truong Thi My Nhung¹, Bo Kyoung Suh¹, Youngsik Woo¹, Yeongjun Suh¹, Eunbyul Cho¹, Sehyung Cho² and Sang Ki Park¹

Abstract

Disrupted-in-schizophrenia 1 (DISC1) is a scaffold protein that has been implicated in multiple mental disorders. DISC1 is known to regulate neuronal proliferation, signaling, and intracellular calcium homeostasis, as well as neurodevelopment. Although DISC1 was linked to sleep-associated behaviors, whether DISC1 functions in the circadian rhythm has not been determined yet. In this work, we revealed that *Disc1* expression exhibits daily oscillating pattern and is regulated by binding of circadian locomotor output cycles kaput (CLOCK) and Brain and muscle Arnt-like protein-1 (BMAL1) heterodimer to E-box sequences in its promoter. Interestingly, *Disc1* deficiency increases the ubiquitination of BMAL1 and de-stabilizes it, thereby reducing its protein levels. DISC1 inhibits the activity of GSK3 β , which promotes BMAL1 ubiquitination, suggesting that DISC1 regulates BMAL1 stability by inhibiting its ubiquitination. Moreover, *Disc1*-deficient cells and mice show reduced expression of other circadian genes. Finally, *Disc1*-LI (*Disc1* knockout) mice exhibit damped circadian physiology and behaviors. Collectively, these findings demonstrate that the oscillation of *DISC1* expression is under the control of CLOCK and BMAL1, and that DISC1 contributes to the core circadian system by regulating BMAL1 stability.

Introduction

Anticipating the time of day is crucial for organisms that live under recurrent sunlight. The molecular circadian clock system maintains the daily cycle of organisms^{1–5}. Transcription–translation feedback loop (TTFL) of circadian genes ensures this cycle, which keeps the circadian clock ticking even without external time cues⁵. In the mammalian TTFL system, the main component is the heterodimer consisting of Circadian locomotor output cycles kaput (CLOCK) and Brain and muscle Arnt-like protein-1 (BMAL1), which binds to E-box sequences of target genes for transcriptional enhancement^{6,7}. In this way, the CLOCK/BMAL1 heterodimer upregulates core clock genes, including Period (*PERs*), Cryptochrome

(*CRYs*), and Nuclear receptor subfamily 1 (*Nr1d1*)⁵. In turn, the action of CLOCK/BMAL1 heterodimer is negatively regulated by PERs and CRYs, thus completing the TTFL^{8,9}.

Timed regulation of circadian proteins is essential to the proper working of the circadian timing system. To this end, the degradation of the molecular circadian components is vital. Newly synthesized circadian proteins must be degraded at the right time for the accurate pacing of the circadian clock. Indeed, several circadian components, including BMAL1, PERs, CRYs, and REV-ERBs, are regulated by degradation pathways¹⁰. Glycogen synthase kinase 3 β (GSK3 β) phosphorylates BMAL1, thereby enhancing its ubiquitination and degradation¹¹. Moreover, casein kinases I δ / ϵ (CKI δ / ϵ) phosphorylate PERs leading to their degradation¹⁰. Chemical inhibition of CKI augments the stability of PERs and induces a more extended circadian period. F-box and leucine-rich repeat protein 3 (FBXL3), an E3 ubiquitin ligase, mediates ubiquitination and subsequent degradation of CRYs¹²,


Correspondence: Sehyung Cho (sehyung@khu.ac.kr) or Sang Ki Park (skpark@postech.ac.kr)

¹Department of Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea

²Department of Physiology, College of Medicine, Kyung Hee University, Seoul, Republic of Korea

Full list of author information is available at the end of the article

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and FBXL3-mutated mice also exhibit longer circadian period¹³. REV-ERB α is phosphorylated by cyclin-dependent kinase 1 (CDK1) and recognized for ubiquitination by F-box and WD repeat domain containing 7 (FBXW7)¹⁴. The loss-of-function of FBXW7 results in a damped circadian amplitude, indicating that the degradation of REV-ERB α is essential for the circadian amplitude. Altogether, these studies indicate that proper degradation of circadian proteins is a critical factor to maintain circadian period and amplitude.

Disrupted-in-schizophrenia 1 (*DISC1*) was first reported as a responsible gene for prevalent psychiatric conditions, including schizophrenia, in a Scottish pedigree¹⁵. Although *DISC1* as a genetic risk factor for schizophrenia has more to be elucidated, *DISC1* plays significant molecular roles, including functions in early brain development, which are important to the molecular basis of psychiatric disorders¹⁶. *DISC1* is a scaffold protein with a large number of interacting partners that perform various functions in the nervous system, such as neuronal migration, neurite outgrowth, spine regulation, and synapse maintenance^{17–20}. Interestingly, *DISC1* is associated with sleep-related phenotypes. Expression of human *DISC1* in fruit flies alters their sleep homeostasis²¹. Furthermore, a mouse model of *DISC1* gain-of-function shows increased wakefulness and decreased REM and NREM sleep²². A close relationship between sleep and the circadian clock has been proposed²³; however, potential direct involvement of *DISC1* in the mammalian circadian system has not been explored yet. In this study, we investigated the relationship between *DISC1* and the circadian system and proposed a modulatory role of *DISC1* in the mammalian molecular clock.

Materials and methods

Animals

All animal-related experiments were approved by Pohang University of Science and Technology Institutional Animal Care and Use Committee (POSTECH-2019-0024 and POSTECH-2019-0025), and animal experiments were conducted with approved guidelines. Wild type C57BL/6J mice were purchased from Hyochang Science (Daegu, South Korea). *Disc1* knockout mouse, named as *Disc1* locus impairment mouse (*Disc1*-LI mouse, C57BL/6J backgrounded), was previously described²⁴ and a kind gift from Dr. Akira Sawa, Johns Hopkins University School of Medicine. In *Disc1*-LI mouse, exons from 1 to 3 of *Disc1* were replaced to Neomycin resistance cassette by homologous recombination^{24,25}. Male wild type (C57BL/6J) mice and *Disc1*-LI mice were housed under 12-h light 12-h dark conditions with *ad libitum* access to food and water. Male mice aged 3 to 4 months were employed for circadian experiments.

Cell culture and transfection

HEK293 cell line, NIH3T3 cell line, and Mouse embryonic fibroblasts (MEFs) were maintained in 5% CO₂ incubator at 37 °C with DMEM high glucose (Welgene, LM 001-05) containing 10% fetal bovine serum (Merck, ES009B-KC) and 1% penicillin/streptomycin (HyClone, SV30010). Cells were transfected with Polyethylenimine (PEI, Polysciences, Inc., 23966-2) as previously described²⁶. Briefly, plasmids were mixed with PEI (1 mg/ml, pH 7.0) in opti-MEM (Gibco, 31985-070) and added to cells after 15 min of incubation.

Antibodies

For antibodies, rabbit anti-*DISC1* (Millipore, ABN-425) was purchased from Millipore; rabbit anti-BMAL1 (Abcam, ab93806) from Abcam; mouse anti- α -tubulin (Proteintech, 66031-1-Ig) from Proteintech; rabbit anti-HA (Bethyl, A190-108A) from Bethyl; rabbit anti-GFP (Invitrogen, A11122) from Invitrogen; rabbit anti-Flag (ThermoFisher, PA1-984B) from ThermoFisher; mouse anti-Flag (Sigma, F1804) from Sigma; mouse anti-c-Myc (Santa Cruz, sc-40) from Santa Cruz; GSK3 β p-Y216 (Invitrogen, 44-604G) from Invitrogen; mouse anti-GSK3 β (Cell Signaling, 9832S) from Cell Signaling. Antibodies were utilized as the first antibody of western blot or precipitating antibody of immunoprecipitation.

Plasmids

Human *DISC1* promoter (-982 to +47 relative to TSS²⁷) was cloned in pGL3-basic (Promega), whose luciferase was modified as destabilized with PEST sequence²⁸. For distal, middle, and proximal promoter regions of *DISC1*, -982 to -624, -623 to -324, and -323 to +47 relative to TSS respectively, were cloned in pGL3-basic with destabilized luciferase. PCR and DpnI-based mutation method was utilized to mutate E-box sequences of *DISC1* promoter. Primers were used for the PCR; for mutant E-box (-718 relative to TSS): 5'-TCTATGACCGTACTC TCCTTC-3' and 5'-GAAGGAGAGTACGGTCATAGA-3'; for mutant E-box (-668 relative to TSS): 5'-AGAGC TACTGTATAGCCCTTC-3' and 5'-GAAGGGCTATA CAGTAGCTCT-3'. Human CLOCK cDNA was cloned in mRFP-C1 with myc tagging at C-terminal. Human BMAL1 cDNA was cloned in pEGFP-C1 (Clontech) and pcDNA3.1 myc-His C (Invitrogen). HA-hDISC1 was a kind gift from Dr. Akira Sawa, Johns Hopkins University School of Medicine, and sub-cloned in pFLAG-CMV2 (Sigma). Human GSK3 β cDNA was cloned in pcDNA3.1 myc-His C (Invitrogen). HA-Ubiquitin was a kind gift from Dr. Chin Ha Chung, Seoul National University, Seoul, Korea. pLL3.7 vector was utilized for shRNA knockdown experiments. Target sequence for control shRNA, 5'-ACTACCGTTGTATAGGTG-3', was previously described²⁹; 5'-GCAGGAGGTGCAAGGCC

TTG-3' for human DISC1 shRNA was previously described³⁰.

Western blot

For western blot, cells or brain samples were lysed in Nonidet P-40 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP40, 5 mM EDTA, 1X protease inhibitor cocktail). Samples were sonicated and centrifuged at 13,000 rpm, 4 °C for 10 min to remove debris. Acquired supernatant was mixed with SDS sampling buffer and denatured at 95 °C for 10 min. Denatured samples were separated with SDS-PAGE gel electrophoresis and transferred to PVDF membrane (Immobilon-PSQ PVDF Membrane, Millipore, ISEQ00010). Membranes were blocked with 5% skim milk and incubated with each appropriate first antibody for overnight at 4 °C, then washed 3 times with TBS-T buffer (20 mM Tris-Cl, pH 7.6, 137.5 mM NaCl, 0.25% Tween 20). Further, membranes were incubated with Horseradish peroxidase (HRP) conjugated second antibody for 1 h, followed by 3 times washing with TBS-T buffer. ECL solution (Bio-rad, 1705061) was applied to membranes, and the band signals were detected by chemiluminescent detector (Azure, c280).

Quantitative RT-PCR

Tri-solution (Bio Science Technology, TS200-001) was used for extracting RNA from cells or brain samples. The RNA concentration was measured by Nanodrop 2000 (Thermo), and 1 µg of RNA was subjected to further experiments. ImProm-II™ Reverse Transcription System (Promega, A3800) and poly T primer were used for reverse transcript of RNA samples. FastStart Universal SYBR Green Master (Rox) (Roche, 04913850001) and StepOnePlus Real-Time PCR System (Applied Biosystems) were utilized to conduct qPCR according to manufacturer's instructions with primers for each gene:

Period1 F: 5'-GCCAAGAAAGATCCGTCGTCAG-3'
 Period1 R: 5'-GGGCTTCTTGCTCCACATGGAC-3' (ref. ³¹)
 Period2 F: 5'-GCTTCTGGTCTGGACTGCAC-3'
 Period2 R: 5'-GAGTGTCTGAGGGCTCGTTG-3' (ref. ³²)
 Cryptochrome1 F: 5'-GTGGATCAGCTGGGAAGAAG-3'
 Cryptochrome1 R: 5'-CACAGGGCAGTAGCAGTGA A-3' (ref. ³³)
 Bmal1 F: 5'-GCAGTGCCACTGACTACCAAGA-3'
 Bmal1 R: 5'-TCCTGGACATTGCATTGCAT-3' (ref. ³⁴)
 Disc1 F: 5'-GAACAGCAGAAGGCTGGGC-3'
 Disc1 R: 5'-GACCTTCCAACACTTCCATGC-3' (ref. ³⁵)
 Tbp F: 5'-GGGAGAATCATGGACCAGAA-3'
 Tbp R: 5'-CCGTAAGGCATCATTGGACT-3' (ref. ³⁶)
 Gapdh F: 5'-CACTGAAGGGCATCTTGG-3'

Gapdh R: 5'-TTACTCCTTGGAGGCCATG-3' (ref. ³⁷)
Gapdh or TATA-Box Binding Protein (*TBP*) was utilized as a reference gene for qRT-PCR according to previous studies^{38–42}.

Mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were obtained from wild type C57BL/6J mouse and *Disc1*-LI C57BL/6J mouse line. Embryos with embryonic day 14.5 were used. Body part of embryos without red blood organs was washed with PBS (Welgene, ML 008-01) and minced with blades. Samples were dissociated further with trypsin-EDTA (HyClone, SH30042.02) and pipetting for 10 min. Complete media (DMEM with 10% FBS and 1% penicillin/streptomycin) was applied to block trypsin activity. Collected cells were centrifuged for 3 min at 1000 rpm and pellets were resuspended with complete media. To dissociate into single cells, cell strainer was applied. Dissociated cells were seeded into culture dishes and media change was conducted after 6 h.

Circadian rhythm experiments

For cultured cell experiments, 1 µM of dexamethasone (Sigma, D4902-25MG) was applied to synchronize cells' circadian phase. After 2 h, cells were changed to fresh complete media (DMEM with 10% FBS and 1% penicillin/streptomycin) and were harvested according to each time point. To obtain mouse brain samples for circadian experiments, mice were entrained to a 12-h light and 12-h dark cycle for 7 days and then to constant darkness to monitor internal circadian phenotypes. After the constant dark cycle had started, mice were kept for 1 day and sacrificed in the next day. 36 h after the constant dark cycle had started was considered circadian time 0. Mice were sacrificed under dim red light according to their circadian times and further dissection for brains was performed.

For long-term monitoring of circadian behavior in live animals, mice were surgically implanted with telemetric probes (E-mitter; Mini-Mitter, Bend) and kept in a light-proof Clean Animal Rack cabinet (Shin Biotech) equipped with an automatic light–dark schedule controller. The light intensity during the light phase was kept at 350–450 lux all over the cabinet. The temperature inside the cabinet was maintained to 23 ± 1 °C, and constant air ventilation was applied all the time. Prior to the actual monitoring, mice were fully entrained to a 12-h light and 12-h dark cycle for at least two weeks. For the next 10 days with the same light–dark cycle, wheel-running activity, body temperature, and home cage activity were continuously recorded at 6-min intervals using ER-4000 system. Data obtained during this period were used for the construction of light–dark profiles of behaviors. Then, mice were released to constant darkness (dark-dark cycle)

for 10 days to determine the free-running period of circadian behaviors. For phase delay assay or phase advance assay, 30 min of light pulse was given to mice on circadian time 14 h or 20 h, respectively. Thereafter, the phase change was measured.

Luciferase assay

Luciferase assay was conducted using Dual Luciferase Assay system (Promega, E1910), according to manufacturer's instruction. Cells were cultured on 12-well plate and transfected with PEL. Prior to cell prep, cells were washed with PBS (Welgene, ML 008-01). 100 μ l of passive lysis buffer (PLB) was applied to each well, and the plate was agitated for 10 min to detach cells from the plate. The lysate was collected and centrifuged for 30 s at 13,000 rpm. The 40 μ l of supernatant was transferred to 96-well white plate (Nunc, 136101). 50 μ l of luciferase assay buffer II was injected into a well of 96-well plate, and shaking was performed for 1 second. Thereafter, Firefly luciferase activity was measured for 10 s. 50 μ l of Stop & Glo Buffer was injected into the same well of 96-well plate, and shaking was performed for 1 s. Thereafter, Renilla luciferase activity was measured for 10 s. Injection of substrates and luminescence detection were programmed and conducted by automatic injector and plate reader (TECAN, infinite M200 PRO).

Immunoprecipitation and ubiquitination assay

Cultured cells, transfected with proper plasmids for each experiment, were lysed with Nonidet P-40 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP40, 5 mM EDTA, 1 \times protease inhibitor cocktail). Brain samples were homogenized in Nonidet P-40 buffer. Samples were sonicated and centrifuged at 13,000 rpm, 4 °C for 10 min. The supernatants were incubated with 500 ng of antibodies via rotation at 4 °C. Protein A beads (Roche, 05015979001) were washed three times with Nonidet P-40 buffer. Antibody incubated samples were added to washed beads and incubated further for 4 h at 4 °C with rotation. Beads, with bound proteins, were precipitated with 1 min of centrifugation at 2000 rpm, 4 °C. Beads were washed three times with Nonidet P-40 buffer and denatured with SDS sampling buffer at 95 °C for 10 min. Samples were further subjected to immunoblotting. For ubiquitination assay, cells had been transfected with plasmids, including HA-ubiquitin; thereafter, 10 mM of MG132 (Sigma, C2211-5MG) was treated for 5 h prior to the cell lysis. Further experimental procedure is identical to immunoprecipitation assay.

Chromatin immunoprecipitation

Cultured cells were transfected with appropriate plasmids. Cells were treated with 0.75% of formaldehyde (Sigma, 252549) dropwise and incubated for 10 min with

slow agitation. 125 mM of glycine was treated to stop cross-linking. After twice of PBS washing, cells were harvested with PBS and centrifuged for 5 min, 1000 \times g, 4 °C. Cells were resuspended with ChIP lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 \times Protease Inhibitor) and incubated for 10 min on ice. Samples were sonicated for 20 min (30% amplitude, sonicate/rest for 3 s/3 s), and cell debris was removed with centrifuge for 10 min, 13,000 rpm, 4 °C. Samples were diluted with mRIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA). 50 μ l of samples were kept as an input. 25 μ g of total DNA samples were used each. Samples were incubated with Protein A beads (Roche, 05015979001) for 4 h at 4 °C with rotation to pre-clear non-specific bindings. Beads were removed by centrifuge for 1 min, 2,000 rpm, 4 °C. Antibody was treated to samples and incubated for 4 h at 4 °C with rotation. New Protein A beads were washed three times with mRIPA buffer, and beads were incubated with samples for overnight. Incubated beads were washed once with Low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), once with High salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) by centrifuge for 1 min, 2000 rpm, 4 °C. Bead-attached samples were eluted from beads by incubation with 120 μ l of Elution buffer (1% SDS, 100 mM NaHCO₃) for 15 min at 30 °C. Beads were removed by centrifuge for 1 min, 2000 rpm. 4.8 μ l of 5 M NaCl and 2 μ l of 10 mg/ml RNase A (Thermo, EN0531) were added to samples and incubated for overnight at 65 °C with agitation. 4 μ l of 10 mg/ml Proteinase K (Sigma, P2308) was added and incubated for 1 h at 60 °C with agitation. Further, DNA was acquired by PCR purification kit. For inputs, 50 μ l of each input was mixed with 70 μ l of Elution buffer and followed the same procedure with bead samples. Promoter targets were quantified with qPCR. For E-boxes of *DISC1* promoter, 5'-G GTTTTGTGCCAAGCCTCTG-3'/5'-AGCTCACCTCC AGGCTAGAA-3' and 5'-GGAGGTGAGCTGCTTAAG GG-3'/5'-CCATGCAAGCTCCTAGGCAA-3' primer sets were utilized for qPCR.

Quantification and statistical analysis

Sample size was chosen based on previous studies. No statistical methods were used for sample size estimation. No randomization was used for animal studies. The investigators were not blinded to the group allocation during the animal experiments. ImageJ (NIH) was used for quantification of band intensity of western blot, and Prism 8 (GraphPad) was used for statistical analyses.

Statistical significances were determined by unpaired *t*-test or paired *t*-test, two-tailed for comparisons of two groups. One-way ANOVA or two-way ANOVA was used for comparisons of multiple groups, followed by Tukey's multiple comparison test or Sidak's multiple comparison test, respectively. *P*-value less than 0.05 was considered a significant difference. If variances of the data were significantly different, we utilized a statistical test that does not assume homogenous variance; Welch's ANOVA, unpaired *t*-test with Welch's correction.

Results

DISC1 shows rhythmic expression relative to circadian time

To elucidate whether *DISC1* is associated with the circadian system, we first examined whether *DISC1* itself has an oscillatory expression pattern as other circadian-related genes. *Disc1* mRNA expression was assessed in NIH3T3 cells after synchronization induced by dexamethasone treatment. The mRNA level of *Disc1* showed an oscillatory pattern that peaked at 24 and 40 h after synchronization (Fig. 1a). Subsequently, we examined this expression pattern on an organism level. We entrained mice to a 12-h light and 12-h dark cycle for a week, and then switched to constant darkness and sacrificed mice according to their circadian time. Remarkably, we revealed the oscillatory mRNA expression of *Disc1* in the hippocampus (Fig. 1b). *Disc1* mRNA peaked at circadian time 20 h (CT20), originally nighttime (~12–24 h) for mice. The mRNA oscillation of *Disc1* was also observed in the suprachiasmatic nucleus (SCN) (Fig. 1c). *Disc1* mRNA peak was CT19. When compared to other circadian genes in SCN, *Disc1* peak time is slower than *Per1* and *Per2*, and faster than *Rev-erba*^{43,44}. Moreover, DISC1 endogenous protein levels also oscillated (Fig. 1d, e). Taken together, *Disc1* expression shows an oscillatory pattern, with the expression level enhanced during late nighttime to early daytime.

CLOCK and BMAL1 heterodimer regulates DISC1 promoter

Next, we attempted to discover the factors that contribute to the oscillatory expression of *DISC1* within the molecular circadian system. As shown in Fig. 1b, the mRNA expression of *Disc1* increases in late nighttime and a little later, roughly similar to the expression pattern of *Cry1*. Since the expression of circadian genes, such as *Cry1*, *Cry2*, and *Per2*, is controlled by CLOCK/BMAL1 heterodimer⁵, we hypothesized that *Disc1* expression could be regulated with a comparable mechanism.

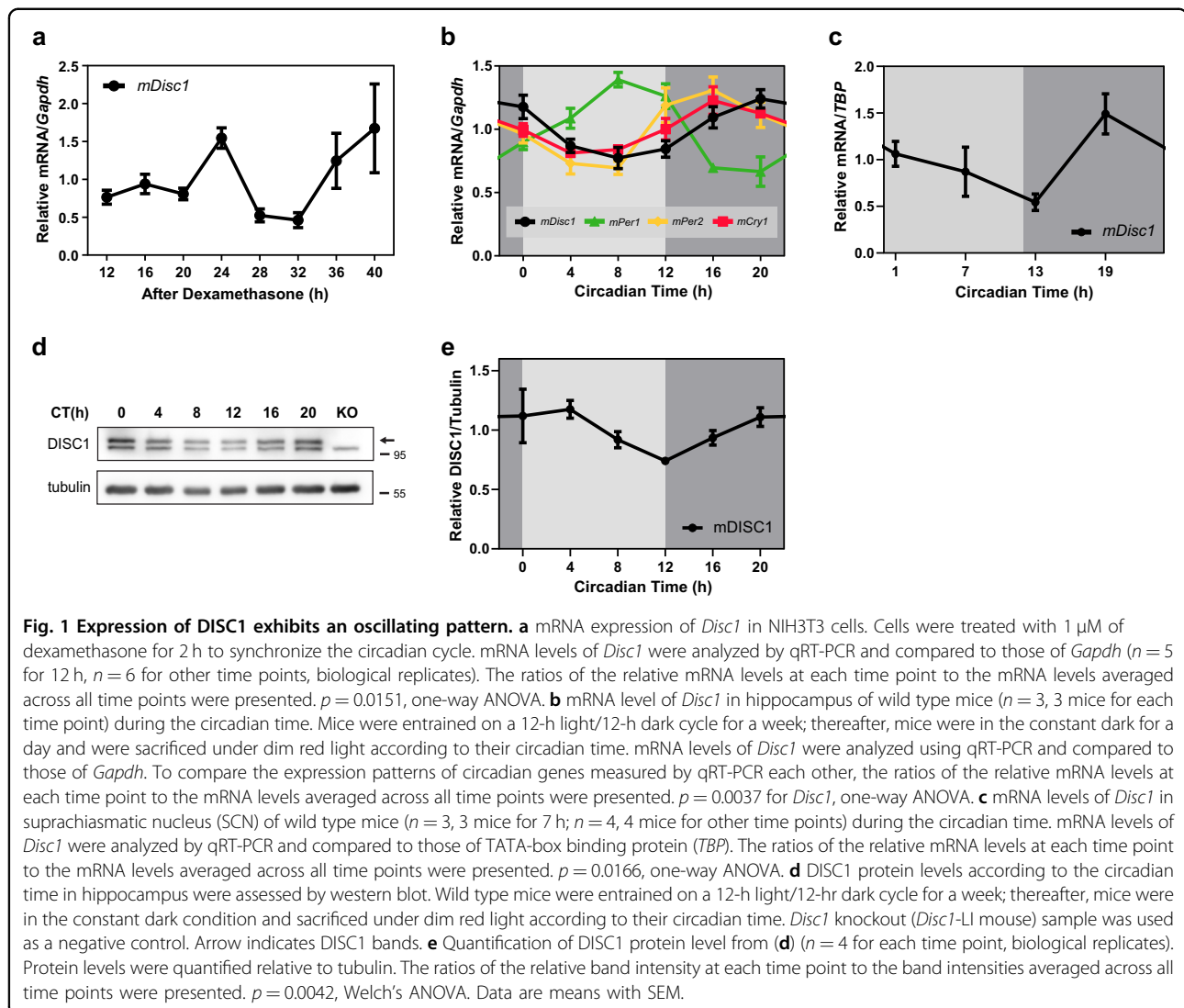
Based on the previous characterization of *DISC1* promoter²⁷, we further analyzed the region from -982 to +47 bp relative to the transcription start site (TSS). *DISC1* promoter was cloned into pGL3 vector containing luciferase destabilized by PEST sequence²⁸. We then assessed the activity of *DISC1* promoter by luciferase assay system.

Interestingly, *DISC1* promoter activity was enhanced when CLOCK and BMAL1 were co-expressed, but not when CLOCK was expressed alone (Fig. 2a). Next, fragments of the *DISC1* promoter were sub-cloned into pGL3 vector. The distal part of *DISC1* promoter (-982 to -624 bp relative to TSS) was highly enhanced by CLOCK and BMAL1 co-expression (Fig. 2b). The middle part of *DISC1* promoter (-623 to -324 bp relative to TSS) was also moderately enhanced by CLOCK and BMAL1 transfection, while the proximal part of *DISC1* promoter (-323 to +47 bp relative to TSS) did not show significant enhancement. Therefore, the distal part of *DISC1* promoter is the most responsive region to CLOCK/BMAL1.

To define the binding sites of CLOCK/BMAL1 heterodimer on *DISC1* promoter, we utilized the 'LASAGNA-Search 2.0' tool^{45,46}. From the prediction based on the previously reported matrix parameter for CLOCK and BMAL1⁴⁷, two candidate binding sites (-718 and -668 bp relative to TSS) were identified (Supplementary Fig. 1). The two predicted sites match with the consensus E-box element, CANNTG, where CLOCK/BMAL1 binds^{48–51}. Then, the predicted E-box sequences of *DISC1* promoter were mutated to 'ACNNTA' to block their activity. *DISC1* promoter with mutations in either one of the two E-box sequences or both showed lower responses to CLOCK and BMAL1 overexpression than the wild type promoter (Fig. 2c). When the distal part of *DISC1* promoter was tested separately, double mutation of E-box sequences abrogated the response to CLOCK and BMAL1 (Fig. 2d). Then, chromatin immunoprecipitation assay (ChIP) was employed to test whether CLOCK/BMAL1 heterodimer physically binds to the E-box sequences of *DISC1* promoter (Fig. 2e). In CLOCK/BMAL1-cotransfected cells, qPCR signals for both E-boxes (-718 and -668 bp relative to TSS) were markedly increased. In addition, we performed ChIP on mutant E-boxes (-718 and -668 bp relative to TSS) of *DISC1* promoter to examine the binding of CLOCK/BMAL1 heterodimer. The qPCR signals for both E-boxes were significantly decreased (Supplementary Fig. 2). Altogether, the results further support the idea that the two E-box sites within the distal part of *DISC1* promoter are critical for the modulation of *DISC1* expression by CLOCK/BMAL1.

DISC1 regulates BMAL1 stability and expression of circadian genes

PERs and CRYs are regulated by CLOCK/BMAL1 through E-box sequences and exhibit oscillatory expression within circadian time⁵. Circadian expression of PERs and CRYs subsequently inhibits CLOCK/BMAL1 via a negative feedback loop. Since *DISC1* expression is regulated by CLOCK/BMAL1 through E-box sequences and displays an oscillatory expression, we hypothesized that



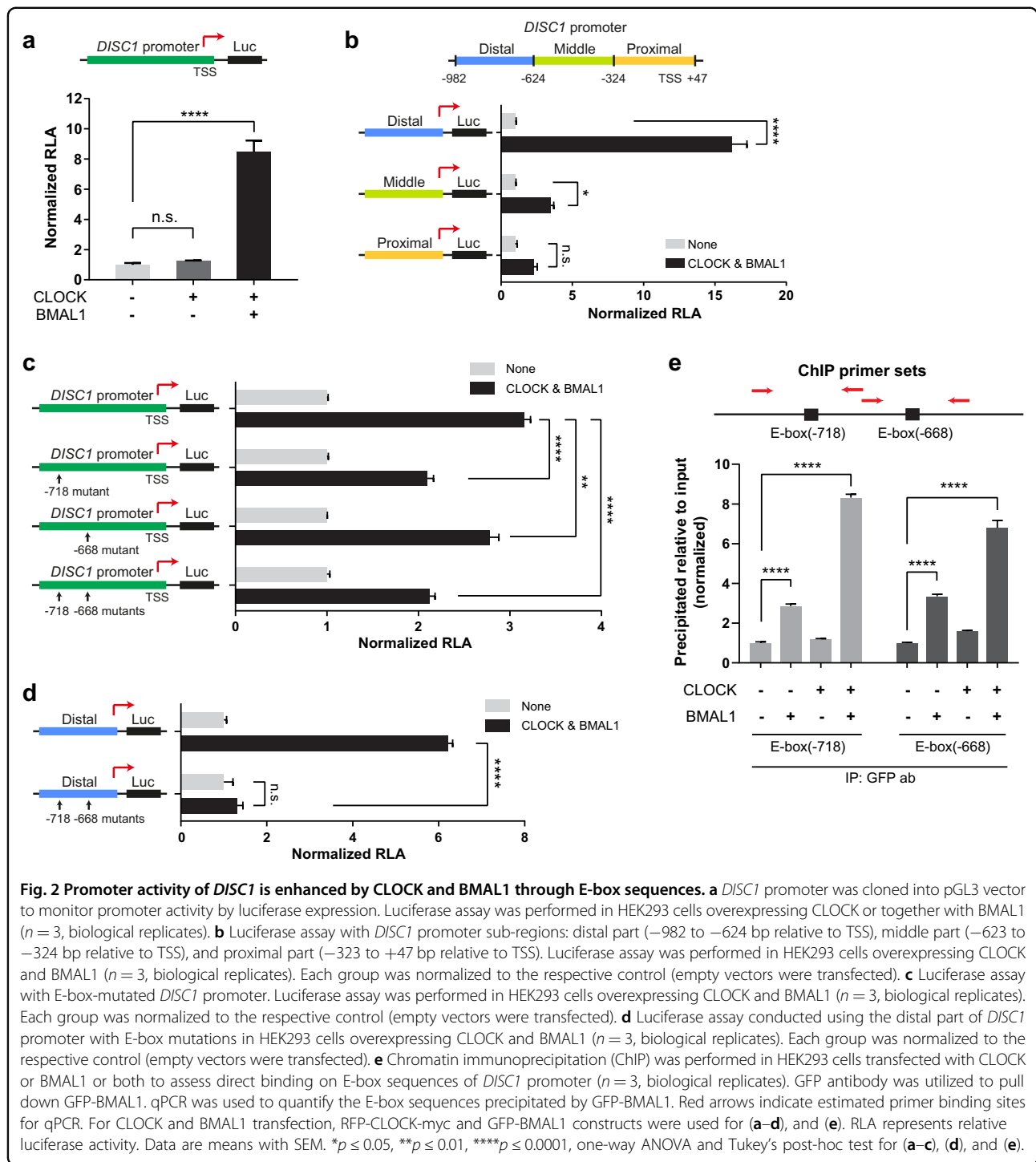
DISC1 protein may also affect the molecular circadian system, and we scrutinized the potential links. Interestingly, we observed that ectopic expression of DISC1 enhanced BMAL1 protein level in HEK293 cells (Fig. 3a, b). Conversely, endogenous BMAL1 protein level was decreased in mouse embryonic fibroblasts (MEFs) derived from *Disc1*-LI mice, a *Disc1*-deficient mouse line²⁴ (Fig. 3c, d).

To verify whether DISC1 deficiency affects BMAL1 oscillation over circadian time, we analyzed BMAL1 protein expression at each circadian time after dexamethasone treatment (Fig. 3e, f). The circadian oscillation of BMAL1 was significantly damped in *Disc1*-LI MEFs, and this effect was higher when BMAL1 levels were at their peak, namely 20 and 24 h after dexamethasone treatment. Moreover, the mRNA levels of circadian genes under the control of BMAL1 were concomitantly reduced in *Disc1*-LI MEFs (Fig. 3g–j). Given that the knockout of

Disc1 damps BMAL1, thereby also decreasing the mRNA expression of *Per2*, *Cry1*, and *Bmal1*, these data strongly suggest that DISC1 may function as a stabilizer of BMAL1.

Disc1 knockout mice display altered circadian behavior

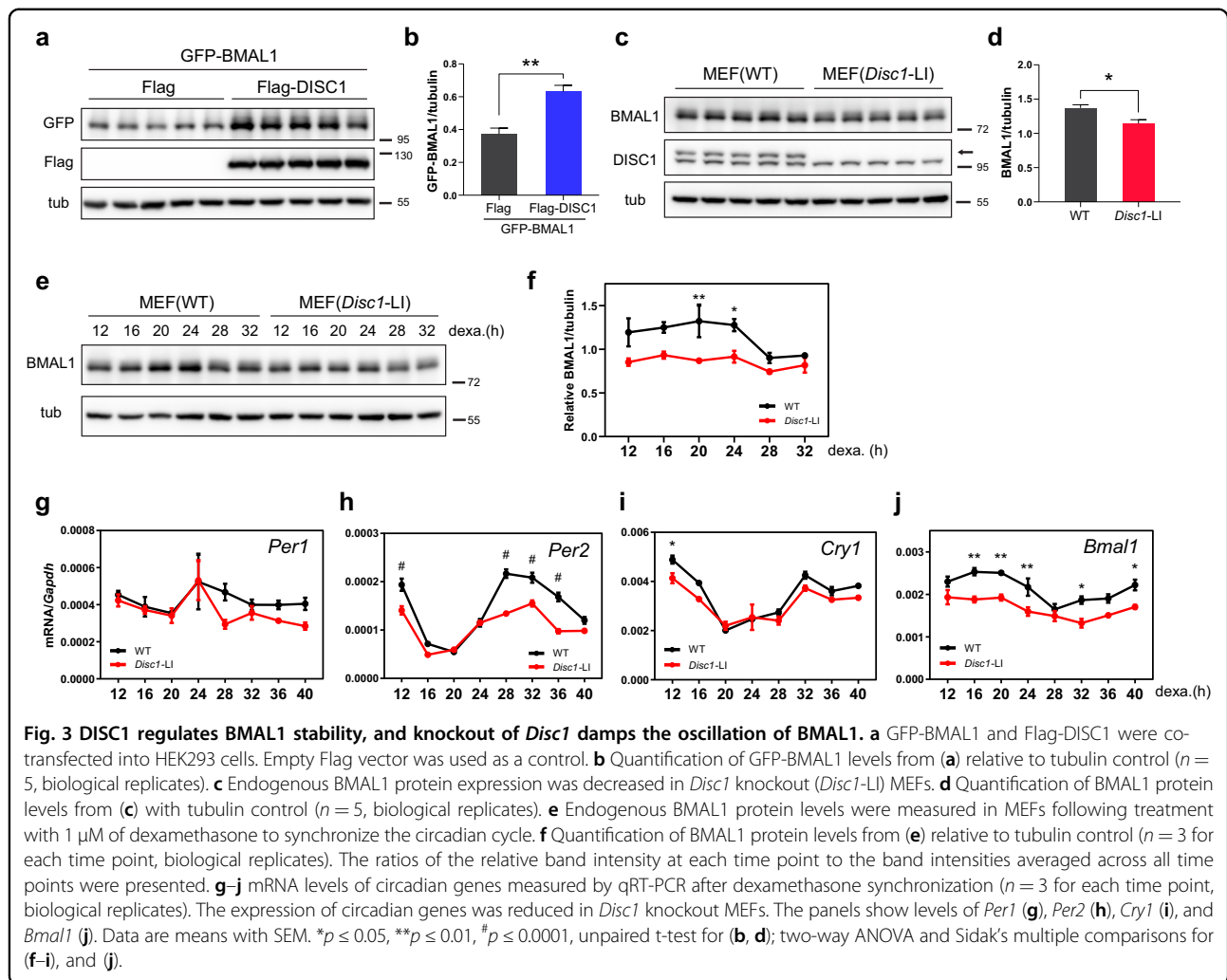
Since *Disc1* knockout decreases the expression of circadian genes and decreases protein levels of BMAL1, we hypothesized that *Disc1* knockout would also affect circadian behaviors. To test this hypothesis, we conducted circadian behavioral tests with *Disc1*-LI mice. We first entrained mice to a 12-h light and 12-h dark cycle prior to the observation of light/dark profile of circadian behavior. Notably, *Disc1*-LI mice exhibited lowered amplitude of circadian behaviors: wheel-running activity (Fig. 4a), body temperature (Fig. 4b), and home cage activity (Fig. 4c). We then monitored the wheel-running activity profile in a constant dark/dark cycle to observe the internal circadian



period of mice, which is represented by the free-running period (Fig. 4d, e). *Disc1*-LI mice displayed a shorter free-running period than wild type mice. Furthermore, we examined phase perturbations from external light cues (Supplementary Fig. 3). We gave 30 min of the light pulse to mice at circadian time 14 h or 20 h for phase delay assay or phase advance assay, respectively. *Disc1*-LI mice

exhibited a tendency toward an increase in phase delay and phase advance experiments. However, both did not reach statistical significance.

We also checked the expression of circadian genes at the transcript level. Consistent with the MEFs data, at circadian time 16 h, *Disc1*-LI mice showed lower expression of *Per2*, *Cry1*, and *Bmal1* in the hippocampus than



wild type mice (Fig. 4f). These results further demonstrate that DISC1 has a modulatory role for circadian rhythm likely through stabilizing BMAL1, a core component of the molecular clock.

DISC1 regulates the stability of BMAL1 by modulating its ubiquitination through GSK3 β

Next, we sought to elucidate how DISC1 regulates the stability of BMAL1. Because DISC1 is known to function as a scaffold protein, we examined the interaction between DISC1 and BMAL1. DISC1 interacted with BMAL1 as shown by co-immunoprecipitation with over-expressed DISC1 and BMAL1 (Fig. 5a). We further confirmed the co-immunoprecipitation in MEFs and mouse brain lysates (Supplementary Fig. 4), suggesting that DISC1 may directly affect BMAL1 function.

Ubiquitination is widely known as a signal for protein degradation⁵². Hence, we conducted ubiquitination assays and observed that ubiquitination of BMAL1 was inhibited upon co-expression of DISC1 (Fig. 5b, c),

consistent with the finding that DISC1 increases BMAL1 levels. Conversely, ubiquitination of BMAL1 was enhanced in *DISC1* knockdown cells (Fig. 5d, e). Thus, we concluded that DISC1 binds to BMAL1 and inhibits its ubiquitination.

The phosphorylation of glycogen synthase kinase 3 β (GSK3 β) Ser9 is inhibitory, while Tyr216 phosphorylation enhances its activity⁵³. DISC1 can inhibit the Tyr216 phosphorylation (p-Y216) of GSK3 β to impede its enzymatic activity³⁰. On the other hand, GSK3 β regulates multiple circadian proteins⁵⁴, such as CRY2^{55,56}, CLOCK⁵⁷, BMAL1¹¹, and REV-ERB α ⁵⁸, by modulating their stability¹⁰. Particularly, BMAL1 phosphorylation by GSK3 β promotes its ubiquitination¹¹. In addition, as shown in previous reports^{11,30}, GSK3 β interacts with DISC1 and BMAL1 in co-immunoprecipitation experiments (Supplementary Fig. 4b). Thus, we tested if DISC1 contributes to the regulation of BMAL1 ubiquitination through GSK3 β . An activity-enhanced form of GSK3 β by mutation of Ser9 to Ala increased the ubiquitination of

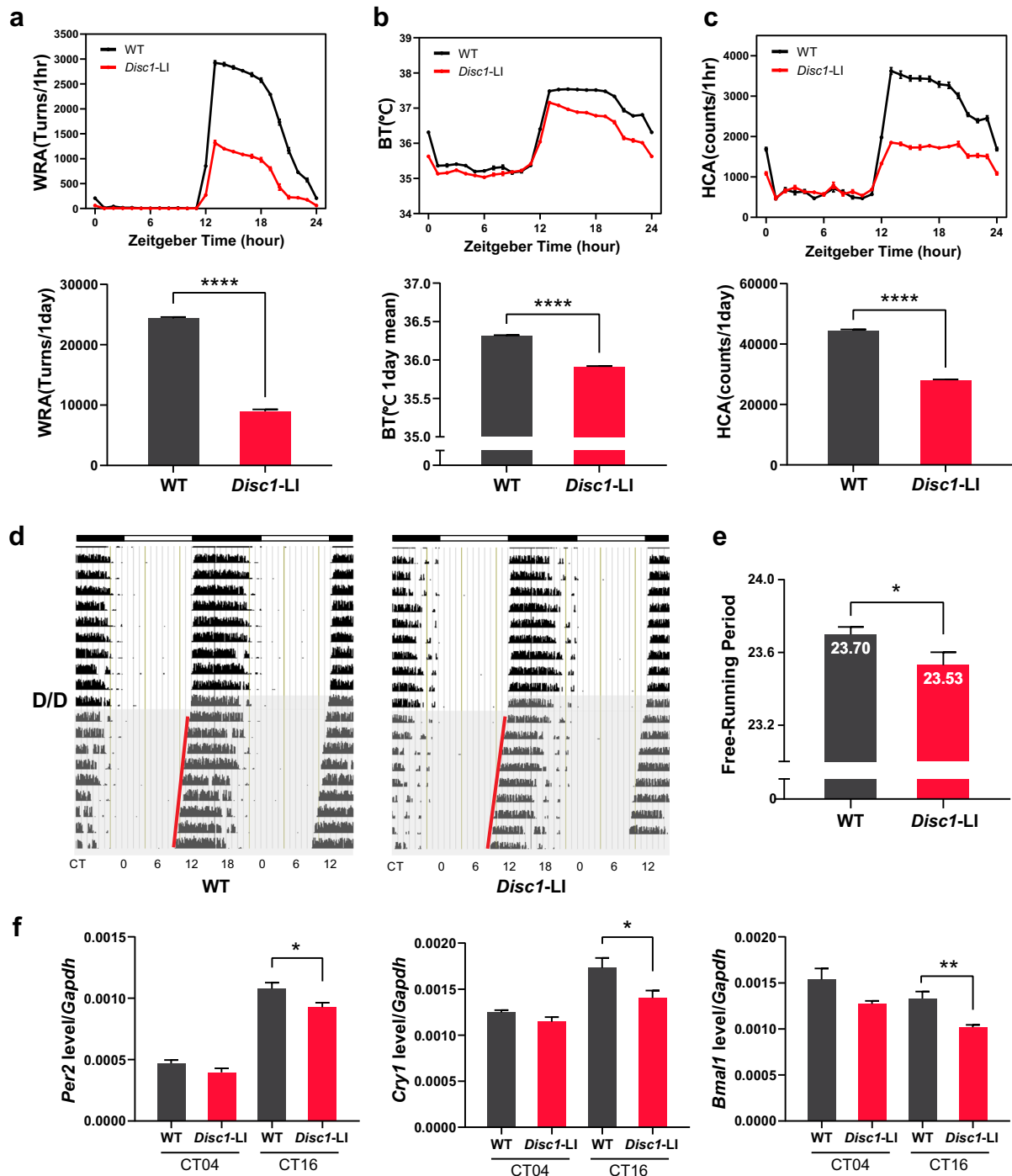
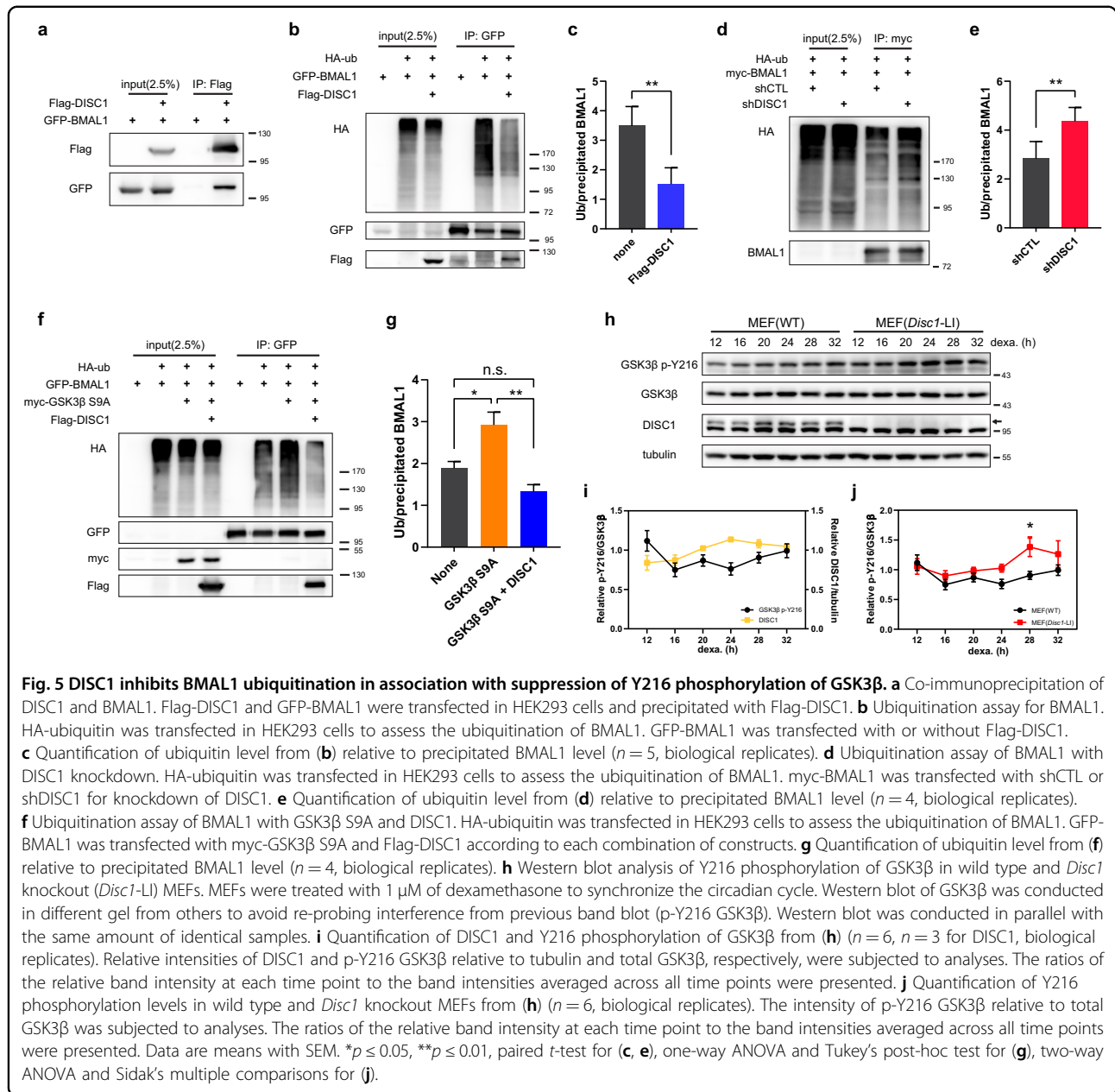


Fig. 4 *Disc1* knockout mice exhibit altered circadian behaviors. **a–c** Overall circadian behaviors during L/D cycle ($n = 10$, biological replicates). Mice were fully entrained to the light/dark (L/D; 12-h/12-h) cycle for at least two weeks. For the next 10 days with the same lighting schedule, light/dark profiles were generated as described in “Methods”. Then, mice were released to a dark/dark (D/D; constant dark) cycle for another 10 days to determine the internal circadian period. **a** WRA, wheel-running activity; **b** BT, body temperature; **c** HCA, home cage activity. Compared to wild type mice, *Disc1* knockout mice (*Disc1*-LI) showed reduced values during the nighttime. Total measured values per one day were depicted as bar graphs for wheel-running and home cage activities. For body temperature, averaged values were used. **d, e** Free-running periods were measured for wild type ($n = 16$, biological replicates) and *Disc1* knockout ($n = 10$, biological replicates) mice under the D/D cycle. *Disc1* knockout mice showed a shorter Free-running period than wild type mice. **f** mRNA levels of circadian genes at circadian time of 4 h (CT04) and 16 h (CT16) ($n = 4$ for 4 h, $n = 5$ for 16 h, biological replicates). Hippocampus was dissected from wild type or *Disc1* knockout mice. Data are means with SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, unpaired *t*-test for **(a)**, **(b)**, **(e)** and **(f)**; unpaired *t*-test with Welch’s correction for **(c)**.



BMAL1 as previously reported (Fig. 5f, g). However, this effect was abolished upon co-expression with DISC1.

To determine the effect of DISC1 on GSK3β over circadian time, we examined the level of p-Y216 in MEFs after dexamethasone synchronization (Fig. 5h). When DISC1 was at its peak, 24 h after synchronization, p-Y216 level was low (Fig. 5i). Subsequently, p-Y216 level gradually increased, while DISC1 level decreased. Moreover, p-Y216 level in *Disc1*-LI MEFs was increased at 28 h after dexamethasone treatment compared to wild type (Fig. 5j). Taken together, these results indicate that DISC1 suppresses p-Y216 of GSK3β according to circadian time, thereby enhancing BMAL1 stability.

Discussion

In this study, we showed that DISC1 has novel functions on the molecular circadian clock, further expanding the repertoire of the diverse roles of DISC1 in various cell types. Interestingly, the functional interaction between DISC1 and the molecular clock is reciprocal; DISC1 modulates the quality of the molecular clock by regulating the GSK3β-mediated BMAL1 stability and, at the same time, *DISC1* expression itself is under control of CLOCK/BMAL1, displaying an oscillating expression pattern according to circadian time (Supplementary Fig. 5). The oscillating level of DISC1 is supposed to affect the oscillating level of BMAL1 protein, which will, in turn, affect

the *DISC1* promoter activity. This regulatory loop is achieved by the presence of E-box elements in the *DISC1* promoter, which are targets of CLOCK/BMAL1 heterodimer. We identified the two major E-boxes controlled by BMAL1/CLOCK, but more cis-regulatory elements participating in the circadian expression of *DISC1* directly or indirectly may still exist. This notion is supported by the observation that the *DISC1* promoter activity was not totally abolished by the mutations of both E-box elements. Presumably, as a result, *DISC1* deficiency leads to damped expressions of circadian genes. The impacts on the circadian genes were quantitatively differential (Fig. 3g–j), likely due to the heterogeneity of the compositions and their collective reactivity of the cis-regulatory elements responsible for circadian control^{59,60}. Further clarification of this novel modulatory pathway will establish a complete view of the molecular clock.

The peak of *DISC1* is similar to the peak of *BMAL1*, occurring ~20–24 h after dexamethasone treatment (Figs. 3f and 5i). Moreover, the expression pattern of *DISC1* in the brain (Fig. 1b, c, e) is also consistent with the previously reported expression pattern of *BMAL1*^{9,61,62}, supporting a protective role of *DISC1* for *BMAL1*. In this regard, the shorter period seen in the *Disc1*-LI mouse appears counterintuitive, given that mice with low *BMAL1* show a more extended period^{63,64} and *Bmal1* knockout mice exhibit arrhythmia^{65,66}. Notably, however, *DISC1* functionally downregulates *GSK3β* by inhibiting p-Y216. Although there are conflicting results on the relationship between *GSK3β* and circadian period^{67,68}, *GSK3β* gain-of-function tends to shorten the circadian period^{69,70}. Thus, it is possible that the shortened period in *Disc1*-LI mice is due to the enhanced activity of *GSK3β*, which is consistent with the notion that *DISC1* prevents *BMAL1* from premature degradation to maintain the robustness of circadian rhythmicity.

We demonstrated that *DISC1* expression oscillates according to circadian time. This finding suggests that the previously known functions of *DISC1*, from embryonic neurodevelopment to adult neurogenesis, may also be regulated by the circadian system. In this respect, the oscillating phosphorylation of Y216, a regulatory hotspot of *GSK3β* activity controlled by *DISC1*³⁰, is noteworthy. The phosphorylation of Y216 is critical for neural progenitor proliferation, which may indicate that *DISC1*-related neurodevelopmental processes, including neuronal signaling, neuronal morphogenesis, and spine regulation¹⁷, are also under circadian control. Moreover, *DISC1* downregulation augments adult neurogenesis in the hippocampus^{71,72} in conjunction with memory formation⁷³. Indeed, memory formation and adult neurogenesis are circadian-related phenomena^{74,75}. For example, the number of BrdU-positive cells in the

hippocampal subgranular zone changes over circadian time, with relatively impaired neurogenesis from late night to early daytime (peak neurogenesis at 15 h)⁷⁵, which corresponds to the level of *DISC1* protein expression in the hippocampus shown in this work (Fig. 1e). Therefore, it will be interesting to test whether *DISC1* acts as an output pathway that links the molecular clock and neurodevelopmental program.

A natural deletion of *Disc1*, resulting in truncation after amino acid residue 528, did not alter the sleep phenotypes⁷⁶, which is counterintuitive to our results from *Disc1*-LI mice. However, it is noteworthy that the truncated *DISC1* still retains the region, amino acid residues 195–228, for the inhibition of *GSK3β* activity³⁰, which is a crucial step of the regulation of *BMAL1* stability by *DISC1* according to the observations in this study. Its more robust circadian phenotype shown in this study is likely to be a reflection of complete *Disc1* loss-of-function. Anyhow, the involvement of *DISC1* in the regulation of the molecular circadian clock is intriguing in that sleep and circadian rhythm disruption (SCRD) is a common feature of various psychiatric disorders⁷⁷. Components of molecular clocks were proposed as factors of SCRD⁷⁸, though the precise molecular mechanisms underlying this phenomenon are still elusive. Although the genetic association of *DISC1* with the various psychiatric conditions is still to be further clarified, the identification of *DISC1* as a novel regulator of the molecular clock may suggest its role in the interface between some aspects of psychiatric disorders and the circadian system. Notably, rhythmic expression of genes, including *PER2* and *CRY2*, is abolished in a group of patients with schizophrenia⁷⁹; similarly, expression of *PER2* and *CRY1* is impaired in some schizophrenia patients⁸⁰. In our study, an interesting correlation was observed in the *Disc1* knockout mouse, where the expression of the circadian genes was also diminished. Thus, although the nature of the correlation needs further validation, the roles of *DISC1* in the molecular clock may provide a novel mechanistic insight into the molecular links between SCRD and psychiatric disorders.

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Author details

¹Department of Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea. ²Department of Physiology, College of Medicine, Kyung Hee University, Seoul, Republic of Korea. ³Present address: SK biopharmaceuticals Ltd, Seongnam-Si, Republic of Korea. ⁴Present address: Department of Pathology, College of Medicine, Hanyang University, Seoul, Korea

Author contributions

S.B.L., Y.K., Y.-U.P., and S.K.P. conceived the study. S.B.L., S.C., and S.K.P. designed experiments. S.B.L., J.P., Y.K., Y.-U.P., T.T.M.N., B.K.S., Y.W., and E.C. performed experiments. S.B.L., B.K.S., Y.W., Y.S., S.C., and S.K.P. analyzed and interpreted data. S.B.L., S.C., and S.K.P. wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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