Ubiquitin-conjugating enzyme UBE2O regulates cellular clock function by promoting the degradation of the transcription factor BMAL1

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Dysregulation of the circadian rhythm is associated with many diseases, including diabetes, obesity, and cancer. Aryl hydrocarbon receptor nuclear translocator-like protein 1 (*Arntl* or *Bmal1*) is the only clock gene whose loss disrupts circadian locomotor behavior in constant darkness. BMAL1 levels are affected by proteasomal inhibition and by several enzymes in the ubiquitin–proteasome system, but the exact molecular mechanism remains unclear. Here, using immunoprecipitation and MS analyses, we discovered an interaction between BMAL1 and ubiquitin-conjugating enzyme E2 O (UBE2O), an E3-independent E2 ubiquitin–conjugating enzyme (*i.e.* hybrid E2/E3 enzyme). Biochemical experiments with cell lines and animal tissues validated this specific interaction and uncovered that UBE2O expression reduces BMAL1 levels by promoting its ubiquitination and degradation. Moreover, UBE2O expression/knockdown diminished/increased, respectively, BMAL1-mediated transcriptional activity but did not affect *BMAL1* gene expression. Bioluminescence experiments disclosed that *UBE2O* knockdown elevates the amplitude of the circadian clock in human osteosarcoma U2OS cells. Furthermore, mapping of the BMAL1-interacting domain in UBE2O and analyses of BMAL1 stability and ubiquitination revealed that the conserved region 2 (CR2) in UBE2O significantly enhances BMAL1 ubiquitination and decreases BMAL1 protein levels. A Cys-to-Ser substitution experiment identified the critical Cys residue in the CR2 domain responsible for BMAL1 ubiquitination. This work identifies UBE2O as a critical regulator in the ubiquitin–proteasome system, which modulates BMAL1 transcriptional activity and circadian function by promoting BMAL1 ubiquitination and degradation under normal physiological conditions.

Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL or BMAL1) is a transcription factor and a core circadian protein located in the positive limb of the transcription/translation feedback loop of the circadian clock (1–5). Abnormal expression of BMAL1 or other core clock proteins may influence the period, amplitude, or phase of the rough 24-h day/night cycle (2). Dysregulation of circadian rhythm is associated with many health problems, such as diabetes, obesity, and cancer (6–8). BMAL1 also has many other biological functions besides its major role in the regulation of circadian rhythm. For example, BMAL1 deletion leads to infertility in both male and female mice (9). BMAL1 deficiency is associated with premature aging and reduced lifespan (10). Recently, it has been suggested that BMAL1 acts as a tumor suppressor and enhances the sensitivity of cancer cells to chemotherapeutic drugs (11). Because BMAL1 has so many important biological functions, it is necessary to discover the upstream regulators that modulate BMAL1 protein level, its transcriptional activity, and biological functions.

It has been reported that BMAL1 can be modified by a variety of post-translational modifications (PTMs),[4] such as acetylation (12, 13), phosphorylation (14–19), and SUMOylation (20–22), which alter protein–protein interaction, subcellular localization, transcriptional activity, and thus its biological functions. The BMAL1-interacting partner CLOCK (circadian locomoter output cycles protein kaput) acts as an acetylase to promote BMAL1 acetylation on a unique lysine reside, facilitating the recruitment of cryptochrome 1 (CRY1) to the CLOCK/BMAL1 heterodimer and repressing its transcriptional activity (12). Several kinases have been identified to regulate BMAL1

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This article contains Tables S1–S4 and Figs. S1–S3.

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[4] The abbreviations used are: PTM, post-translational modification; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DUB, deubiquitinating enzyme; CR, conserved region; CC, coiled coil; UBC, ubiquitin-conjugating domain; NLS, nuclear localization sequence; PSM, peptide spectral match; CHX, cycloheximide; qPCR, quantitative PCR; Ub, ubiquitin; UPS, ubiquitin–proteasome system; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PEI, polyethyleneimine; ANOVA, analysis of variance; WB, Western blotting.

phosphorylation. Casein kinase 2 mediates the BMAL1 phosphorylation at Ser-90 (17), whereas Akt regulates its phosphorylation at Ser-42 (23), resulting in the alteration of BMAL1 nuclear localization. Inhibition of Ser-90 phosphorylation leads to the impaired BMAL1 nuclear accumulation and disrupted circadian behavior (17). BMAL1 phosphorylation at Ser-42 causes its nuclear exclusion by disrupting the BMAL1 interaction with DNA and 14-3-3 protein, thereby suppressing its transcriptional activity (23). S6K1-mediated BMAL1 phosphorylation stimulates protein synthesis through interfering with the translational machinery by influencing the elongation step (15). Although glycogen synthase kinase-3β phosphorylates the BMAL1 N terminus (Ser-17 and Thr-21), which subsequently leads to BMAL1 ubiquitination (16), the E3 ubiquitin ligase responsible for BMAL1 ubiquitination in that case is unknown.

Ubiquitination is one major PTM that regulates protein stability, localization, and thus biological functions (24). During protein ubiquitination, E3 ligases recognize the specific substrates and covalently attach ubiquitin molecules to the side chain of lysine (or less frequently other) residues of the substrates to form different types of ubiquitination in the assistance of ubiquitin-activating enzymes E1s and ubiquitin-conjugating enzymes E2s. If the type of ubiquitination, such as the Lys-48–linked polyubiquitination, is recognized by the 26S proteasome for subsequent degradation, the stability and biological functions of the modified proteins will be affected (24). In addition, deubiquitinating enzymes (DUBs) remove the conjugated ubiquitin molecule from the substrates to reverse the effect caused by ubiquitination. Several E3 ligases and DUBs (25–29) were identified to modulate the protein level or localization of core clock proteins, thus modulating circadian behavior. These E3 ligases and DUBs mainly regulate the ubiquitination of the core clock proteins in the negative limb of transcription/translation feedback loop. Only one E3 ligase, ubiquitin–protein ligase E3A (UBE3A) (30), and two DUBs, ubiquitin-specific protease 2 (USP2) (28) and probable ubiquitin C-terminal hydrolase FAF-X (USP9X) (31), have been reported to regulate the BMAL1 protein level through modulating its ubiquitination. Because BMAL1 is a cytosol–nucleus shuttling protein that exists in tissues from many organs with biological rhythms, we expect that more E2 activating enzymes and E3 ligases may be present to regulate BMAL1 and its biological functions.

Ubiquitin-conjugating enzyme UBE2O was first discovered as a large E2 enzyme containing 1292 amino acids (32). It is composed of two conserved regions (CRs), one coiled coil (CC) domain, one ubiquitin-conjugating domain (UBC), and two nuclear localization sequences (NLSs) (33). Although this protein has been discovered for more than 15 years, its biological functions are still elusive. Later, it was shown that UBE2O has hybrid E2/E3 activities (33–35). UBE2O regulates the ubiquitination and biological functions of several proteins through the formation of monoubiquitination (34) and multi-monoubiquitination (33), which are not designated for proteasomal degradation. Recently, it was revealed that UBE2O is highly expressed or down-regulated in multiple cancers and can degrade proteins, such as AMP-activated protein kinase-α2 (AMPKα2) (36–38), mixed-lineage leukemia (MLL) (39), and transcription factor c-Maf (40), suggesting its roles in the regulation of protein functions and disease pathogenesis through the ubiquitin–proteasome system (UPS) (41). It has also been reported that UBE2O directly recognizes unassembled orphan proteins to mediate their ubiquitination and degradation for the maintenance of protein homeostasis (42). In addition, UBE2O can target ribosomal proteins for their degradation and thus remodel the proteome during terminal erythroid differentiation (43). However, whether UBE2O also regulates other substrates and circadian function under normal physiological conditions is unknown.

In this work, we used immunoprecipitation (IP) and MS to identify the BMAL1 interactome in the UPS. We then used immunoblotting to validate the interaction between BMAL1 and UBE2O. Detailed experiments uncovered the regulation of BMAL1 protein level by UBE2O and the underlying molecular mechanism and revealed the role of UBE2O in the modulation of the BMAL1 transcriptional activity. We further identified the major UBE2O domain that interacts with BMAL1 and affects its stability and found the critical cysteine residue that regulates BMAL1 ubiquitination. Moreover, we used the circadian clock as an example to examine the influence of UBE2O on the biological function of BMAL1 upon *UBE2O* knockdown. This work identifies the hybrid E2/E3 enzyme UBE2O as a new regulatory factor which modulates BMAL1 protein level and its associated biological functions.

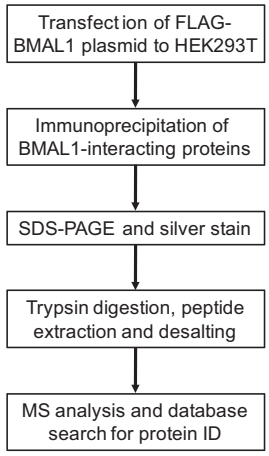
Results

Identification and validation of the interaction between UBE2O and BMAL1

It has been shown that BMAL1 has diverse roles in the regulation of protein synthesis, cellular senescence, reproductive ability, and cancer progression. Ubiquitination and degradation represent a major pathway to modulate protein level and thus protein activity. However, only a couple of enzymes in the UPS have been found to regulate BMAL1 ubiquitination, which cannot fully explain the fact that BMAL1 participates in diverse cellular processes in cells from different tissues. Here, we thought that identification of the BMAL1 interactome might uncover new regulators for BMAL1, which may influence its biological functions. To do so, we carried out an IP and MS analysis (Fig. 1*A*). From the comparison of peptide spectral matches (PSMs) between the mock and BMAL1 immunoprecipitates, we identified 24 BMAL1-interacting proteins that were potentially involved in the UPS (Fig. 1*B*). Among them was an (E3-independent) E2 ubiquitin–conjugating enzyme, UBE2O. From the proteomic data, two unique tryptic peptides derived from UBE2O were detected (Table S1). An MS/MS spectrum of one tryptic peptide provided annotated *b*-ions and *y*-ions for its confident identification (Fig. 1*C*).

We further validated the interaction between UBE2O and BMAL1 using four biochemical experiments. In the first experiment, FLAG-BMAL1 was expressed without or with Myc-UBE2O in HEK293T cells, and FLAG-BMAL1 was immunoprecipitated for immunoblotting (Fig. 1*D*). In the second experiment, SF-UBE2O plasmid along with the control or FLAG-BMAL1 plasmid was transfected into HEK293T cells, and SF-UBE2O

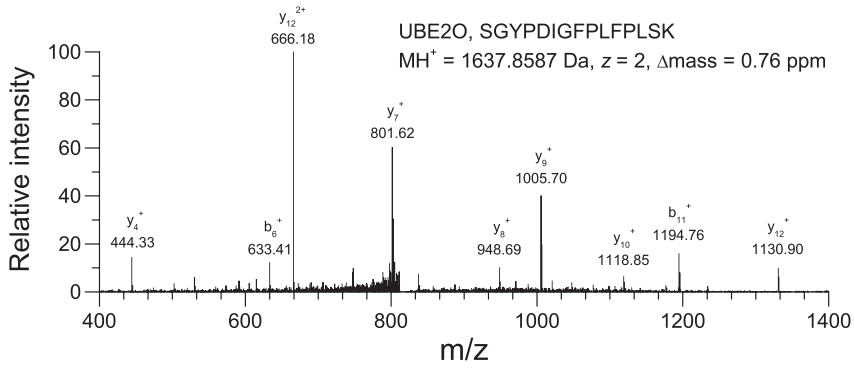
A



B

#	Accession	Description	PSM_{expt}
1	IPI00018398.4	26S protease regulatory subunit 6A	2
2	IPI00185374.4	26S proteasome non-ATPase regulatory subunit 12	11
3	IPI00012268.3	26S proteasome non-ATPase regulatory subunit 2	5
4	IPI00011603.2	26S proteasome non-ATPase regulatory subunit 3	2
5	IPI00163230.5	COP9 signalosome complex subunit 6	1
6	IPI00014310.3	Cullin-1	1
7	IPI00002922.5	F-box-like/WD repeat-containing protein TBL1XR1	1
8	IPI00746059.2	HECT, UBA and WWE domain containing 1	5
9	IPI00456695.1	Isoform 2 of 26S proteasome non-ATPase regulatory subunit 1	2
10	IPI00604431.1	Isoform 2 of Cullin-associated NEDD8-dissociated protein 1	1
11	IPI00029669.3	Isoform 2 of Zinc finger protein 174	1
12	IPI00219760.1	Isoform 3 of Zinc finger protein 207	1
13	IPI00555749.1	Proteasome 26S ATPase subunit 5 variant (Fragment)	1
14	IPI00021926.3	proteasome 26S ATPase subunit 6	3
15	IPI00105598.3	Proteasome 26S non-ATPase subunit 11 variant (Fragment)	2
16	IPI00746205.1	proteasome activator subunit 2	1
17	IPI00556291.1	Proteasome beta 1 subunit variant (Fragment)	1
18	IPI00888942.1	similar to Zinc finger protein 679	3
19	IPI00168084.2	Ubiquitin carboxyl-terminal hydrolase 38	1
20	IPI00640279.1	Ubiquitin carboxyl-terminal hydrolase L5	1
21	IPI00783378.3	**Ubiquitin-conjugating enzyme E2O**	3
22	IPI00645078.1	Ubiquitin-like modifier-activating enzyme 1	1
23	IPI00477971.3	WD repeat-containing protein SAZD	1
24	IPI00296977.1	Zinc finger protein 44 (KOX 7), isoform CRA_a	1

C



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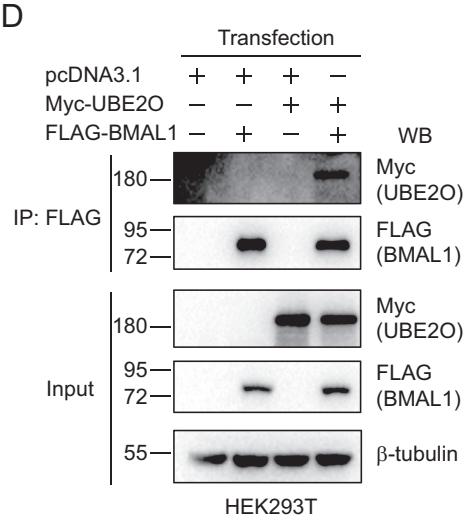

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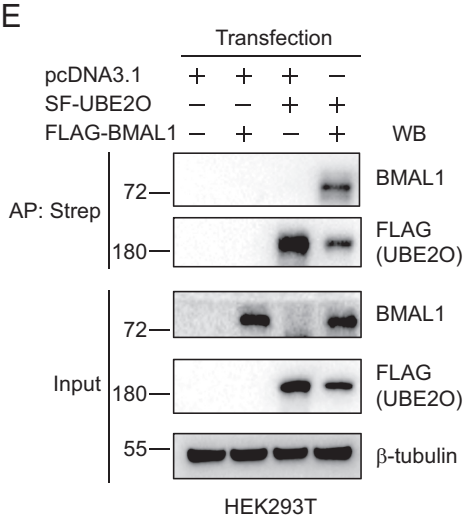

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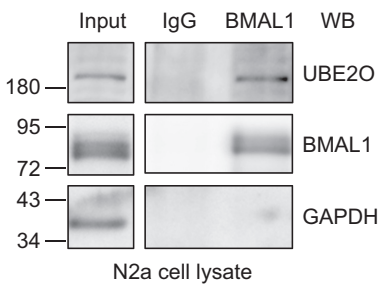

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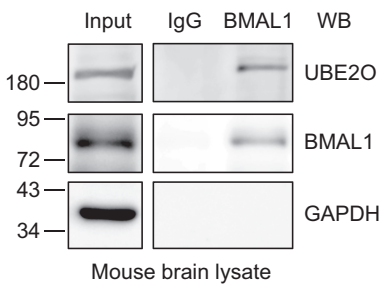

was pulled down with Strep-tactin agarose beads for immunoblotting (Fig. 1*E*). Our results clearly confirmed the interaction between BMAL1 and UBE2O in two reciprocal affinity purification experiments. To further validate the interaction of the two endogenous proteins, in the other two experiments, BMAL1 was immunoprecipitated with an anti-BMAL1 antibody from mouse neuroblastoma N2a cell lysate (Fig. 1*F*) and mouse brain lysate (Fig. 1*G*) for immunoblotting. Consistent with the overexpressed system, the results demonstrated that endogenous UBE2O was also co-immunoprecipitated with a BMAL1 antibody but not with the control IgG in both samples. Together, using MS and biochemical approaches, we discovered that BMAL1 interacts with UBE2O in cells and animal tissue.

UBE2O regulates BMAL1 protein level in cells

Although UBE2O could catalyze the formation of monoubiquitination and multi-monoubiquitination on its substrates, recently it was found that UBE2O has both E2 and E3 activities (33–35) and could target proteins for ubiquitination and degradation (36–40, 42, 43). Therefore, we sought to explore whether UBE2O can also regulate BMAL1 protein level in cells. To test this, we transfected the control or SF-UBE2O plasmid into HEK293T cells and found that the endogenous BMAL1 protein level was markedly attenuated upon UBE2O expression (Fig. S1). A gradient transfection of SF-UBE2O resulted in a dose-dependent reduction of endogenous BMAL1 protein level in HEK293T cells (Fig. 2*A*). However, when we carried out the similar experiment for CLOCK, the BMAL1 binding partner, we did not detect the decrease in CLOCK protein level upon a gradient expression of UBE2O (Fig. 2*B*). These results suggest that the reduction of BMAL1 protein level by UBE2O is specific.

To further validate the regulation of UBE2O on BMAL1 protein level, we used siRNAs to knock down *UBE2O* in HEK293T cells and carried out immunoblotting experiments for the whole-cell lysates. All three siRNAs efficiently knocked down *UBE2O*, and the reduction of UBE2O protein level resulted in an increase of endogenous BMAL1 protein level in HEK293T cells (Fig. 2*C*). To rule out a possible cell type–specific effect, we conducted the similar experiment in N2a cells. First, we tested three sets of siRNAs targeting the murine *Ube2o* and found that the first set had the highest knockdown efficiency (Fig. S2). Three biological replicates using the first set of siRNAs demonstrated that knockdown of *Ube2o* in N2a cells also elevated BMAL1 protein level (Fig. 2*D*). Together, the above experiments demonstrated that UBE2O could negatively regulate the BMAL1 protein level in cells.

UBE2O promotes BMAL1 ubiquitination and reduces its stability

Next we sought to explore the potential mechanism by which UBE2O regulates BMAL1. Because UBE2O is a hybrid E2/E3 enzyme, we tested whether this regulation could act through protein ubiquitination and degradation. First, we examined the effect of UBE2O on the BMAL1 degradation. To do so, we cotransfected the FLAG-BMAL1 plasmid with a control or SF-UBE2O plasmid into HEK293T cells in 10-cm plates and then divided the transfected cells to 6-well plates. Cells were further treated with cycloheximide (CHX) (200 μg/ml) for the indicated time, and the whole-cell lysates were immunoblotted. Our experiments showed that UBE2O expression enhanced BMAL1 degradation (Fig. 3*A*). The half-life of BMAL1 was reduced from ~12 to ~5 h upon UBE2O expression (Fig. 3*B*), suggesting that UBE2O might regulate BMAL1 through the UPS.

Next, we examined the influence of proteasome inhibition on the BMAL1 protein level in the absence or presence of UBE2O. In the absence of proteasome inhibitor MG132, UBE2O reduced BMAL1 protein level, similarly as shown in Fig. 2*A* and Fig. S1. However, in the presence of MG132, the reduction in BMAL1 caused by UBE2O expression was completely abolished, and the BMAL1 protein level was returned to the level without UBE2O expression (Fig. 3*C*). Statistics from three biological replicates showed that BMAL1 protein level after MG132 treatment was significantly different from that in the absence of MG132, and MG132 eliminated the effect of UBE2O on the BMAL1 protein level. This result again suggests that the regulation of BMAL1 by UBE2O acts through the UPS.

To further validate this mechanism, we transfected FLAG-BMAL1 plasmid along with the control or Myc-UBE2O plasmid into HEK293T cells and treated the cells with MG132. Immunoprecipitation of BMAL1 and immunoblotting of ubiquitin revealed that BMAL1 ubiquitination was significantly increased upon UBE2O expression (Fig. 3*D*). These data indicate that UBE2O indeed reduces BMAL1 protein level through the UPS.

UBE2O reduces the BMAL1 transcriptional activity

BMAL1 is a transcription factor that forms a heterodimer with CLOCK to activate the expression of the downstream genes, such as *E-box* controlled genes *PER1* and *CRY1* (44, 45). To examine whether the reduced BMAL1 protein level caused by UBE2O affects its transcriptional activity, we carried out two experiments. In the first experiment, we transfected pcDNA3.1 or Myc-UBE2O plasmid into HEK293T cells and performed

Figure 1. UBE2O interacts with BMAL1. *A*, flow chart for the identification of BMAL1-interacting proteins by IP-MS. *B*, list of IP-MS–identified BMAL1-interacting proteins that are associated with the UPS. The accession number, protein name, and peptide spectral matches are provided. Note that PSM of the listed proteins is 0 for the control immunoprecipitate. *C*, a representative MS/MS spectrum of a UBE2O tryptic peptide identified from MS analysis of the affinity-purified BMAL1-interacting proteins. The *b*- and *y*-ions are annotated, and the peptide sequence, MH^+, charge state (*z*), and Δmass are shown in the spectrum. *D*, BMAL1 co-immunoprecipitates UBE2O. Myc-UBE2O was cotransfected with pcDNA3.1 or FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent. Cell lysates were prepared 48 h after transfection, and FLAG-BMAL1 was purified with FLAG M2 affinity gel. The immunoprecipitates and whole-cell lysates were immunoblotted for the indicated antibodies. *E*, BMAL1 was detected in the UBE2O pulldown sample. Strep-FLAG-UBE2O (SF-UBE2O) was cotransfected with pcDNA3.1 or FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent. Cell lysates were prepared 48 h after transfection, and UBE2O was pulled down with Strep-tactin agarose beads. The purified samples and whole-cell lysates were immunoblotted. *F* and *G*, BMAL1 interacts with UBE2O endogenously. Endogenous BMAL1 was immunoprecipitated with a BMAL1 antibody from N2a cell lysates (*F*) or mouse brain cell lysates (*G*). Immunoprecipitates and cell lysates were immunoblotted for UBE2O, BMAL1, and/or GAPDH.

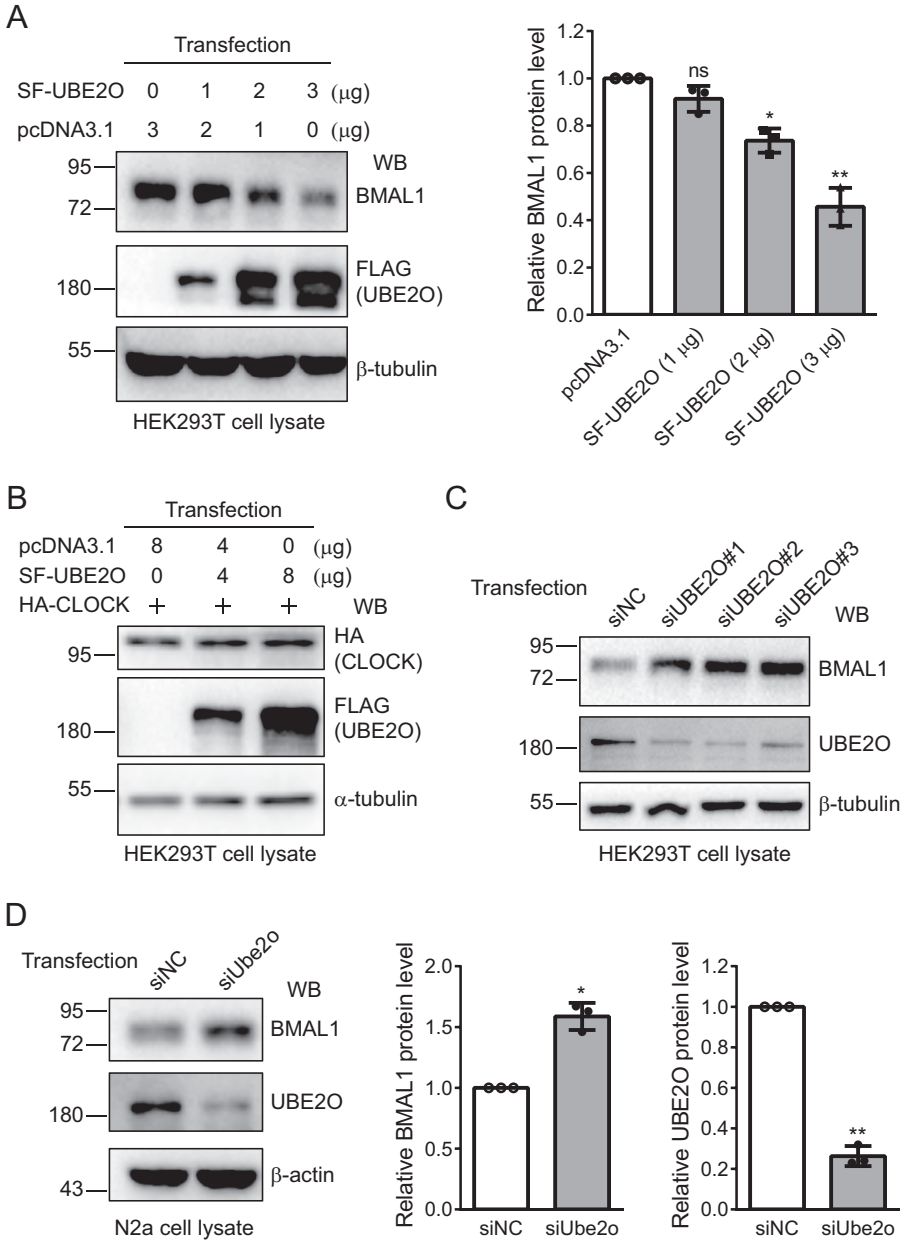

Figure 2. UBE2O regulates BMAL1 protein level. *A*, UBE2O reduces the protein level of endogenous BMAL1 in a dose-dependent manner. HEK293T cells in 6-well plates were transfected with the indicated amount of SF-UBE2O and/or pcDNA3.1 plasmid using PEI transfection reagent for 48 h. Cell lysates were blotted for BMAL1, FLAG, and β-tubulin. Three biological replicates were carried out, and densitometric quantification was performed with ImageJ. Student's *t* test was used to calculate the *p* value against the control sample transfected with the pcDNA3.1 vector. *Error bars*, S.D. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$. *B*, UBE2O does not affect the CLOCK protein level. HEK293T cells were transfected with HA-CLOCK plasmid and equally split into three 6-cm plates 12 h after transfection. Cells were further transfected with either pcDNA3.1 (balance plasmid) or SF-UBE2O plasmid. Cell lysates were prepared 48 h after the second transfection and immunoblotted for HA (CLOCK), FLAG (UBE2O), and α-tubulin. *C*, *UBE2O* knockdown increases BMAL1 protein level in HEK293T cells. Control or three *UBE2O*-specific siRNAs were transfected into HEK293T cells using Lipofectamine 2000 for 48 h. Cell lysates were immunoblotted for the indicated proteins. *D*, *Ube2o* knockdown increases BMAL1 protein level in N2a cells. Control or *Ube2o*-specific siRNAs were transfected into N2a cells using Lipofectamine 2000. Cell lysates were prepared 48 h after transfection and immunoblotted for the indicated proteins. The protein levels were quantified for data from three biological replicates, and data are expressed as mean ± S.D. Statistical analyses were performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

qPCR experiments for *BMAL1*, *PER1*, *CRY1*, *RORA*, *NR1D1* (*REV-ERBα*). The *BMAL1* mRNA level was not affected upon UBE2O expression (Fig. 4*A*), further supporting the idea that the reduced BMAL1 protein level by UBE2O is caused by the increased protein degradation and reduced protein stability. However, when we examined the expression of several BMAL1 downstream target genes *PER1*, *CRY1*, *RORA*, and *NR1D1*, all of them had the reduced mRNA level (Fig. 4*A*). To further verify this result, we knocked down *Ube2o* in mouse N2a cells and performed qPCR experiments for *Ube2o*, *Bmal1*, and its downstream target genes in the second experiment. As expected, after *Ube2o* knockdown, *Bmal1* mRNA level was not altered (Fig. 4*B*). However, all of the tested BMAL1 downstream target genes had increased mRNA level, which is in agreement with the finding that UBE2O expression down-regulates BMAL1 protein level and decreases its transcriptional activity (Figs. 2*A* and 4*A*).

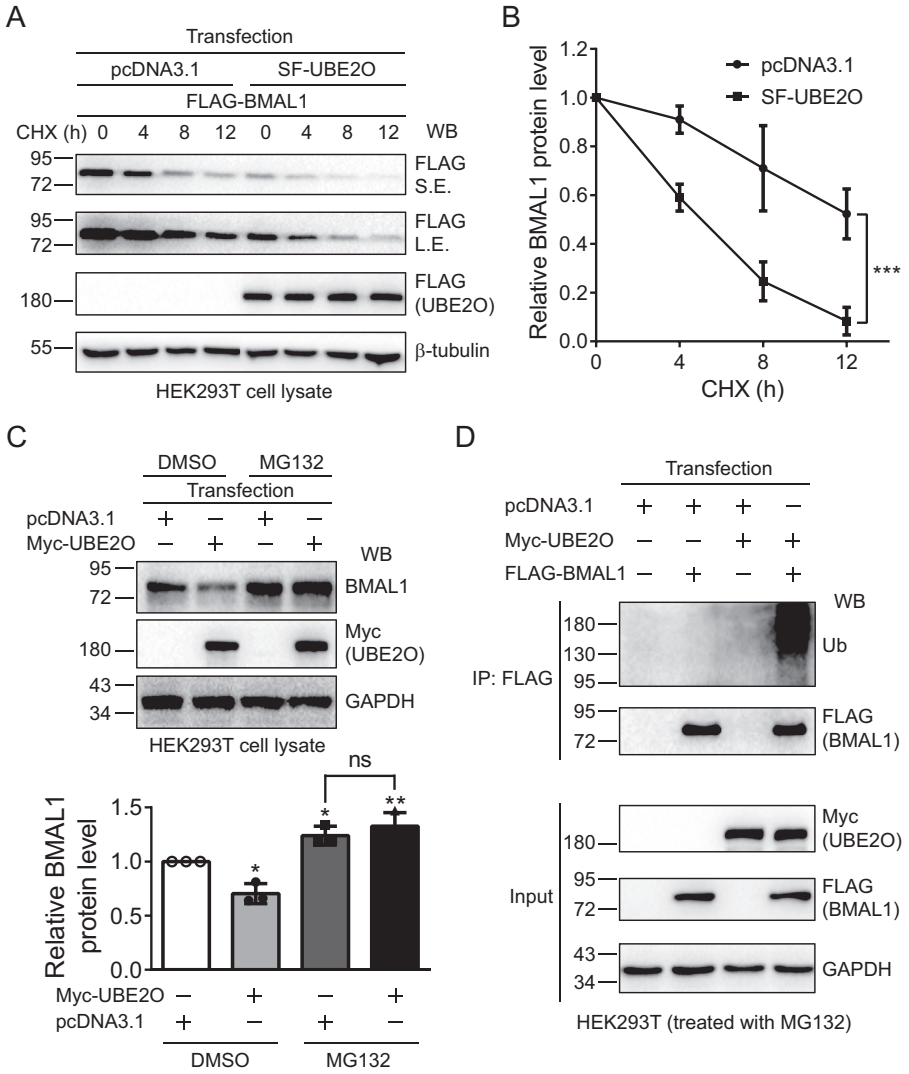

Figure 3. UBE2O reduces BMAL1 stability by promoting its ubiquitination. *A*, UBE2O reduces BMAL1 stability. pcDNA3.1 or SF-UBE2O plasmid was cotransfected with the FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent and split into four plates 12 h after transfection. At 48 h post-transfection, cells were treated with CHX (200 μg/ml) for the indicated time. Cell lysates were immunoblotted for FLAG and β-tubulin. *S.E.*, short exposure; *L.E.*, long exposure for FLAG-BMAL1. *B*, densitometric quantification of Western blotting images from three biological replicates of *A*. Two-way ANOVA was used to calculate the *p* value, and mean ± S.D. (*error bars*) values are shown. ***, $p < 0.001$. *C*, proteasomal inhibition rescues the BMAL1 protein level reduced by UBE2O expression. pcDNA3.1 or Myc-UBE2O plasmid was transfected into HEK293T cells using PEI transfection reagent. At 48 h post-transfection, cells were treated with DMSO or MG132 (10 μM for 12 h), respectively, and lysed. Cell lysates were immunoblotted with the indicated antibodies. Student's *t* test was used to calculate the *p* values for data from three biological replicates, and mean ± S.D. values are depicted in the *bar graph*. *, $p < 0.05$; **, $p < 0.01$ compared with the first bar (transfected with pcDNA3.1 empty vector and treated with DMSO); *ns*, not significant. *D*, UBE2O promotes BMAL1 ubiquitination. pcDNA3.1 or Myc-UBE2O plasmid was cotransfected with the FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent. At 48 h post-transfection, cells were treated with MG132 (10 μM for 12 h) and lysed. FLAG-BMAL1 was immunoprecipitated with FLAG M2 affinity gel. The immunoprecipitates and cell lysates were immunoblotted for the indicated antibodies.

CR2 in UBE2O is the major domain responsible for the reduction of BMAL1 protein level

Next, we asked which domain in UBE2O is responsible for the reduction of BMAL1. We constructed several UBE2O truncation plasmids based on its functional domains (Fig. 5*A*) and examined the effect of their expression on BMAL1 protein level in HEK293T cells. Our results showed that not only did the full-length UBE2O reduce BMAL1 protein level, but also several UBE2O truncations containing the CR2 domain attenuated the BMAL1 protein level markedly. The smallest domain that still has significant reduction in BMAL1 protein level is the CR2 domain (Fig. 5*B*), although these UBE2O truncations were expressed at slightly different levels.

To further validate the effect of CR2 domain on the regulation of BMAL1 protein level, we performed a gradient expression of Myc-CR2 and found that the endogenous BMAL1 protein level was gradually reduced with statistical significance when more than 1 μg of plasmid was transfected into one well of a 6-well plate of HEK293T cells (Fig. 5*C*). Together, these biochemical experiments suggest that CR2 is the major domain responsible for the BMAL1 reduction.

UBE2O CR2 domain interacts and ubiquitinates BMAL1

To further validate the interaction between UBE2O and BMAL1, we carried out immunofluorescence experiments in HEK293 cells to examine their potential colocalization (Fig. 6*A*,

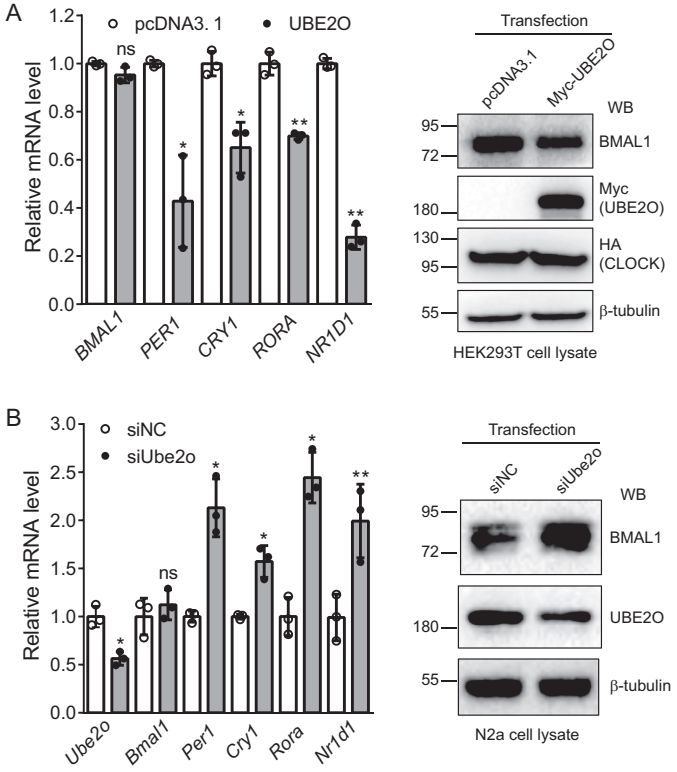

Figure 4. UBE2O regulates the BMAL1 transcriptional activity. *A*, UBE2O attenuates the mRNA level of *PER1*, *CRY1*, *RORA*, and *NR1D1* but not *BMAL1*. pcDNA3.1 or Myc-UBE2O plasmid was cotransfected with HA-CLOCK plasmid into HEK293T cells using PEI transfection reagent for 48 h. Total RNA was isolated from the first half of the samples with TRIzol reagents. The first-strand cDNA was synthesized, and qPCR was performed. The second half of the samples were lysed for immunoblotting of BMAL1, Myc (UBE2O), HA (CLOCK), and β-tubulin. Student's *t* test was used to perform statistical analysis of the three technical and three biological replicates, and mean ± S.D. (*error bars*) values are plotted in the *bar graph*. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$ compared with the sample transfected with pcDNA3.1 empty vector. *B*, knockdown of *Ube2o* with siRNA in N2a cells increases the expression of BMAL1 downstream target genes. N2a cells were transfected with siNC (negative control) or siUbe2o with Lipofectamine 2000. Samples were prepared as described in *A*, and qPCR was performed with primers specific for mouse mRNA. Student's *t* test was used for statistical analysis of data from three technical and three biological replicates, and mean ± S.D. values are shown in the *bar graph*. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$ compared with the sample transfected with the pcDNA3.1 empty vector.

two top rows). Although BMAL1 is mostly localized to the nucleus and the full-length UBE2O mainly lies in the cytoplasm, a small amount of BMAL1 and UBE2O was also detected in the cytoplasm or in the nucleus, respectively. In addition, BMAL1 is a cytosol–nucleus shuttling protein (46, 47). This information and our immunofluorescence experiments suggest that BMAL1 interacts with UBE2O in cells. Interestingly, when we examined the localization of CR2 truncation, we found that, similar to BMAL1 localization, CR2 was almost completely localized in the nucleus (Fig. 6*A*, *two bottom rows*). The difference in the localization of the full-length UBE2O and its CR2 truncation may explain the fact that the full-length UBE2O reduces the BMAL1 protein level to a slightly lesser degree than the CR2 domain does (Fig. 5*B*).

To further validate the effect of CR2 domain on BMAL1 protein level, we carried out an immunoprecipitation and immunoblotting experiment to determine the interaction between the UBE2O CR2 domain and BMAL1. In the FLAG-BMAL1 immunoprecipitate, we clearly detected the UBE2O CR2 truncation, although its expression level was much weaker than the negative control in the cell lysate, indicating the presence of the interaction between BMAL1 and UBE2O CR2 domain (Fig. 6*B*). We also constructed the C-terminal fragments CC+UBC+NLS2 and CR2+CC+UBC+NLS2 (Fig. 5*A*) and examined their interaction with BMAL1 along with the CR2 truncation to examine the specificity of the interaction between CR2 and BMAL1. Our results clearly demonstrated that FLAG-BMAL1 co-immunoprecipitated the CR2 truncation and the CR2-containing truncation CR2+CC+UBC+NLS2 but not the CC+UBC+NLS2 truncation, which does not contain the CR2 domain (Fig. 6*C*). These results indicate that the CR2 domain is the major domain contributing to the interaction between UBE2O and BMAL1.

We further tested the effect of the CR2 truncation on BMAL1 ubiquitination and detected the increased ubiquitination level upon expression of CR2 truncation (Fig. 6*D*), which is in concert with the more significant reduction in BMAL1 protein level compared with the full-length BMAL1 (Fig. 5*B*). To explore how UBE2O CR2 domain promotes the BMAL1 ubiquitination, we mutated four cysteines (Fig. 6*E*) to serines, one at a time or in combination, in the CR2 domain and transfected these plasmids with HA-ubiquitin (Ub) and FLAG-BMAL1 plasmids into HEK293T cells followed by MG132 treatment. Immunoblotting of cell lysates confirmed the similar expression level of different CR2 mutants, BMAL1, and ubiquitin conjugates. Evaluation of BMAL1 ubiquitination in the immunoprecipitates found that unlike the WT CR2 truncation, which enhances BMAL1 ubiquitination, the C617S mutant did not affect BMAL1 ubiquitination (Fig. 6*F*). Mutating additional Cys at 566, 585, and 598 to Ser did not further reduce BMAL1 ubiquitination. However, other single point mutants (C566S and C598S) promoted BMAL1 ubiquitination in a degree similar to the WT CR2 truncation. It should be noted that for some unknown reason, the C585S mutant was not expressed in HEK293T cells, and therefore this mutant was not included in this experiment. These results suggest that CR2 might act as an E2 or E3 to promote the BMAL1 ubiquitination. Together with the previous discovery that a large UBE2O domain containing the CR2 region has the E3 activity (34), it is highly possible that the CR2 domain functions as an E3 ligase to ubiquitinate BMAL1 in the assistance of endogenous E2s. Our experiments further find that Cys-617 is the critical residue responsible for its enzymatic activity.

Full-length UBE2O and the CR2 truncation rescue the effect of UBE2O knockdown on the BMAL1 protein level

In Fig. 2 (*C* and *D*) we found that knockdown of *UBE2O* (*Ube2o*) in HEK293T (or N2a) cells increased the BMAL1 protein level. We further examined whether the full-length UBE2O or the CR2 truncation can rescue the effect of *UBE2O* knockdown on BMAL1 protein level to assess the specificity of the siRNA knockdown in both cell lines. To do so, we first transfected the control or *UBE2O* (or *Ube2o*) siRNAs to HEK293T cells (or N2a cells) and then transfected the Myc-UBE2O or Myc-CR2 plasmid. Western blotting of cell lysates with the UBE2O or Myc antibodies confirmed the knockdown of endog-

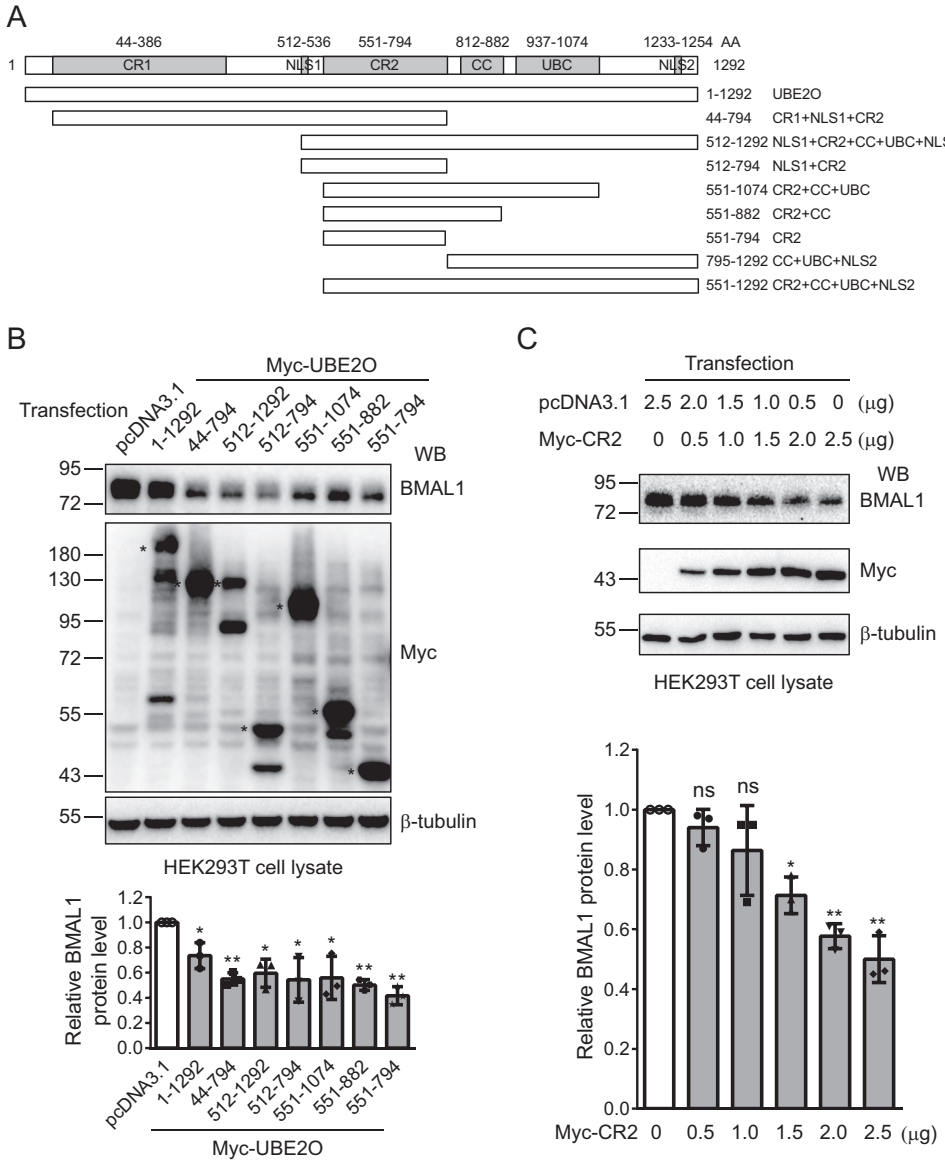

Figure 5. The CR2 domain in UBE2O is the major domain responsible for the reduction of BMAL1 protein level. *A*, schematic representation of UBE2O domains and its truncations. *B*, CR2 domain reduces BMAL1 protein level. pcDNA3.1, Myc-UBE2O, or six UBE2O truncation plasmids were transfected into HEK293T cells using PEI transfection reagents. Cell lysates were prepared 48 h after transfection and immunoblotted for endogenous BMAL1, Myc (for UBE2O and its truncations), and β-tubulin. The *asterisks* at the *left* of the bands indicate the expected bands for UBE2O and its truncations. Student's *t* test was used to calculate the *p* value against the first *bar* (transfected with the pcDNA3.1 empty vector), and mean ± S.D. (*error bars*) values are presented in the *bar graph*. *, $p < 0.05$; **, $p < 0.01$. *C*, gradient expression of UBE2O CR2 truncation progressively reduces BMAL1 protein level. An increased amount of Myc-CR2 plasmid was transfected into HEK293T cells in 6-well plates using PEI transfection reagent. pcDNA3.1 empty vector was used to balance the total amount of transfected plasmid. Cell lysates were obtained 48 h after transfection and immunoblotted for endogenous BMAL1, Myc (CR2), and β-tubulin. Densitometric quantification was carried out for data from three biological replicates, and Student's *t* test was used for statistical analyses (compared with the sample transfected with the pcDNA3.1 empty vector). Data are presented as mean ± S.D. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$.

enous *UBE2O* (or *Ube2o*) and the expression of exogenous UBE2O and CR2 truncation (Fig. 7 (*A* and *B*), *two middle images*). Examination of BMAL1 protein level found that *UBE2O* (or *Ube2o*) knockdown increased BMAL1 similarly as observed in Fig. 2 (*C* and *D*). However, further expression of exogenous UBE2O and the CR2 truncation reduced the BMAL1 protein level that was enhanced by the *UBE2O* (or *Ube2o*) knockdown (Fig. 7 (*A* and *B*), *top images*). These experiments in HEK293T and N2a cells clearly demonstrate that both the full-length UBE2O and the CR2 truncation could rescue the effect of *UBE2O* (or *Ube2o*) knockdown, further confirming the specificity of the siRNA knockdown and the mechanism by which the full-length UBE2O and its CR2 domain regulate the BMAL1 protein level and ubiquitination.

UBE2O knockdown alters the amplitude of the circadian clock in U2OS cells

Because UBE2O attenuates the BMAL1 transcriptional activity, we sought to test whether UBE2O affects its biological function. To do so, we used *PER2*-luciferase U2OS cells and knocked down *UBE2O* with a specific set of siRNAs (Fig. S3). We further synchronized the cells using dexamethasone and measured the bioluminescence signal in the luminometer. Both the raw data and the detrended data showed the increase of the

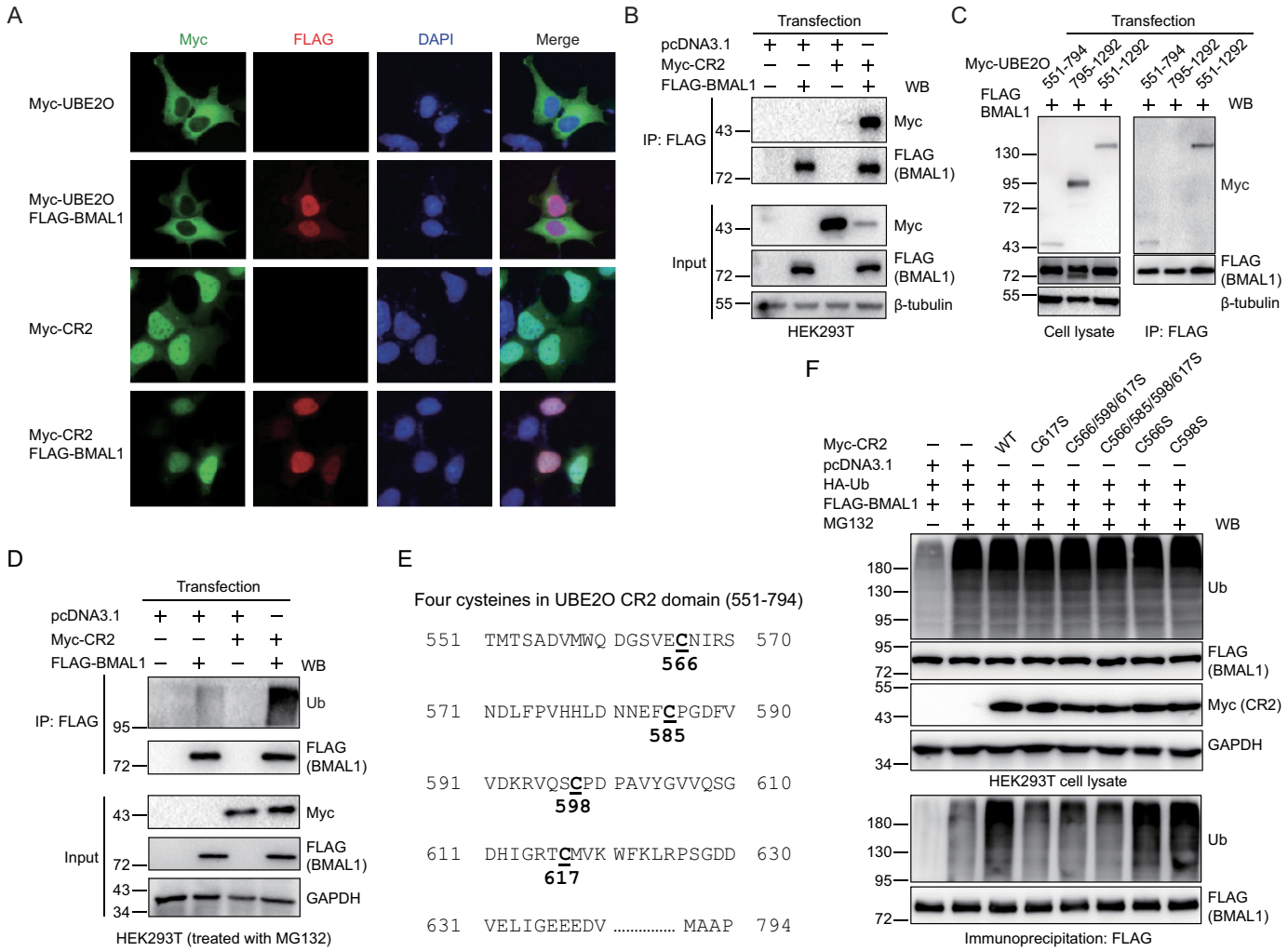

Figure 6. UBE2O CR2 domain interacts with, colocalizes with, and ubiquitinates BMAL1. *A*, CR2 colocalizes with BMAL1 in the nucleus. Myc-UBE2O or Myc-CR2 plasmid was cotransfected with pcDNA3.1 or FLAG-BMAL1 plasmid into HEK293 cells in a 24-well plate using PEI transfection reagent. At 24 h post-transfection, cells were fixed, permeabilized, and stained for Myc (UBE2O or CR2; *green*), FLAG (BMAL1; *red*), and DAPI (*blue*). *B*, UBE2O CR2 domain interacts with BMAL1. Myc-CR2 plasmid was cotransfected with pcDNA3.1 or FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent. Cell lysates were obtained 48 h after transfection, and BMAL1 was purified with FLAG M2 affinity gel. The immunoprecipitates and whole-cell lysates were immunoblotted. *C*, a UBE2O truncation lacking the CR2 domain does not interact with BMAL1. HEK293T cells were transfected with FLAG-BMAL1 and three UBE2O truncation plasmids (551–794, CR2; 795–1292, CC+UBC+NLS2; 551–1292, CR2+CC+UBC+NLS2). Cells were lysed 48 h after transfection, and FLAG-BMAL1 was immunoprecipitated with FLAG M2 affinity gel. The immunoprecipitates and cell lysates were immunoblotted for the indicated antibodies. *D*, the UBE2O CR2 domain enhances BMAL1 ubiquitination. pcDNA3.1 or Myc-CR2 plasmid was cotransfected with FLAG-BMAL1 plasmid into HEK293T cells using PEI transfection reagent. At 48 h post-transfection, cells were treated with MG132 (10 μM) for 12 h and lysed, and FLAG-BMAL1 was immunoprecipitated with FLAG M2 affinity gel. The immunoprecipitates and cell lysates were immunoblotted with the indicated antibodies. *E*, the location of four cysteines (Cys-566, -585, -598, and -617) in the CR2 domain of UBE2O. *F*, C617S mutation abolishes the CR2-mediated BMAL1 ubiquitination. HEK293T cells in two 10-cm plates were transfected with HA-Ub and FLAG-BMAL1 plasmids and were equally split 6 h after transfection. At 24 h post-transfection, cells were further transfected with either pcDNA3.1, Myc-CR2, or each of its Cys-to-Ser mutants for 36 h and treated with MG132 (10 μM) for 12 h. Cells were lysed, and BMAL1 was immunoprecipitated with FLAG M2 affinity gel. Both the cell lysates and immunoprecipitates were immunoblotted for the indicated antibodies. The experiment was repeated twice, and similar results were obtained.

amplitude of the circadian clock upon *UBE2O* knockdown (Fig. 8*A*). The relative amplitude and circadian period were obtained from three biological replicates. Upon *UBE2O* knockdown, the amplitude of the circadian rhythm was increased about 50% (Fig. 8*B*). However, the period was almost not affected.

Discussion

As a transcription factor, BMAL1 activates the expression of many genes and plays important roles in the regulation of aging, reproductive ability, and cancer development (9–11). The transcriptional activity of BMAL1 could be modulated by PTMs, such as acetylation, phosphorylation, and SUMOylation (12–22). Although the UPS is a major pathway for protein degradation, only one E3 ligase, UBE3A (30), and two DUBs, USP2 (28) and USP9X (31), have been discovered to affect the BMAL1 ubiquitination. Using proteomic and biochemical approaches, we discovered a new regulator, an E3-independent E2 ubiquitin–conjugating enzyme, UBE2O, that interacts with and subsequently ubiquitinates BMAL1, resulting in the reduction of BMAL1 protein level. UBE2O also modulates the BMAL1 transcriptional activity, leading to the alteration of the circadian function in cells. This discovery added another layer of complexity in the regulation of BMAL1 functions, such as the modulation of circadian clock, by the UPS.

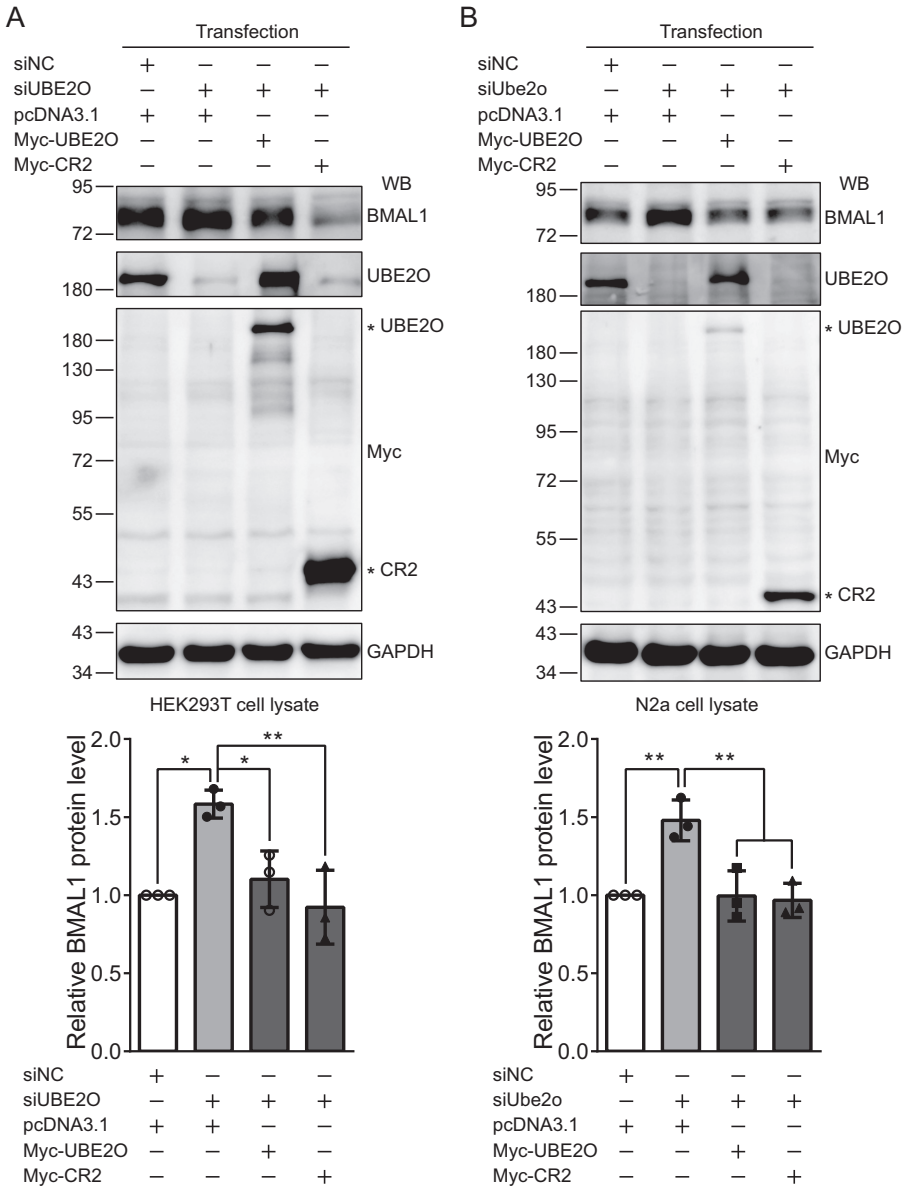

Figure 7. Full-length UBE2O and the CR2 truncation rescue the effect of *UBE2O* knockdown on BMAL1 protein level. HEK293T cells (*A*) or N2a cells (*B*) were first transfected with the control or *UBE2O* (*Ube2o*)-specific siRNAs using RNAiMAX. At 24 h post-transfection, cells were further transfected with pcDNA3.1, Myc-UBE2O, or Myc-CR2 plasmid. Cells were lysed 48 h after the second transfection, and cell lysates were immunoblotted with the indicated antibodies. *Asterisks* on the *right* indicate the full-length UBE2O or the CR2 truncation. Quantification was performed for data from three biological replicates, and data are presented as mean ± S.D. (*error bars*). Student's *t* test was used for statistical analyses. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$.

It has been found that UBE2O also has both E2 and E3 activities (33–35). Although UBE2O promotes the ubiquitination that is not targeted for proteasomal degradation (33, 34), three proteins, AMPKα2, MLL, and c-Maf, can be ubiquitinated and degraded by UBE2O in cancer cells (36–40). In addition, the unassembled orphan proteins and ribosomal proteins are also targeted for proteasomal degradation by UBE2O for proteostasis (42) and reticulocyte differentiation (43). Here, we found that UBE2O could also ubiquitinate BMAL1 and subsequently reduce BMAL1 stability and affect its biological function under normal physiological conditions. Earlier findings showed that UBE2O could promote the formation of different types of ubiquitination, such as monoubiquitination (34), multi-monoubiquitination (33), and polyubiquitination (36). Although our experiments did not explicitly demonstrate the type of chain linkage on BMAL1, the facts that the proteasomal inhibition increased BMAL1 ubiquitination and attenuated the decrease of BMAL1 protein level induced by UBE2O indicate that the ubiquitin chain linkage formed on BMAL1 by UBE2O is the type targeted for proteasomal degradation, such as the Lys-48–linked polyubiquitin chains. Our experiment further supports the previous discovery that UBE2O has the E3 ligase activity. Moreover, we demonstrated that UBE2O regulates circadian function in cells through mediating the BMAL1 ubiquitination, although we cannot completely exclude a role of other UBE2O substrates on the regulation of circadian function.

It is very interesting that our experiments with the UBE2O truncation found that CR2 of UBE2O plays an important role in promoting BMAL1 ubiquitination and degradation. A survey of the amino acid sequence found that this domain contains four

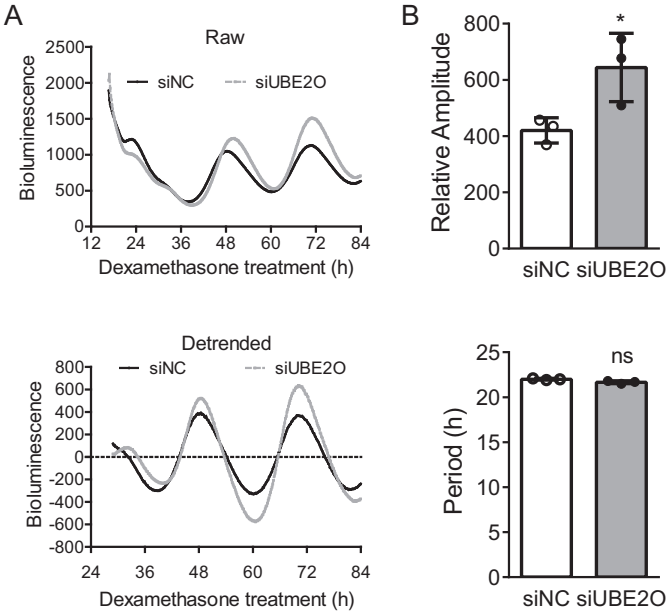

Figure 8. UBE2O alters the amplitude of the circadian clock in U2OS cells. *A*, *UBE2O* knockdown alters the circadian clock. *PER2*-luciferase U2OS cells were transfected with control or *UBE2O*-specific siRNAs for 24 h, synchronized with recording DMEM containing 100 nM dexamethasone. Bioluminescence signals were recorded in a luminometer. The raw and detrended data are depicted. *B*, quantification of the relative amplitude and period obtained from bioluminescence signals of three biological replicates. Statistics were performed with Student's *t* test, and mean ± S.D. (*error bars*) values are depicted in the *bar graph*. *, $p < 0.05$; *ns*, not significant.

cysteine residues, which may be responsible for its E3 ligase activity, in concert with a previous discovery that a larger domain-containing CR2 region has E3 ligase activity (34). Experiments with multiple Cys-to-Ser mutants identified the critical Cys (Cys-617) in the CR2 domain, responsible for the regulation of BMAL1 ubiquitination, indicating that this Cys plays important roles in modulating its E3 ligase activity. In this experiment, we did not knock out the endogenous UBE2O when the CR2 domain was transfected. Therefore, it might be possible that the CR2 domain coordinates with the full-length endogenous UBE2O or other E2s to modulate the ubiquitination of its substrates. In the experiment demonstrating the specificity of *UBE2O* knockdown, we found that both the full-length UBE2O and CR2 domain could rescue the effect of *siUBE2O* on the BMAL1 protein level, suggesting that CR2 decreases BMAL1 protein level in a manner similar to the full-length protein. It should be noted that we cannot completely rule out the possibility that other CR2-associated E2 ubiquitin–conjugating enzymes, E3 ligases, or adaptor proteins might be involved in the regulation of BMAL1 ubiquitination, although this possibility is unlikely.

Our experiments found that the full-length UBE2O, which contains two NLSs, is localized mostly in cytoplasm, whereas the CR2 domain, which does not contain any apparent NLS, is mostly localized in the nucleus. Three possible mechanisms may explain this ostensible discrepancy for the CR2 truncation. One is that CR2 is translocated to the nucleus after binding to a nuclear transporter or an interacting partner containing an NLS in cytoplasm. The second possibility is that CR2 is so small that it may freely enter the nucleus, bind to its interacting partners, and then be retained in nucleus. The third possibility is that the CR2 domain may contain an undiscovered NLS, which directs the nuclear localization of the CR2 truncation. Our data suggest that the modulation of UBE2O on BMAL1 might occur in the nucleus and that this regulation may be tightly modulated by the exposure of its NLSs under certain circumstances, such as the presence of a specific binding partner or stimulus. In addition, based on the degree of its regulation on endogenous BMAL1 protein level, the UBE2O CR2 domain may have a more profound effect on its biological functions than the full-length UBE2O. Although we found that the CR2 domain alone could enhance BMAL1 ubiquitination and its protein level, the minimal UBE2O sequence responsible for the regulation of BMAL1 is unknown. If such a short peptide exists or small molecules that disrupt the interaction between BMAL1 and UBE2O are discovered, they may be used to modulate the regulation between UBE2O and BMAL1, thus affecting the diverse biological functions in which BMAL1 participates.

The biological functions of UBE2O are only beginning to unfold 15 years after its discovery. Recently, it was found that UBE2O was amplified in multiple cancers and was correlated with patient survival rate in breast cancer, lung cancer, and gastric cancer (36). It was also reported that CLOCK and BMAL1 were required for the growth of leukemia stem cells (48) and that BMAL1 elevated the sensitivity of paclitaxel to tongue squamous cell carcinoma and inhibited tumor growth (11). These discoveries converge two previously unrelated signaling pathways, supporting the fact that dysregulation of BMAL1 or circadian rhythm is closely associated with cancer development.

Experimental procedures

Materials

Human embryonic kidney 293 (HEK293), 293T, and mouse neuroblastoma N2a cells were from American Type Culture Collection (ATCC). *PER2*-luciferase U2OS cells were obtained from a previous work (49). Primers for PCR and qPCRs were synthesized by GeneWiz or Synbio Technologies (Tables S2 and S3). Human and mouse *UBE2O*-specific and control siRNAs were from RiboBio (Table S4).

TRIzol reagent, ChamQ SYBR qPCR Master Mix, and HiScript First Strand cDNA Synthesis kit were from Vazyme Biotech Co. Antibodies were from the following companies: mouse FLAG antibody and FLAG M2 affinity gel from Sigma; rabbit FLAG, Myc, and α-tubulin from Hua'An Biotechnologies; BMAL1 and ubiquitin from Santa Cruz Biotechnology, Inc.; UBE2O from CusaBio; GAPDH and β-tubulin from Ruiying Biological; secondary antibodies for rabbit or mouse IgG from Beyotime Biotechnology; Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG from Invitrogen (Thermo Fisher Scientific).

UBE2O and its truncations were constructed using standard molecular biology techniques, and Strep-FLAG (SF) or Myc tags were added to their N termini. pCMV-Tag 2B-FLAG-BMAL1 and pcGN-HA-CLOCK plasmids were from a previous study (50). All of the constructed plasmids were confirmed by Sanger sequencing.

Cell culture and transfection

HEK293, 293T, and N2a cells were cultured in high-glucose DMEM or Opti-MEM (Gibco) supplemented with 10% FBS (PAN Biotech, Lonsera, and Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were transfected with the indicated plasmids or siRNAs using polyethyleneimine (PEI; Sigma), Lipofectamine 2000 (Life Technologies, Inc.), or RNAiMAX (Life Technologies) transfection reagents according to the manufacturer's instructions, and growth medium was changed 6 h after transfection. Cells were used for subsequent experiments at the indicated time.

Immunoprecipitation

Cells were washed with ice-cold PBS twice and lysed on ice for 30 min in the modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) supplemented with 10% glycerol and freshly prepared protease inhibitor mixture tablet (Roche Applied Science). Cell lysates were obtained after centrifugation at 4 °C for 10 min at 16,000 $\times$ *g*.

Immunoprecipitation of FLAG-BMAL1 was carried out according to a method described previously (51). Briefly, 20 μl of prewashed FLAG M2 affinity gel was added to the cell lysate obtained from each sample and incubated at 4 °C overnight on a four-dimensional rotator. The affinity gel was washed five times with TBST (TBS with 0.1% Tween 20) and eluted twice with 50 μl of TBST containing 200 μg/ml FLAG peptide (DYKDDDDK; ChinaPeptides). Cells from 10 10-cm plates were used for the FLAG M2 immunoprecipitation and MS analysis, and cells from one 10-cm plate (for each condition) were used for immunoprecipitation and immunoblotting. SF-UBE2O was pulled down with Strep-tactin agarose beads (IBA Life Sciences) according to a method described previously (52). Briefly, cell lysates were incubated with prewashed Strep-tactin agarose beads for 4–6 h at 4 °C and washed five times with radioimmune precipitation assay buffer followed by eluting twice with 5 mM desthiobiotin (Sigma).

MS analysis

The FLAG M2 immunoprecipitates from cells transfected with the control or pCMV-Tag 2B-FLAG-BMAL1 plasmids were separated on SDS-PAGE. Bands from silver-stained gel of the control and experimental samples were excised. Proteins were digested with trypsin according to a method described previously (53). The extracted tryptic peptides were purified with a C18 ZipTip and analyzed on an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific) according to a method described previously (54). The MS/MS spectra were searched using Proteome Discoverer (version 1.4) against the International Protein Index (IPI) database (55) to obtain the protein and peptide identity. The false discovery rate was set to 1% using a decoy database search strategy (56).

CHX treatment

The control or SF-UBE2O plasmid was cotransfected with FLAG-BMAL1 plasmid into HEK293T cells in 10-cm plates. Cells were split to 6-well plates 12 h after transfection, and growth medium containing CHX (200 μg/ml) was replaced 48 h after transfection. Cells were washed twice with ice-cold PBS and lysed at the indicated time after the addition of CHX for subsequent analysis.

Immunoblotting analysis

Immunoblotting experiments were carried out according to a method described previously (57). Briefly, cell lysates or immunoprecipitates were mixed with a proper amount of 5$\times$ SDS sample loading buffer, heated at 98 °C for 10 min, and centrifuged at 16,000 $\times$ *g* for 10 min. The supernatant was loaded and separated on SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane (Millipore). The membrane was briefly washed with double-distilled water and blocked with 5% nonfat milk in TBST for 1 h. The membrane was incubated with primary antibodies for 1–2 h at room temperature and washed three times with TBST on a plate shaker for 10 min. The membrane was further incubated with secondary antibodies followed by extensive washing. The protein bands were visualized with Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore), and signals were recorded in a ChemiDoc MP (Bio-Rad) or Tanon 5200 imaging system. The densitometric quantification was performed with ImageJ.

Immunofluorescence measurement

A previously published method (58) was used to perform the immunofluorescence experiment. Briefly, Myc-UBE2O or Myc-CR2 plasmid was cotransfected with the control or FLAG-BMAL1 plasmid into HEK293 cells in a 24-well plate. Twenty-four hours later, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 2% FBS in PBS for 1 h at room temperature. Cells were then incubated with anti-FLAG (mouse) and anti-Myc (rabbit) antibodies for 3 h, washed three times with TBST, and incubated again with Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 donkey anti-mouse IgG for 1 h. After washing three times, cells were stained with DAPI (Sigma) for 5 min. The images were taken under an inverted microscope (Olympus IX71).

qPCR

The control, UBE2O plasmid, or *UBE2O*-specific siRNAs were transfected into HEK293T or N2a cells with Lipofectamine 2000 transfection reagent. Cells were detached 48 h after transfection, and total cellular RNA was isolated with TRIzol reagent. cDNA was synthesized using the HiScript first-strand cDNA synthesis kit. cDNA template, primers, and 2$\times$ SYBR Green qPCR Master Mix were mixed, and qPCR was performed on an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific). The relative cDNA abundance was calculated using the $\Delta\Delta C_t$ method, and β-actin was used as the loading control. The means and S.D. were plotted with GraphPad Prism for data obtained from three biological replicates.

Bioluminescence recording

A bioluminescence experiment was conducted according to a method reported previously (50). Briefly, *PER2*-luciferase

U2OS cells in 35-mm plates were transfected with the control or *UBE2O*-specific siRNAs. At 24 h post-transfection, cells were synchronized with recording DMEM (Sigma) containing B27 supplement (Gibco), 0.1 mM luciferin (Promega), antibiotics, and 100 nM dexamethasone (Sangon Biotech). Bioluminescence was recorded in real time with the 32-channel luminometer (Actimetrics, LumiCycle). Three independent experiments were performed, and the raw data and detrended data were depicted. The data were fitted with damped sinusoidal function to acquire the period and amplitude. In all other experiments, cells were not synchronized with dexamethasone.

Statistics

Statistics were performed using Student's *t* test, one-way ANOVA, or two-way ANOVA as indicated in the figure legend. All average values were presented as means ± S.D.

Author contributions—S. C., J. Y., Y. Z., L. Z., and G. X. designed the research; S. C., J. Y., Y. Z., L. Z., C. D., Q. L., and Z. H. performed the research; S. C., J. Y., Y. Z., L. Z., and G. X. analyzed the data; S. C., J. Y., Y. Z., L. Z., Y. X., and G. X. wrote and/or edited the paper.

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