E3 ubiquitin ligase HRD1 modulates the circadian clock through regulation of BMAL1 stability

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Abstract. Circadian rhythm serves an essential role in numerous physiological functions. Circadian oscillations are organized by circadian clock components at the molecular level. The precision of the circadian clock is controlled by transcriptional-translational negative feedback loops, as well as post-translational modifications of clock proteins, including ubiquitination; however, the influence of E3 ligases on clock protein ubiquitination requires further investigation. The results of co-immunoprecipitation and immunofluorescent localization, indicated that the endoplasmic reticulum transmembrane E3 ubiquitin ligase HRD1, encoded by the synoviolin 1 gene, interacted with brain and muscle ARNT-like 1 (BMAL1) and enhanced BMAL1 protein ubiquitination. In addition, the results of western blotting and reverse transcription-quantitative PCR suggested that HRD1 promoted K48-associated polyubiquitination of BMAL1 and thus mediated its degradation via the ubiquitin-proteasome system. Furthermore, gene knockdown and gene overexpression assays revealed that HRD1-dependent degradation of BMAL1 protein regulated the expression of BMAL1 target genes and the amplitude of circadian oscillations in mammalian cells. The findings of the current study indicate that HRD1 may influence the regulation of circadian rhythm via modulation of BMAL1 stability.

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Introduction

Circadian rhythm influences various behaviors and physiological characteristics of the body, including movement, sleep, body temperature, endocrine function and metabolic rhythms, in both invertebrate and vertebrate species. Dysregulation of the circadian rhythm is associated with numerous diseases, including diabetes, obesity and cancer (1-4). Circadian oscillations result from transcriptional-translational negative feedback loops that are organized by circadian clock components at the molecular level. Primarily, two core circadian clock transcriptional factors [brain and muscle ARNT-like1 (BMAL1) and clock circadian regulator (CLOCK)] form heterodimers and rhythmically bind to enhancer boxes in the promoters of target genes, such as the negative regulators periods 1-3 (PER 1-3) and cryptochromes 1 and 2 (CRY 1 and 2). Subsequently, PERs and CRYs suppress the transcriptional activity of BMAL1 and CLOCK (5-8). The precision of the circadian clock is further influenced by clock protein posttranslational modifications, including ubiquitination, phosphorylation, acetylation and SUMOylation, that are critical for the maintenance of normal physiological function (9-11).

Ubiquitination is one of the most important post-translational modifications for proteins and the ubiquitin-proteasome system (UPS) is responsible for the degradation of various proteins (12). Dysfunction in the UPS can result in multiple diseases, including metabolic diseases, cancer and neurological diseases (13,14). The UPS is composed of three classes of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. E3 ligases are complementary to specific substrates (15,16). Previous studies have indicated that ubiquitination is associated with the regulation of the circadian clock (7,17). Notably, two F-box E3 ligases, F-box and leucine rich repeat protein 3 and F-box and leucine rich repeat protein 21 pseudogene, cooperate to regulate the degradation of CRY proteins and the oscillation of the circadian clock (18-20). An additional two E3 ligases, ARF-BP1 and PAM, have been revealed to form a complex that mediates degradation of the circadian heme receptor REV-ERBα (also transcriptionally activated by BMAL1/CLOCK heterodimers), which in turn inhibits *BMAL1* transcription (21-24). Furthermore, BMAL1 is degraded

via ubiquitination mediated by E3 ligase UBE3A (25). E3 ligase HRD1 (HRD1), an endoplasmic reticulum (ER) transmembrane protein, is an E3 ubiquitin ligase encoded by the synoviolin 1 gene (26-28). HRD has been suggested to influence ER-associated degradation (ERAD), which is a protein quality control system that targets misfolded ER-associated proteins for ubiquitination and subsequent degradation (29). As HRD1 is a well-established E3 ligase that mediates substrate ubiquitination (26-28), it was hypothesized that the UPS may influence HRD1-mediated ubiquitination of BMAL1.

The results of the present study suggested that HRD1 enhanced the ubiquitination of BMAL1 and promoted its degradation via the UPS. Furthermore, the results suggested that HRD1-dependent degradation of BMAL1 protein regulated the expression of BMAL1 target genes and the amplitude of circadian oscillations in mammalian cells, which indicated that HRD1 may influence circadian rhythm.

Materials and methods

Plasmids. HA-Ub [wild-type (WT)], HA-Ub (K48R) and HA-Ub (K63R) plasmids were kindly provided by Dr Hui Zheng (Soochow University, Suzhou, China). The 3xFLAG-BMAL1, PGL3-*BMAL1*-luciferase and PGL3-*PER1*-luciferase plasmids were kindly provided by Dr Ying Xu (Soochow University). The 3xFLAG-HRD1 plasmid was kindly provided by Dr Shengyun Fang (University of Maryland Biotechnology Institute, Rockville, USA). For pEGFP-N3-BMAL1 plasmid construction, the full length BMAL1 cDNA was created by subcloning the PCR product with the primers 5'-CGGAATTCC ATGGCGGACCAGAGAATG-3' and 5'-GCGTCGACCAGC GGCCATGGCAAGTC-3', which was then inserted into the pEGFP-N3 (Takara Bio-USA, Inc.) vector at *Eco*RI/*Sal*I sites. A previously developed mutant FLAG-HRD1 vector that lost the E3 ubiquitin ligase activity was used in the present study, in which the RING finger domain was deleted (ΔRING) (30).

Cell culture, transfection and drug treatment. 293 T-cells or mouse neuroblastoma cells (Neuro-2a; N2a) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with penicillin (100 mg/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO_2. 30-50% seeding densities of $1x10^5$ cultured cells/well in 12-wells were transfected with 2 μg plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h of transfection at 37°C with 5% CO_2, $3x10^5$ cells were harvested for immunoblot analyses or treated with proteasome inhibitor MG132 (10 μM) or control for 14 h for immunoprecipitation assays. In order to analyze protein stability, transfected cells were treated with cycloheximide (CHX; Sigma-Aldrich; Merck KGaA;100 μg/ml) for 0, 2, 4, 6 or 8 h before harvesting. Cells were incubated with 100 nM bafilomycin A1 (Baf; Sigma-Aldrich; Merck KGaA) for 24 h or 10 μM MG132 (Sigma-Aldrich; Merck KGaA) for 12 h before harvesting. N2a cells were synchronized to the same stage of cell cycle using horse serum treatment. N2a cells were shifted to a DMEM containing 50% horse serum (Gibco; Thermo Fisher Scientific, Inc.) and incubated for

2 h, after which the serum-rich DMEM was replaced with serum-free medium.

Antibodies. The following primary antibodies were used: Mouse monoclonal antibodies against FLAG (Sigma-Aldrich; Merck KGaA; cat. no. F9291; 1:1,000), GAPDH (Chemicon; Merck KGaA; cat. no. SAB2108668; 1:5,000), GFP (Santa Cruz Biotechnology, Inc.; cat. no. (B-2):SC-9996; 1:1,000), HA (Santa Cruz Biotechnology, Inc.; cat. no. sc-7392; 1:500), BMAL1 (Santa Cruz Biotechnology, Inc.; cat. no. SC-365645; 1:500), Ub (Santa Cruz Biotechnology, Inc.; cat. no. sc-8017; 1:500); rabbit polyclonal antibodies against HRD1 (Abgent; cat. no. ap2184a; 1:500) and Histone 2B (Abcam; cat. no. ab52599; 1:500).

The following secondary antibodies were used: Horseradish peroxidase-conjugated sheep anti-mouse antibody (GE Healthcare; cat. no. NA931; 1:5,000) and anti-rabbit antibody (GE Healthcare; cat. no. NA934; 1:5,000) for 2 h at room temperature. The proteins were visualized with an ECL detection kit (Thermo Fisher Scientific, Inc.).

Immunoblot analysis. Cells were harvested and then lysed in cell lysis buffer [50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% sodium deoxycholate; 1% Nonidet P-40; protease inhibitor cocktail (Roche Diagnostics)]. The bicinchoninic acid method was used to determine the protein concentration and the quantity of protein loaded in each lane. 8% SDS-PAGE was used for the separation of FLAG, HA and Ub proteins. 13.5% SDS-PAGE was used for the separation of BMAL1, GAPDH and HRD1 proteins. SDS-PAGE were transferred onto a PVDF membrane (EMD Millipore). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, followed by incubation with the primary antibodies for 12 h at 4°C and secondary antibodies for 2 h at room temperature. Densitometric analyses of immunoblots from three independent experiments were performed using Adobe Photoshop CS5 (Adobe, Inc.), and the resulting data were analyzed using Origin 6.0 (OriginLab Corporation). Graphs indicate the quantification of the blots and were prepared according to a previously reported method (31).

Immunocytochemistry. HEK293 cells transfected with EGFP-BMAL1 and FLAG-HRD1 were washed with PBS twice and fixed with 4% paraformaldehyde for 10 min at room temperature. After treatment with 0.25% Triton X-100 for 15 min, the cells were blocked with 4% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Cells were washed with PBS and incubated with anti-FLAG antibody (Sigma-Aldrich; Merck KGaA; cat. no. F9291; 1:1,000) or GFP antibody (Santa Cruz Biotechnology, Inc.; cat. no. (B-2):SC-9996; 1:1,000) in PBS overnight at 4°C. Next, the cells were incubated with Alexa Fluor 594 donkey anti-mouse secondary antibodies (Thermo Fisher Scientific, Inc.; cat. no. A32744; 1:300) for 2 h and then the nuclei were stained with DAPI (Sigma-Aldrich; Merck KGaA; cat. no. D9542; 2 μg/ml) for 10 min. Finally, the cells were observed with an inverted system microscope Ti2-E (Nikon, Japan).

Immunoprecipitation assay. 293 cells or N2a cells were co-transfected with various combinations of tagged plasmids detailed in the figure legends. After 24 h, the transfected

cells were treated with 10 μM/ml MG132 (Sigma-Aldrich; Merck KGaA) for 14 h. The cells were then harvested and lyzed in cell lysis buffer (50 mM Tris-HCl pH 7.5 buffer containing 150 mM NaCl, 1% NP-40 and 0.5% deoxycholate) supplemented with the protease inhibitor cocktail (Roche Diagnostics) at 4˚C. Cells were sonicated with 200 W amplitude and duration of 10 sec on ice, and centrifuged at 3,000 x g for 30 min at 4˚C. Meanwhile, protein G-Sepharose beads (Roche Diagnostics) were incubated with anti-Flag antibody (Sigma-Aldrich; Merck KGaA; cat. no. F9291; 1:1,000) or anti-GFP antibody (Santa Cruz Biotechnology, Inc.; cat. no. (B-2):SC-9996; 1:1,000) and 0.01% BSA (Sigma-Aldrich; Merck KGaA) at 4˚C for 12 h. After they were washed with ice-cold PBS, the beads were incubated with the supernatants for 6 h on ice. After incubation, the beads were washed with ice cold PBS, six times, and then the bound proteins were eluted with SDS sample buffer and analyzed by with immunoblotting.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from N2a cells was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and then 500 ng of each RNA sample was reverse-transcribed at 16˚C into cDNA using a PrimeScript RT Master Mix (Takara Bio, Inc.). qPCR was performed with the following thermocycling conditions: 95˚C for 10 min, 95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 40 sec, for 40 cycles using Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with their relevant primers using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following sequences of primers were used: Mouse *GAPDH*: Forward, 5'-CATGGCCTTCCGTGTTCCTA-3' and reverse, 5'-CCTGCTTCACCACCTTCTT-3'; mouse *HRD1*: Forward, 5'-CGTGTGGACTTTATGGAACGC-3' and reverse, 5'-CGGGTCAGGATGCTGTGATAAG-3'; mouse *BMAL1*: Forward, 5'-TCCAGTCTTGGCATCAATGAGT-3' and reverse, 5'-CCTAATTCTCAGGGCAGCAGAT-3'; mouse *DBP*: Forward, 5'-CGTGGAGGTGCTTAATGACCTTT-3' and reverse, 5'-CATGGCCTGGAATGCTTGA-3'; mouse *PER1*: Forward, 5'-TGGCTCAAGTGGCAATGAGTC-3' and reverse, 5'-GGCTCGAGCTGACTGTTCACT-3'. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (32).

Nuclear and cytoplasmic fractionation assay. The 293 cells that had been transfected with BMAL1-EGFP along with empty control vector or FLAG-tagged HRD1 for 24 h were lysed in fractionation buffer (320 mM sucrose; 3 mM CaCl$_2$; 2 mM MgAc; 0.1 mM EDT; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride and 0.5% NP-40) for 20 min on ice. After centrifugation at 600 x g for 15 min at 4˚C, the supernatant was collected as the cytoplasmic fraction. The pellet was washed once with fractionation buffer without NP-40 and lysed in cell lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5% deoxycholate, 1% NP-40 and protease inhibitor cocktail (Roche Diagnostics)] as the nuclear fraction. Histone 2B (H2B) served as a nuclear marker and GAPDH served as a cytoplasmic marker.

Luciferase reporter gene assay. 293 cells were transfected using Lipofectamine® 2000 (Invitrogen; Thermo

Fisher Scientific, Inc.) with 2 μg pGL3-*BMAL1*-Luciferase (500 ng/μl) or PGL3-*PER1*-Luciferase (500 ng/μl) construct and co-transfected with various combinations of tagged plasmids (500 ng/μl). The *Renilla* luciferase-expressing plasmid pRL-CMV (500 ng/μl) was co-transfected into cells to normalize the variations in transfection efficiency. After 36 h of transfection, the cells were harvested and treated with passive lysis buffer (Promega Corporation). The activities of both firefly and *Renilla* luciferase were measured using a dual luciferase assay kit (Promega Corporation) through a Microplate reader Infinite M1000 Pro (Tecan Group, Ltd.) according to the manufacturer's instructions (Promega Corporation). The absolute values of firefly luminescence were normalized to those of *Renilla* and the ratios were presented as the median of three transfected experiments, as described previously (31).

Statistical analysis. Quantitative data are presented as the mean ± SEM. Statistical analysis of the data was performed by a paired Student's t-test for two group comparisons and one-way ANOVA with Tukey's test for multiple group comparisons. $P < 0.05$ was considered to be statistically significant.

Results

E3 ligase HRD1 decreases BMAL1 protein levels. To investigate the degradation pathway of BMAL1, 293 cells were treated with the proteasome inhibitor, MG132, and the autophagy inhibitor, Baf. It was revealed that treatment with MG132, but not Baf, significantly increased the protein levels of BMAL1 in comparison to vehicle (Fig. 1A). This suggests that BMAL1 protein is prone to degradation via the UPS rather than lysosomes. To confirm whether other E3 ligases besides UBE3A were involved in BMAL1 degradation, 293 cells were transfected with several E3 ligase plasmids. Among those E3 ligases, HRD1 reduced the protein levels of BMAL1 compared to empty vector (Fig. 1B). Increased expression of HRD1 was seen in cells transfected with FLAG-HRD1 compared with FLAG vector alone (Fig. 1C). In addition, the reduction of BMAL1 protein due to HRD1 overexpression could be rescued following treatment with the proteasome inhibitor MG132 (Fig. 1D and E), suggesting that HRD1-mediated BMAL1 reduction by the proteasome system. To further confirm the effect of HRD1 on BMAL1, short interfering RNA (siRNA) was used to knock down HRD1 in different cell lines. Depletion of HRD1 markedly increased endogenous BMAL1 levels in N2a cells (Fig. 1F) as well as in 293 cells (Fig. 1G). The current results indicated that HRD1 may degrade BMAL1 protein.

HRD1 regulates the stability of BMAL1 protein. As HRD1 is an E3 ligase, it was hypothesized that HRD1-mediated reduction of BMAL1 protein was relevant to its ubiquitin ligase activity. The C-terminal RING finger domain of HRD1 is essential for its activity as an E3 ubiquitin ligase (28). Hence, the effect of a RING finger domain deletion mutant of HRD1 (ΔRING) on BMAL1 degradation was investigated. By contrast to wild-type HRD1, overexpression of the mutant HRD1 did not result in degradation of BMAL1 (Fig. 2A). To further confirm the finding that HRD1 promoted the degradation of BMAL1, 293 cells were transfected with different doses
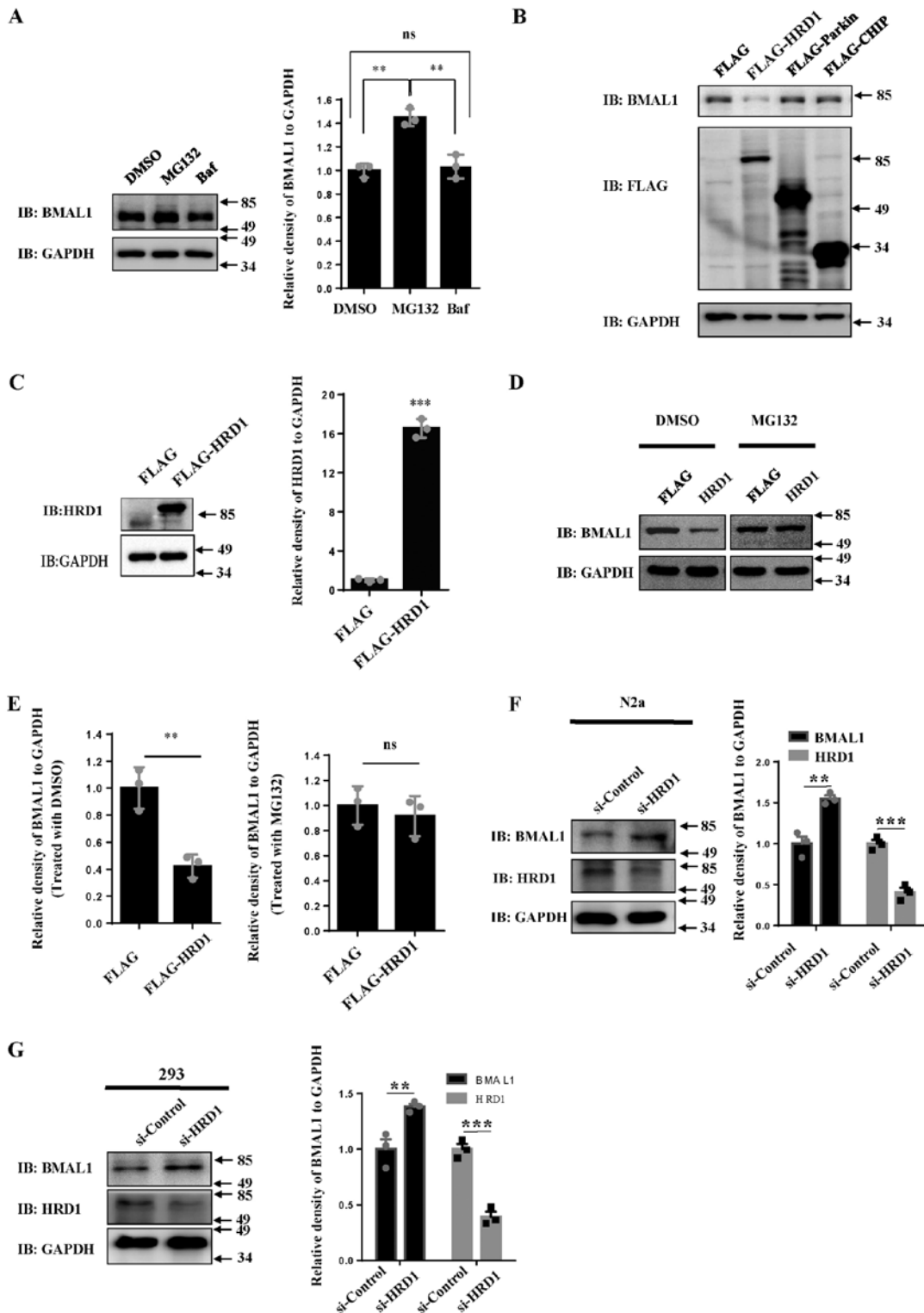
Figure 1. HRD1 decreases BMAL1 protein levels. (A) 293 cells were treated with MG132 (10 μM) or Baf (100 μM) for 14 h and the levels of endogenous immunoblotting BMAL1 were determined by western blot analysis. The relative levels of BMAL1 to GAPDH were quantified. (B) 293 cells were transfected with empty control vector or FLAG-tagged HRD1, Parkin or CHIP, respectively. After 24 h of transfection, cell lysates were subjected to western blotting. 293 cells were transfected with empty control vector or FLAG-tagged HRD1. After 24 h of transfection, the cells were treated with MG132 (10 μM) or vehicle. The levels of (C) HRD1 and (D) BMAL1 were determined by western blotting. (E) The relative levels of BMAL1 to GAPDH were analyzed. (F) N2a cells and (G) 293 cells were transfected with si-control or si-HRD1. After 72 h, the cell lysates were subjected to western blotting. The relative levels of BMAL1 to GAPDH were analyzed. **P<0.01, ***P<0.001. Data were collected from 3 repeats, indicated by the squares and circles. Baf, bafilomycin A1; BMAL1, brain and muscle ARNT-like 1; DMSO, dimethyl sulfoxide; HRD1, endoplasmic reticulum transmembrane E3 ubiquitin ligase HRD1; ns, no statistical significance; si, small interfering.

of FLAG-tagged HRD1. The abundance of BMAL1 decreased in association with an increase in FLAG-HRD1 (Fig. 2B). The increased expression of BMAL1 in cells transfected

with BMAL1-EGFP compared with EGFP vector only is shown in Fig. 2C. It was revealed that the degradation rate of BMAL1 protein was faster in cells transfected with HRD1,
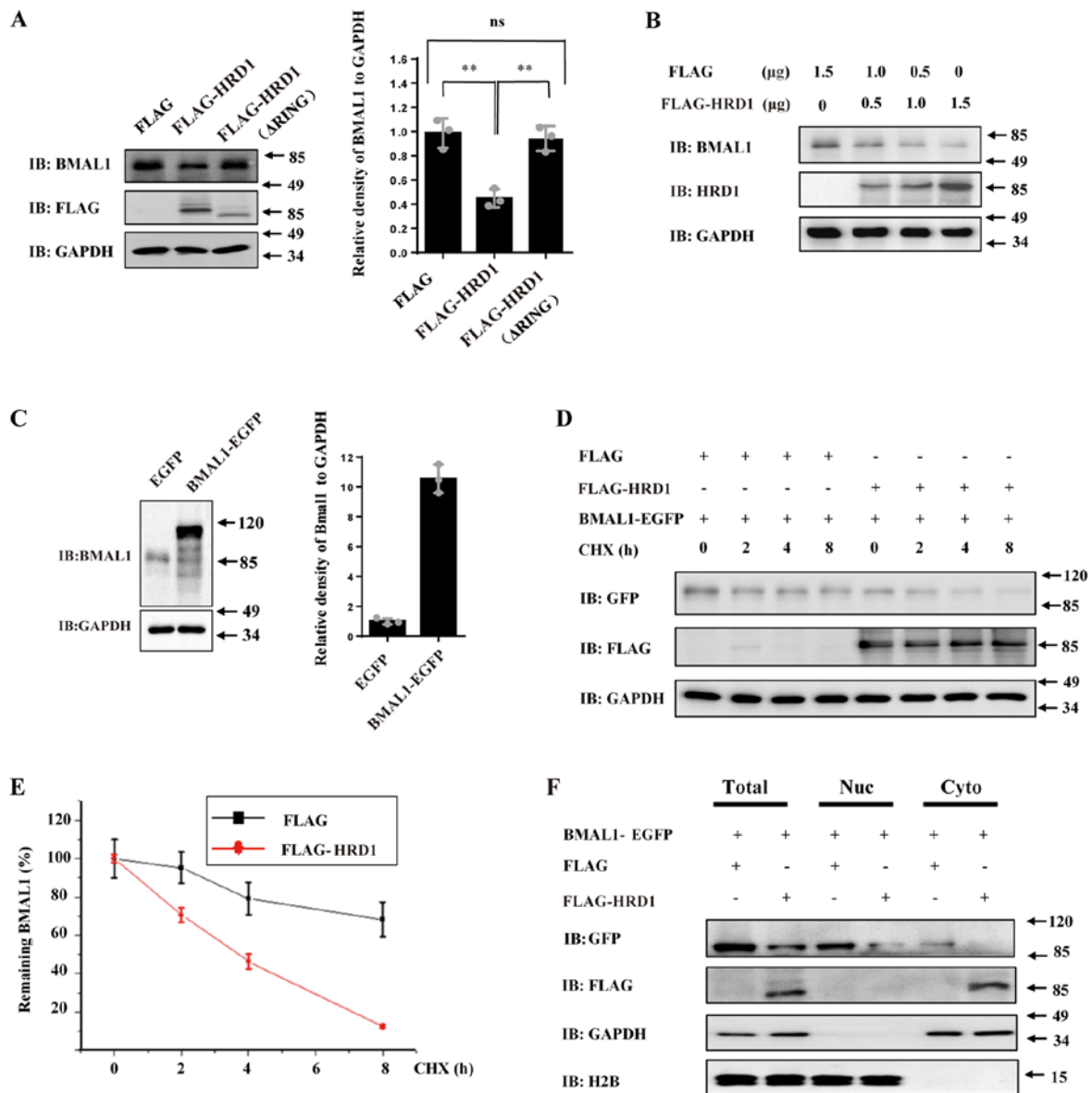
Figure 2. HRD1 regulates the stability of BMAL1 protein. (A) 293 cells were transfected with empty control vector, FLAG-tagged HRD1 or FLAG-tagged mutant HRD1 (ΔRING), respectively. After 24 h of transfection, cell lysates were subjected to western blotting. The relative levels of BMAL1 to GAPDH were analyzed. (B) 293 cells were transfected with a mixture of empty control vector and quantities of FLAG-tagged HRD1 as indicated. The levels of endogenous BMAL1 protein and FLAG-HRD1 were determined by western blotting. (C) 293 cells were transfected with empty control vector or EGFP-tagged BMAL1 for 24 h. The protein levels of BMAL1 were determined by western blotting. (D) 293 cells were co-transfected with BMAL1-EGFP along with empty control vector or FLAG-tagged HRD1. After transfection for 24 h, the cells were treated with 125 μg/ml CHX and harvested at the indicated timepoints. The protein levels of BMAL1-EGFP and FLAG-HRD1 were determined by western blotting. (E) The relative levels of BMAL1 to GAPDH at the different CHX treated times in (D) were analyzed. (F) 293 cells were co-transfected with BMAL1-EGFP along with empty control vector or FLAG-tagged HRD1. After 24 h of transfection, the cells were subjected to nuclear and cytoplasmic fractionation assay. **P<0.01. n=3 for all experiments. BMAL1, brain and muscle ARNT-like 1; CHX, cycloheximide; EGFP, enhanced green fluorescent protein; HRD1, endoplasmic reticulum transmembrane E3 ubiquitin ligase HRD1; ns, no statistical significance.

compared with the control cells, in a pulse-chase experiment (Fig. 2D and E), indicating that HRD1 may accelerate BMAL1 protein degradation. A subcellular fractionation assay was performed to investigate whether HRD1 differentially mediated subcellular BMAL1 protein degradation. Notably, HRD1 significantly reduced BMAL1 protein levels in both the nucleus and the cytoplasm (Fig. 2F).

HRD1 interacts with BMAL1 and regulates ubiquitination of BMAL1. Subsequently, whether HRD1 interacts with BMAL1 in cells was investigated. Immunoprecipitation using a BMAL1 antibody indicated that there were interactions between endogenous HRD1 and BMAL1 in 293 cells (Fig. 3A). In addition, the interactions between HRD1 and BMAL1 in FLAG-HRD1 overexpressing cells were confirmed using anti-FLAG antibody; however, CLOCK was not determined to bind to HRD1 (Fig. 3B). As displayed in Fig. 3C, it was revealed that overexpression of FLAG-HRD1 significantly increased the polyubiquitination of BMAL1-EGFP. Immunocytochemistry to detect the distribution of HRD1 and BMAL1 was also performed. Although EGFP-BMAL1 was mainly distributed in the nucleus, it was partly localized to the cytoplasm and co-localized with FLAG-HRD1 (Fig. 3D).
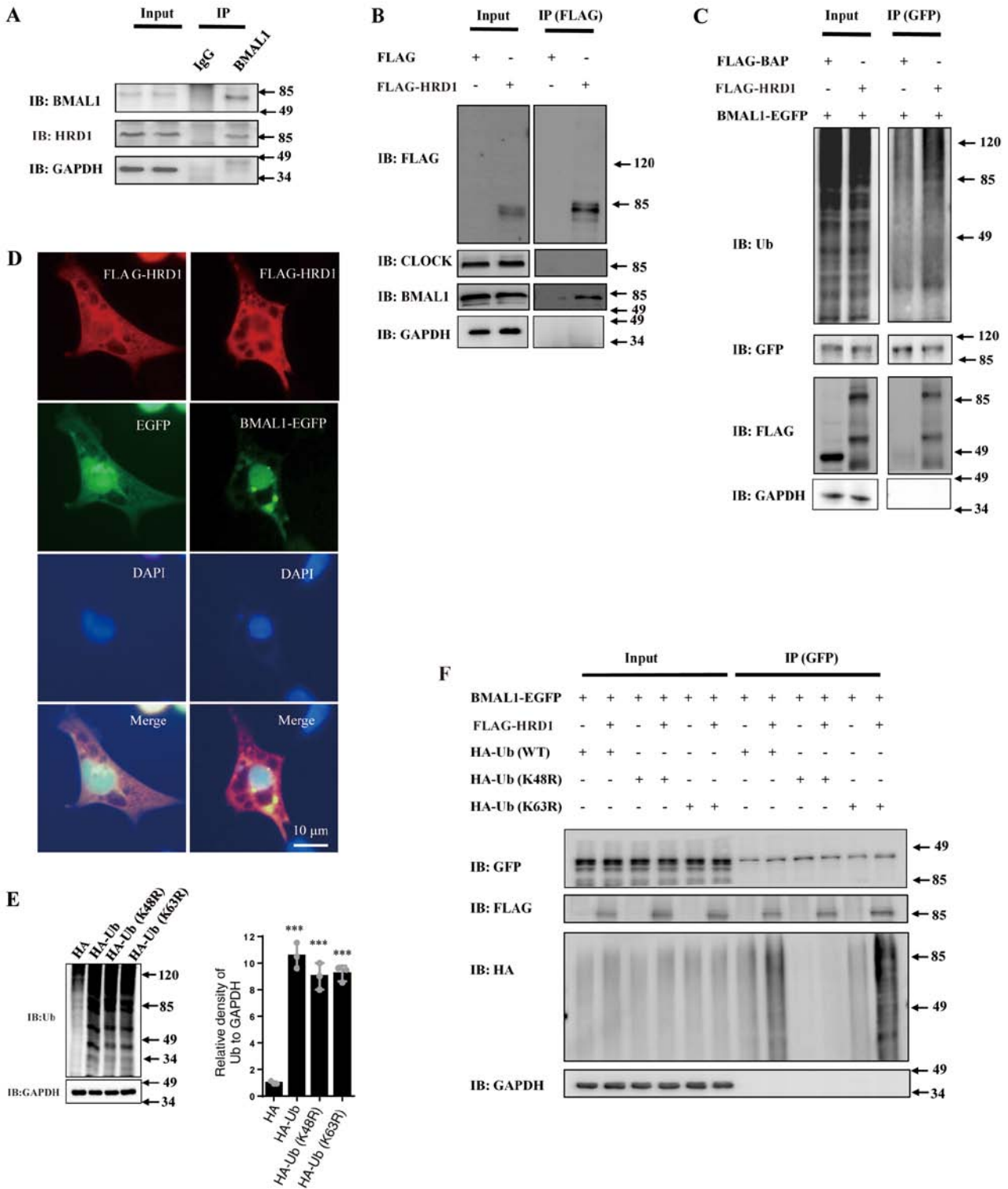
Figure 3. HRD1 interacts with and increases BMAL1 ubiquitination. (A) 293 cells were treated with 10 μM MG132 for 14 h, and the cell lysates were subjected to immunoprecipitation using an anti-BMAL1 antibody. The levels of proteins in the cell lysates (input) and the eluted bound proteins (IP) were assessed by western blotting. Input lane represents whole cell lysate and IP lane represents immunoprecipitation of bound eluted proteins. GAPDH proteins were consider to indicate equal loading for all figures. (B) 293 cells were transfected with empty control vector or FLAG-tagged HRD1 for 24 h and then were treated with 10 μM MG132 for 14 h. Then cell lysates were subjected to immunoprecipitation assay using an anti-FLAG antibody. The cell lysates and the bound proteins were assessed by western blotting. (C) 293 cells were co-transfected with BMAL1-EGFP-N3 along with empty control vector or FLAG-tagged HRD1 for 24 h and then treated with 10 μM MG132 for 14 h. The cell lysates were then subjected to immunoprecipitation assay using an anti-EGFP antibody. The cell lysates and the bound proteins were assessed by western blotting. (D) 293 cells were co-transfected with FLAG-HRD1, EGFP or BMAL1-EGFP and 48 h after transfection, immunocytochemistry was performed with anti-FLAG antibodies. (E) 293 cells were transfected with HA-Ub (WT), its mutant types HA-Ub (K48R or K63R) or empty HA vector for 36 h. The protein levels of Ub were determined by western blotting. (F) 293 cells were co-transfected with BMAL1-EGFP, FLAG or FLAG-HRD1 along with HA-Ub (WT) or its mutant types of HA-Ub (K48R or K63R) as indicated for 24 h and then were treated with MG132 (10 μM) for 14 h. The cell lysates were then subjected to immunoprecipitation assay using an anti-GFP antibody. The cell lysates and the bound proteins were analyzed by western blotting. ***P<0.001 vs. HA; n=3 for all experiments. BMAL1, brain and muscle ARNT-like 1; CHX, cycloheximide; EGFP, enhanced green fluorescent protein; HRD1, endoplasmic reticulum transmembrane E3 ubiquitin ligase HRD1; ns, no statistical significance; Ub, ubiquitin; WT, wild-type.

The type of ubiquitin chains conjugated to BMAL1 by HRD1 was investigated. Cells were co-transfected with GFP-tagged BMAL1 and FLAG-tagged HRD1 with either HA-Ub (WT), or Lys site mutant of Ub (K48R or K63R) and underwent a ubiquitination assay. Expression levels of the various HA-Ub vectors are presented in Fig. 3E. Both wild-type and K63R mutant Ub were able to form polyubiquitin chains conjugated to BMAL1 and the numbers of polyubiquitin chains conjugated to BMAL1 were significantly increased by HRD1 overexpression(Fig. 3F). By contrast, HRD1 did not induce K48R Ub to form polyubiquitin chains conjugated to BMAL1. The present results indicate that the E3 ligase HRD1 promotes K48-linked polyubiquitination of BMAL1 and mediates its degradation via the UPS.

HRD1 regulates CLOCK gene expression and circadian rhythm via targeting of BMAL1. BMAL1 and CLOCK form heterodimers to bind to the E-box motifs in promoters of target genes, such as *PERs*, *CRYs* and *DBP*, and thus activate their transcription (5,6). It was hypothesized that overexpression of HRD1, which promotes the degradation of BMAL1, may influence the transcription of those genes. Although BMAL1 protein levels were markedly reduced in FLAG-HRD1 transfected cells, *BMAL1* mRNA levels were not significantly altered by overexpression of HRD1, according to qPCR analysis (Fig. 4A), indicating that HRD1 does not influence the mRNA level of BMAL1. However, the mRNA levels of *PER1* and *DBP* were significantly decreased (Fig. 4B and C) with FLAG-HRD1 transfection in comparison to a control, indicating that HRD1 may suppress the transcription of BMAL1 downstream genes. To further investigate these findings, the activity of *BMAL1* and *PER1* promoters were measured using a dual-luciferase reporter system. Overexpression of HRD1 did not affect the activity of *BMAL1* promoter (Fig. 4D), but it strongly repressed the activity of *PER1* promoter (Fig. 4E and F) when co-transfected with BMAL-EGFP and FLAG-CLOCK. The expression of FLAG-CLOCK is shown in Fig. 4E. The results indicated that HRD1 may regulate the expression of clock genes such as *PER1* and *DBP* via regulation of BMAL1 protein levels. In addition, BMAL1 is a core clock transcription factor, which is critical for circadian rhythms (3,6). Therefore, whether HRD1 influences the circadian rhythm of clock gene transcription was investigated. To study the effect of HRD1 on the circadian rhythm of clock gene transcription, N2a cells, which are frequently used in circadian studies, were used (33-35). When N2a cells were synchronized by horse serum treatment, *PER1* was expressed in a manner consistent with the circadian rhythm. Notably, the oscillation amplitude of *PER1* mRNA levels was lower in N2a cells harboring FLAG-HRD1 compared with in control cells (Fig. 4G). In conclusion, the present results indicate that HRD1, a novel E3 ubiquitin ligase, interacted with BMAL1 and enhanced the ubiquitination modification of BMAL1 protein (Fig. 4H).

Discussion

Circadian oscillation relies on the molecular transcriptional-translational feedback loop (36,37). The positive components of this system, BMAL1 and CLOCK, are necessary for promoting the expression of clock genes (5,21,36,37). Post-translational modification has been revealed to regulate the stability of clock proteins and, thus, serves an essential role in the maintenance of circadian rhythm. Ubiquitin-conjugating enzyme E2O, an E3-independent E2 ubiquitin-conjugating enzyme, reduces BMAL1 levels by promoting its ubiquitination and degradation (38). E3 ubiquitin ligase, tumor necrosis factor receptor-associated factor 2 (TRAF2), interacts with BMAL1 to reduce its stability through ubiquitination (39). BMAL1 preferentially interacts with the zinc finger domain, but not the conventional substrate recognition domain in TRAF2 (39). Additionally, ubiquitin carboxyl-terminal hydrolase FAF-X, a deubiquitinating enzyme, was found to modulate the ubiquitination and degradation of BMAL1 (40). Although several ubiquitinating and deubiquitinating enzymes have previously been identified to mediate BMAL1 degradation, it is still of interest to investigate novel E3s that influence BMAL1 protein degradation. In the current study, it was revealed that an ERAD-associated E3 ligase HRD1 influenced BMAL1 degradation.

BMAL1 is a well-established transcriptional factor and a nucleocytoplasmic shuttling protein (41-44). A nuclear localization signal and nuclear export signal have been identified at the N-terminus of BMAL1, which suggests it may serve cytoplasmic functions (43). Previously, it was reported that BMAL1 influences the regulation of translation (45). BMAL1 regulates protein translation by interacting with a number of translation-associated factors and ribosomal proteins in the cytoplasm (45). The present results also suggested that HRD1, an ER transmembrane protein may interact with BMAL1 whereas other E3 ligases, such as Parkin and CHIP, did not influence BMAL1 degradation. The specificity of HRD1 in the regulation of BMAL1 degradation provides further evidence that HRD1 is a BMAL1-specific E3 ligase. Furthermore, nuclear and cytoplasmic fractionation assays indicated that both nuclear and cytoplasmic BMAL1 levels were decreased by the overexpression of HRD1. It is possible that degradation of BMAL1 by HRD1 in the cytoplasm decreases the BMAL1 protein level, resulting in a reduction in BMAL1 transport into the nucleus.

Polyubiquitin chains are formed at different lysine residues of ubiquitin molecules (46,47). Different topologies of polyubiquitin chains have different three-dimensional structures and functions. Two well-characterized forms are K48-linked and K63-linked polyubiquitin chains. K48-linked polyubiquitin chains are typically considered to target substrates to the proteasome, while K63-linked polyubiquitin chains function in numerous cellular pathways, such as vesicle trafficking, aggresome formation, nuclear transport and NF-κB signaling (46,47). In the current study, it was revealed that BMAL1 was rarely ubiquitinated with the K48R mutant of ubiquitin when HRD1 was overexpressed. By contrast, the K63R mutant of ubiquitin did not influence BMAL1 ubiquitination via overexpression of HRD1 compared with the wild-type ubiquitin. The current data demonstrate that the K48 site, but not the K63 site, of ubiquitin influences HRD1-mediated BMAL1 polyubiquitination.

BMAL1 and CLOCK form heterodimers to activate the transcription of key clock genes such as PERs and CRYs, as well as many clock-mediated genes (2,4,6,37). The present
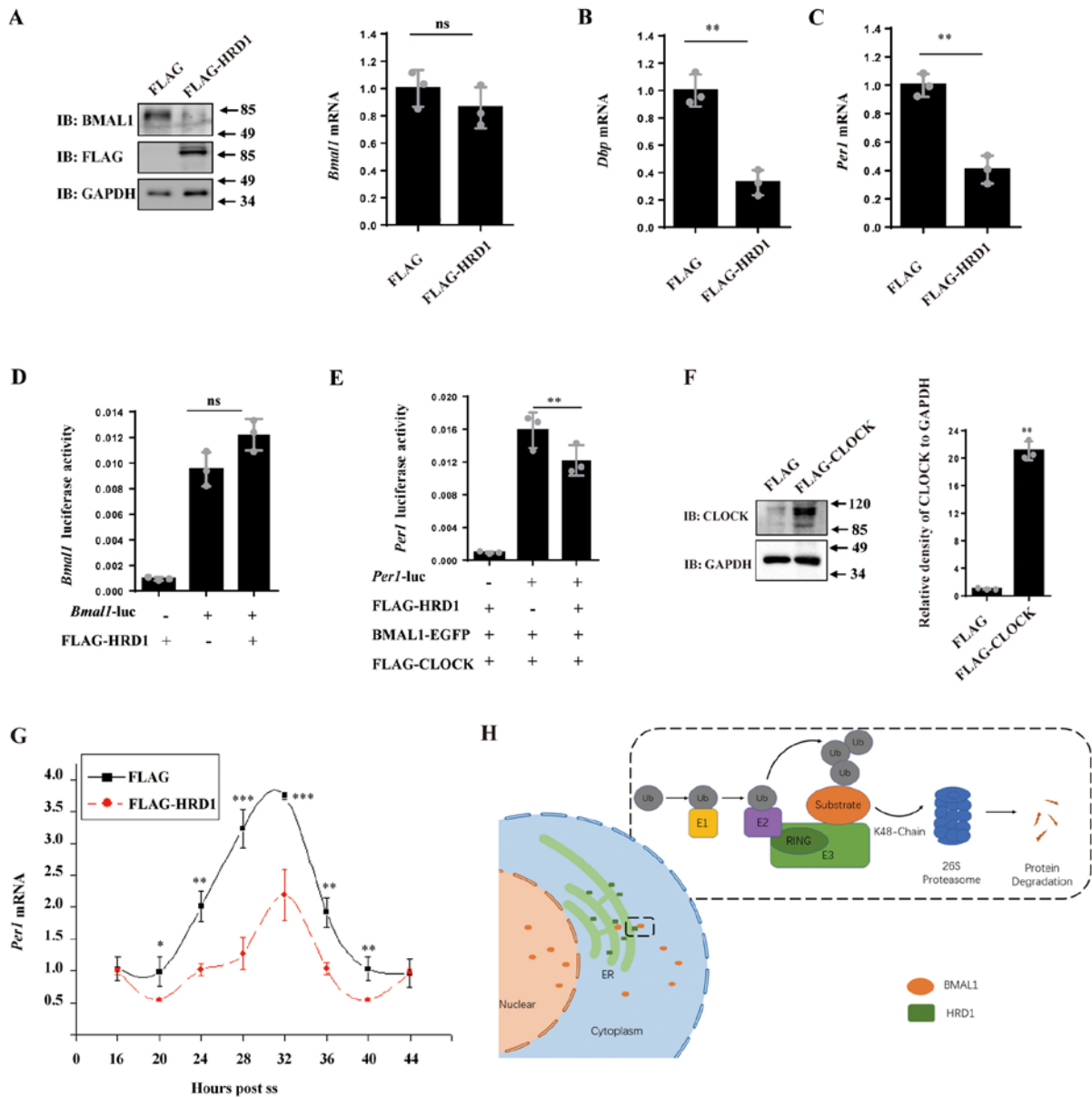
Figure 4. HRD1 influences CLOCK gene expression by degrading BMAL1 protein. (A) N2a cells were transfected with empty control vector or FLAG-tagged HRD1 for 24 h. The levels of endogenous BMAL1 and FLAG-HRD1 protein were determined by western blot analysis and the mRNA levels of *BMAL1* were determined by RT-qPCR. The mRNA levels of (B) *DBP* and (C) *PER1* were determined using RT-qPCR. **P<0.01. (D) 293 cells were co-transfected with *BMAL1*-Luc or the PGL3-Basic negative control along with empty control vector or FLAG-tagged HRD1 for 24 h. The activity of the *Bmal1* promoter was analyzed. (E) 293 cells were co-transfected with BAML1-EGFP and FLAG-CLOCK, a PGL3-*PER1*-Luc or the PGL3-Basic negative control along with empty control vector or FLAG-tagged HRD1. The activity of the *PER1* promoter was analyzed. **P<0.01. (F) 293 cells were transfected with empty control vector or FLAG-tagged CLOCK for 24 h. The levels of CLOCK protein were determined by immunoblot assay **P<0.01 vs. FLAG. (G) N2a cells were transfected with empty control vector or FLAG-tagged HRD1. The mRNA levels of *PER1* were determined by RT-qPCR. *P<0.05, **P<0.01, ***P<0.001 vs. FLAG-tagged HRD1. (H) HRD1 promoted K48-linked poly-ubiquitination of BMAL1 and subsequently mediated its degradation by the ubiquitin-proteasome system. n=3 for all experiments. BMAL1, brain and muscle ARNT-like 1; CHX, cycloheximide; EGFP, enhanced green fluorescent protein; HRD1, endoplasmic reticulum transmembrane E3 ubiquitin ligase HRD1; RT-q, reverse transcription quantitative; ns, no statistical significance; Luc, luciferase reporter; ss, synchronized with 50% horse serum.

study indicated that immunoprecipitation of endogenous HRD1 was able to detect BMAL1. Immunoprecipitation was also performed to immunoprecipitate endogenous HRD1 to detect BMAL1. The HRD1 antibody used was not available for the immunoprecipitation experiment. It was also revealed that, HRD1 may bind to BMAL1, but not CLOCK, which indicates the specificity of HRD1 to certain circadian clock proteins. Meanwhile, overexpression of HRD1 significantly suppressed the expression of PER1 and DBP1, which indicates

that a decrease in BMAL1 levels found in BMAL1/CLOCK heterodimers formed by HRD1 results in the dysfunction of circadian rhythm regulation. The current data indicate that HRD1 modulates molecular circadian rhythm via regulation of BMAL1 protein stability.

In conclusion, the results of the present study indicate that the E3 ligase HRD1 is an endogenous regulator of the circadian clock that serves its function via modulation of BMAL1 stability.

Authors' contributions

DG performed the majority of the experiments, analyzed the data, and drafted the manuscript. YZ and HW performed immunoblot assays and analyzed the data. GW analyzed the data and revised manuscript, CW analyzed and interpreted the data, and revised manuscript. HR designed experiments, analyzed the data and revised manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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