**Title:** Differential Effects of SUMO1/2 on Circadian Protein PER2 Stability and Function

**Short Title:** PER2 SUMOylation in circadian regulation

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**Keywords:** Period2/SUMO/phosphorylation/ubiquitination/circadian/post-translational modification

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## Abstract

Circadian rhythm control by clock proteins, including Period 2 (PER2), is strongly impacted by posttranslational modifications (PTMs) controlling clock protein stability, localization and activity. PER2 function is regulated by casein kinase 1 (CK1)-mediated phosphorylation and ubiquitination but little is known about other PER2 PTMs. We found that PER2 can be SUMOylated by both SUMO1 and SUMO2; however, SUMO1 versus SUMO2 conjugation had dramatically different effects on PER2 turnover and subcellular localization. PER2-SUMO2 conjugation facilitated PER2 ubiquitination and degradation. In contrast, SUMO1 conjugation mediated by the E3 SUMO-protein ligase RanBP2 led to enhanced CK1-mediated $PER2^{S662}$ phosphorylation and also increased PER2 nuclear accumulation, chromatin association and suppression function. PER2 K736 was an important determinant of both SUMO1- and SUMO2-conjugation. A $PER2^{K736R}$ mutation was sufficient to alter circadian periodicity, reduce PER2 interaction with β-TrCP or CRY1, decrease PER2 nuclear entry and abolish PER2-mediated transcriptional suppression of REV-ERBα, REV-ERBβ and RORγ. Together, our data revealed differential effects of SUMO isoforms on PER2 function and demonstrated the effect of SUMOylation on PER2 coordination of the circadian regulatory system.

## Introduction

The circadian clock controls many rhythmic physiological processes such as hormonal oscillation, metabolism and immune function that are essential to maintain homeostasis [1,2]. At the molecular level, the circadian clock establishes these biological rhythms through interconnected transcription-translation feedback loops (TTFLs). The core loop is composed of *BMAL*, *CLOCK*, Period (*PER1*, *PER2*) and Cryptochrome (*CRY1*, *CRY2*). BMAL-CLOCK heterodimers transcriptionally activate the expression of PER and CRY and other circadian output genes. In turn, PER and CRY suppress their own expression and expression of other circadian output genes controlled by the BMAL-CLOCK complex [3,4]. PER2 suppresses BMAL/CLOCK mediated transcription by displacing BMAL/CLOCK/CRY complexes from target promoters. This displacement is not only important for inhibition of circadian gene expression, but also essential for reactivation of the TTFL [5]. This core loop regulates expression of retinoic acid receptor–related orphan receptor (ROR) and REV-ERBα/β (NR1D1, NR1D2) which form a secondary loop that stabilizes the core loop by regulating BMAL1 and CRY1 transcription [6-8].

The robustness and stability of circadian rhythm is also reinforced by post-translational modifications (PTMs) that determine the level, activity and subcellular localization of core clock

proteins [9-14]. For example, phospho-null mutation at PER2 Serine 662 enhances PER2 repressor function but increases PER2 turnover in the nucleus. Loss of PER2 S662 phosphorylation (typically the result of a PER2 S662G substitution) causes human Familial Advanced Sleep Phase Syndrome (FASPS) which is characterized by a dramatically shortened circadian period with a 4-hour advance of sleep, temperature, and melatonin rhythms [15,16]. Besides phosphorylation and ubiquitination, the core clock proteins are also subject to other PTMs such as small ubiquitin-related modifier (SUMO)ylation, acetylation and O-linked β-N-acetylglucosamine modification [12].

In addition to its circadian role, PER2 exhibits tumor suppressive functions in many cancers [17-19]. Suppression of PER2 at both gene expression and protein levels facilitates tumor growth, invasion and cancer malignancy [19-22]. Since PER2 protein level oscillates and PER2 shuttles between nucleus and cytoplasm, PTMs that affect PER2 stability and subcellular localization can determine both its circadian transcriptional suppression activity and tumor suppressor activity.

Among the known PER2 PTMs, phosphorylation has been most intensively studied [13]. Two important serine phosphorylation sites (S662, S480) have been identified. Phosphorylation at S662 (the FASP site) affects PER2 subcellular localization and therefore influences transcription of PER2 itself and its downstream genes [16,23]. Phosphorylation at S480 facilitates PER2-β-TrCP interaction leading to PER2 proteasomal degradation (β-TrCP phosphodegron) [24]. Recent research has suggested that casein kinase 1 (CK1) is responsible for PER2 phosphorylation at both S662 and S480 [25]; however, the factors that determine which site will be phosphorylated remain unknown. Other PTMs also can regulate PER2 protein stability and subcellular localization. For example, O-GlcNAcylation in a cluster of serines around S662 competes with phosphorylation to regulate PER2 repressor activity [26]. Acetylation of PER2 at lysine (K) residues protects PER2 from ubiquitination; whereas deacetylation of PER2 by SIRT1 leads to degradation [27]. Despite these investigations of PER2 PTMs, little is known about PER2 SUMOylation.

The effects of SUMOylation on protein function are multifaceted. SUMOylation can alter protein-protein interactions, protein subcellular localization or protein activity and can participate in transcriptional regulation [28,29]. Usually, SUMO modification does not promote protein degradation. However, for a subset of proteins, conjugation with multiple SUMOs is critical for recognition by SUMO-targeted ubiquitin ligases (STUbLs), leading to proteasomal degradation [30]. At least three major SUMO proteins in higher eukaryotes have been identified: SUMO1 and SUMO2/3. SUMO2 and SUMO3 are 97% identical, but they share less than 50% sequence identity with SUMO1. SUMO2/3 often form SUMO chains on the substrates, whereas SUMO1 typically appears as monomers or acts as a chain terminator on SUMO-2/3 polymers [31]. However, SUMO1

and SUMO2/3 may still be able to act redundantly as SUMOylation events which typically use SUMO1 can be compensated by SUMO2 or SUMO3 in SUMO1-deficient mice [32]. Conversely, conjugation by SUMO1 or SUMO2/3 can impart different fates to the SUMOylated protein. SUMO1 conjugation typically affects cellular processes including nuclear transport, cell cycle control and response to virus infection [33], antagonizes ubiquitination [34] and modulates protein-protein interaction [35]. In contrast, SUMO2/3 is thought to mainly participate in cellular response to stress [36] and can facilitate targeted protein ubiquitin-mediated degradation [37]. Recently, SUMO1 and SUMO2/3 have been found to have opposite functions in mouse lens cells at different stages of development by differentially regulating the transcriptional activity of Specificity Protein 1 (SP1) [38]. Whether or not other transcriptional regulators are similarly affected by SUMO1 versus SUMO2/3 conjugation is unknown.

We found that PER2 K736 is critical for both SUMO1 and SUMO2 conjugation. However, SUMO1 versus SUMO2 conjugation had dramatically different effects on PER2 protein turnover and subcellular localization. Contrary to previous hypotheses, PER2 SUMO1 conjugation acted upstream of and was required for CK1-mediated S662 phosphorylation to control PER2 nuclear entry and transcriptional repressor activity. In contrast, SUMO2 promoted PER2 degradation. These data show that SUMOylation is an important layer of PER2 regulation which impacts PER2 repressor function and coordination of the circadian regulatory system.

## Results and Discussion

### *PER2 can be SUMOylated*

Analysis of endogenous PER2, SUMO1 and SUMO2 levels in HEK-293T cells at different circadian times (CT) after serum shock showed that PER2 had a rhythmic oscillation from high protein abundance at CT1 and CT5 to lower protein levels from CT13 to CT21 (Fig EV1A, Top) while SUMO1 and SUMO2 did not show significant rhythmic changes (Fig EV1A). To test whether PER2 is affected by SUMOylation, HEK-293T cells were treated with N-ethylmaleimide (NEM), a SUMO protease inhibitor that stabilizes SUMO-modified proteins [39], at CT1, CT5, CT13 and CT17. NEM treated samples had increased PER2 abundance, particularly a higher molecular weight PER2 species, compared to un-treated samples (Fig 1A, lane 3, 4 vs. lane 7, 8, indicated by the arrows). Immunoprecipitation (IP) confirmed that PER2 was SUMO-conjugated (Fig 1B). PER2-SUMOylation was further confirmed by detection of SUMO-conjugated PER2 in HEK-293T cells ectopically expressing tagged-PER2 and SUMO1 or SUMO2 (Fig 1C, lane 3 and 6). Importantly, co-expression of SUMO1 or SUMO2 with SENP1, a SUMO protease, eliminated the PER2 band

shift (Fig 1C, lane 4 and 7), confirming that PER2 could be SUMOylated by both SUMO1 and SUMO2.

***SUMO2 promotes PER2 protein degradation***

Interestingly, the signal of PER2-SUMO2 modification was less prominent than that of PER2-SUMO1 (Fig 1C, lane 6 vs. lane 3), suggesting that SUMO2 modification may lead to PER2 ubiquitination and proteasomal degradation. Consistent with this hypothesis, treatment with the proteasome inhibitor MG132 resulted in accumulation of PER2-SUMO2 conjugates (Fig 1D, lane 12 vs. lane 9). Also, PER2 protein levels decreased in a dose-dependent manner upon SUMO2 expression (Fig 2A). Immunoblot (IB) assay using whole cell lysates from cycloheximide (CHX)-treated cells further showed that SUMO2, but not SUMO1, significantly shortened PER2 protein half-life (Fig EV1B). To demonstrate that SUMO2-conjugation promoted PER2 degradation via ubiquitination, Co-IP assay was performed using HEK-293T cells ectopically expressing FLAG-PER2 and GFP-SUMO2. Proteasome inhibition by MG132 resulted in accumulations of not only PER2-SUMO2 conjugates, but also ubiquitinated-PER2 species (Fig EV1C, lane 5, 6 vs. lane 2, 3). In contrast, SUMO1 did not affect PER2 protein stability (Fig 2A).

***Lysine 736 is important for PER2 SUMOylation and required to maintain correct circadian period***

The majority of known SUMO substrates are SUMOylated at a lysine in the consensus motif $\psi$KxD/E (where $\psi$ is a large hydrophobic residue) [40]. However, SUMOylation can also occur at lysine residues outside this motif. Two putative PER2 SUMOylation sites at K87 and K1163 were identified using the GPS-SUMO bioinformatics database [41] (Fig 2B). An additional SUMOylation motif, QKEE [42], was also identified at K736 by manual inspection of the PER2 sequence. PER2 K736 and K1163 are conserved between human and mouse (Fig S1).

To determine which of these putative PER2 SUMOylation sites are functionally important, a series of K to Arginine (R) PER2 mutations at K87, K736 and/or K1163 were created. The K736R mutation clearly inhibited SUMO2-mediated PER2 degradation while K87R and K1163R had no effect (Fig EV2A). We further tested PER2 double and triple mutants and found that whenever K736 was mutated, PER2 protein became more stable regardless of whether K87 or K1163 could be SUMOylated (Fig EV2B). These data indicated that K736 could be an important SUMOylated site of PER2. Consistent with this, liquid chromatography–tandem mass spectrometry (LC-MS/MS) using lysates prepared from HEK-293T cells transiently expressing FLAG–PER2 and EGFP-SUMO1 identified a SUMO peptide branch at K736 of PER2 (Fig 2C). In addition, denatured-IP assays confirmed that the PER2 K736R mutation significantly decreased both SUMO1 and SUMO2 conjugation (Fig 2D, 2E). While the mass spectrometry data demonstrated that K736 is itself

SUMOylated, we do not rule out the possibility that mutation of K736 also affects SUMOylation at other unidentified sites. Also consistent with the critical importance of PER2 K736, co-IP showed a significant decrease in PER2$^{K736R}$ interaction with β-TrCP (Fig 2F). β-TrCP-containing Skp–Cullin–F-box (SCF) complexes have been shown to mediate PER2 degradation in response to S480 phosphorylation [24,43]. Together these results demonstrated that PER2 K736 is critical for PER2 SUMOylation.

PER2 K736 was also critical for maintenance of correct circadian period. Stable clones of HEK-293T cells harboring the PER2 K736R mutation were generated by CRISPR/CAS editing (Fig EV3). Analysis of circadian rhythm using a PER2 luciferase reporter showed that the PER2$^{K736R}$ cells had lower circadian peak amplitude and their peak time was shifted forward by about 3 circadian hours compared to PER2$^{WT}$ cells (Fig 2G, 2H). These observations demonstrated that PER2 K736, and K736-dependent SUMOylation, were required for proper circadian regulation.

***PER2 K736-dependent SUMO1 conjugation is a signal for CK1-mediated phosphorylation and accumulation in the nucleus.***

In contrast to the effect of SUMO2 to destabilized PER2, subcellular fractionation showed that SUMO1 expression promoted PER2 cytoplasm-to-nuclear translocation and chromatin association (Fig 3A). This differed from observations in SUMO2-expressing cells where the majority of PER2 that was not degraded remained in the cytoplasm (Fig 3A). We therefore sought to determine the mechanisms of PER2 SUMO1 conjugation and how it influenced PER2 nuclear translocation.

Phosphorylation can affect PER2 localization [16,23]. Rhythmic changes in PER2 phosphorylation are controlled by coordination between casein kinase (CK1) and phosphoprotein phosphatase 1 (PPP1) and are crucial for modulating circadian rhythm [24,44-47]. Two functional CK1 targeted phosphorylation sites in human PER2, S480 (S478 in mPer2) and S662 (S659 in mPer2), have been identified [23,24]. Comparing our data with previous results, it was intriguing to note that PER2 K736-mediated SUMO1-conjuation and S662 phosphorylation both led to increased PER2 nuclear retention (Fig 3A) [23]. Conversely, K736-mediated SUMO2-conjugation and S480 phosphorylation both promoted PER2 ubiquitination and proteasomal degradation (Fig 2A, EV1B, EV1C) [24,43]. To determine whether there is a relationship between PER2 SUMO1 versus SUMO2 conjugation and PER2 phosphorylation, we first used the CK1δ/ε inhibitor PF670462 to determine whether loss of phosphorylation prevents PER2 SUMOylation (Fig 3B). This analysis found that inhibition of CK1 reduced PER2 S662 phosphorylation but did not affect PER2 SUMOylation (Fig 3B). However, PER2$^{K736R}$ had reduced S662-phosphorylation and slightly decreased CK1 interaction (Fig 3C). PER2$^{K736R}$ also led to a decrease in soluble nuclear and

chromatin-bound protein compared to $PER2^{WT}$ (Fig 3D). Taken together, these data indicate that K736-mediated SUMO1 conjugation may serve as a critical signal upstream of CK1 to promote CK1 phosphorylation of S662 and thereby promote PER2 nuclear entry.

***PER2 SUMO1 conjugation mediated by RanBP2 promotes CK1 phosphorylation of PER2 S662 and CRY1/GAPVD1 interactions required for PER2 nuclear translocation.***

Unlike ubiquitination, E3 ligases are not required for protein SUMOylation as the SUMO E2-conjugating enzyme UBC9 is able to conjugate SUMO onto target proteins [48,49]. In the case of PER2, however, both SUMO1 and SUMO2 can be conjugated on the same K736 site. Therefore, a specific SUMO E3-ligating enzyme may be required for specific SUMO isoform conjugation to determine PER2 protein fate. As SUMO1 conjugation promoted PER2 nuclear entry, we hypothesized that the nucleoporin RanBP2, an E3 enzyme known to mediate SUMO1 conjugation to target proteins for nuclear import [28,50], could be responsible for PER2-SUMO1 conjugation. Consistent with this hypothesis, depletion of RanBP2 in HEK-293T cells (Fig 4A) ectopically expressing MYC-PER2 and GFP-SUMO1 or GFP-SUMO2 resulted in a significant decrease in PER2-SUMO1 conjugation (Fig 4B, 4C upper panel). The effect of RanBP2 depletion on SUMO2 was less pronounced (Fig 4B, 4C lower panel). In addition, CK1-PER2 interaction and PER2 S662-phosphorylation were also significantly decreased upon RanBP2 depletion (Fig 4D). Together, these results suggest that RanBP2-mediated PER2-SUMO1 conjugation is critical for CK1-mediated PER2 phosphorylation at S662 to promote PER2 nuclear entry.

PER2 nuclear entry is facilitated by dimerization with CRY1 [23,51-53] and interaction of the PER2/CRY/CKI complex with GAPVD1, a Rab GTPase guanine nucleotide exchange factor involved in endocytosis and cytoplasmic trafficking [54]. Consistent with these previous observations, $PER2^{K736R}$ had significantly decreased CRY1 and GAPVD1 interaction (Fig 4E, 4F). Taken together, these results indicate that PER2 SUMOylation by SUMO1 is an important signal to promote the subsequent S662 phosphorylation and protein interactions that needed for PER2 translocation into the nucleus.

***SUMOylation is critical for PER2 mediated transcriptional suppression.***

As a key player in the core circadian TTFL, changes in PER2 subcellular localization and protein stability will affect the expression of circadian-regulated genes, particularly those in auxiliary feedback loops such as nuclear receptors REV-ERBs and RORs [6,55]. Therefore, we examined the expression of REV-ERBα, REV-ERBβ, RORα and RORγ in HEK-293T cells overexpressing $PER2^{WT}$ or $PER2^{K736R}$. REV-ERBα, REV-ERBβ and RORγ, but not RORα, were repressed upon

PER2$^{WT}$ expression. In contrast, PER2$^{K736R}$ caused all of these genes to be expressed at higher level than both control cells and PER2$^{WT}$ expressing cells (Fig 4G). This confirmed that K736 modification was essential for PER2 transcriptional repression and was consistent with SUMO1-mediated PER2 nuclear entry (Fig 3). In the core TTFL, PER2 dimerizes with CRY1 to suppress its own expression. Consistent with this, and with our findings that SUMO1 enhanced PER2-CRY1 interaction, transient reporter assays demonstrated that co-expression of PER2$^{WT}$ with SUMO1 repressed PER2 promoter activity (Fig 4H). Further repression was observed when a higher level of SUMO1 was expressed (Fig 4H). In line with the observation of SUMO1-mediated PER2 nuclear translocation (Fig 3), these further indicate that PER2 modification by SUMO1 is critical for its transcriptional suppressor function in regulating circadian genes.

Recent evidence indicates that many SUMO targets can be modified by both SUMO1 and SUMO2/3 [56]. However, the mechanisms balancing SUMO1 versus SUMO2/3 conjugation are still not clear. One possibility is that differential regulation is primarily due to the relative expression level of SUMO isoforms [38]. Another possibility is that SUMO1 and SUMO2/3 may have different conjugation site preferences [56,57]. In the case of PER2, K736 is important for both SUMO1 and SUMO2 conjugation (Fig 2D, 2E). PER2 SUMO1 modification is strongly promoted by RanBP2 (Fig 4) and important for CK1-mediated PER2 phosphorylation at S662. In contrast, PER2 S480 phosphorylation and SUMO2 modification both promote PER2 degradation. However, S480 is more distal to K736 than S662 and it is not known how SUMO2 conjugation at K736 may affect S480 phosphorylation (or vice versa). The SUMO E3 ligase responsible for PER2-SUMO2 conjugation also remains unknown and its identification will be of interest for future research.

In summary, our experiments identified SUMOylation as a key post-translational modification governing PER2 protein stability, transcriptional suppressor function (Fig 5) and maintenance of circadian regulation. PER2 K736 was found to be critical for SUMOylation by multiple SUMO isomers. RanBP2-mediated SUMO1 conjugation promotes PER2 phosphorylation, cytosol-to-nuclear translocation and thereby enhances its transcriptional repression activity. Conversely, PER2 K736-mediated SUMO2 conjugation is essential to facilitate PER2 protein interaction with β-TrCP and its subsequent turnover via ubiquitination-mediated degradation. Thus, SUMO1 versus SUMO2 conjugation forms a critical crossroads controlling the PER2 fate and influences PER2 activities in circadian regulation and tumor suppression. Discovery of this crossroads determining PER2 function provides new insights into the regulation of PER2 specifically as well as the function of SUMOylation in circadian regulation more broadly.

**Materials and Methods**

***Cell line*** Immortalized human embryonic kidney cell HEK-293T and phenotypically normal breast epithelial cell MCF-10A were cultured using the standard growth medium according ATCC in a humidified 37 °C incubator supplemented with 5% $CO_2$. Serum shock was performed with DMEM supplemented with 50% horse serum (GIBCO) for 2 h when cells reached ~80% confluence. The medium was then replaced with DMEM supplemented with 0.1% FBS. At the time indicated, the cells were washed twice with ice-cold PBS and whole-cell lysates prepared using RIPA lysis buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.05% SDS, 1 mM PMSF, 1x protease inhibitor, with or without 10 mM NEM).

***Generation of PER2$^{K736R}$ knock-in mutant*** An all-in-one CRISPR vector, pAll-Cas9.Ppuro (RNAi core facility, Academia Sinica, Taiwan), was digested with BsmBI and ligated with annealed oligonucleotides (GGCTGCACACACACAGAAGG) for sgRNA expression to target the PER2 coding region at the K736 residue. The 5' mismatched G of PER2 sgRNA was created for optimal U6 transcriptional initiation. A single- stranded oligodeoxynucleotide (ssODN) (5'-AAGGAGGTACTCGCTGCACACACACAGCGCGAGGAGCAGAGCTTCCTGCAGAAGTTCAAA-3') was used to generate the K736R mutation. The underlined bases indicate the mutated codon encoding Arginine (R). Cells were transfected with all-in-one CRISPR plasmid and ssODNs donor template. After selection with puromycin, genomic DNA from selected cells was sequenced using a target-specific sequencing primer (5'-GCACTAGCCTGGGACTATTC-3') to validate the knock-in mutation.

***Plasmids and reagents*** pcDNA3.1Myc-hPER2 plasmid was generously provided by Dr. Randal Tibbetts (University of Wisconsin, USA) [44]. pEGFP.C3.SUMO.GG plasmids were kindly provided by Dr. Mary Dasso (NIH, USA). pcDNA3.1.HA.Ub plasmid was kindly provided by Dr. Hsiu-Ming Shih (Academia Sinica, Taiwan). A series of mutant PER2 plasmids including S480A, S480D, K83R, K1163R, K736R, K83/1163R, K83/736R, K736/1163R, K83/736/1163R were made using site-directed mutagenesis (Agilent) with primer sets listed in Appendix Table S1. Depending on the experiments, these cDNAs were sub-cloned into various tagged vectors including pcDNA3.0.HA (Invitrogen), 3XFLAG-CMV-7.1-2 (Sigma) and pcDNA3.1.Myc.HisA/pcDNA3.1.Myc.HisC (Invitrogen) for transient expression in mammalian cells. pGL3.PER2.Luc promoter (-948~+424) was generated as previously described [22]. All cloned and mutated genes were verified by sequencing. The lentiviral shCtrl (TRC2.Scramble, ASN0000000003), shRANBP2#1 (TRCN0000272800) and shRANBP2#2 (TRCN0000272801) were purchased from the National RNAi Core Facility (Taipei, Taiwan). *In vitro* translation was performed using TnT® Quick Coupled Translation System (Promega). Transient transfection was done by Mirus TransIT-LT1 Reagent (Mirus Bio, USA) according to the manufacturer's instructions or by electroporation. Subcellular protein fractionation was performed using a kit for cultured cells from Thermo Fisher Scientific (78840). Proteasome inhibitors MG132, SUMO protease inhibitor N-Ethylmaleimide (NEM), cycloheximide and D-luciferin were from Sigma-Aldrich. CK1δ/ε inhibitor PF 670462 was purchased from TOCRIS.

***RNA isolation, reverse transcription and real-time (q)PCR*** Total RNA from cell culture was isolated using Trizol reagent or RNeasy kit (Qiagen) and reverse-transcribed with Quant iNova Reverse Transcription kit (Qiagen). qPCR was performed using Quant iNova SYBR Green PCR kit (Qiagen) and primer sets for gene expression and analyzed on a Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher). Primers used in this study were listed in Appendix Table S2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for mRNA expression. Expression levels were calculated according to the relative ΔCt method.

***Co-immunoprecipitation (Co-IP)*** Whole-cell lysates were prepared using a TNE lysis buffer (10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 10 μM MG132, 1 mM PMSF, 10 mM NEM and 1x protease inhibitors) followed by sonication and centrifugation at 12,000 × g at 4°C. 1 ml of the crude whole-cell extract was incubated with 1-2 μg of antibody as indicated or control IgG antibodies at 4°C overnight. Then, 50 μl prewashed protein A/G agarose was added to the mixture and incubated at 4°C for 4 h with gentle agitation. After extensive washing with diluted NP-40 lysis buffer (0.1-0.5% NP-40), PER2 interacting proteins were eluted with SDS buffer and analyzed by immunoblot. Antibodies and agarose used for PER2 IP were listed in Appendix Table S3.

***Denatured-IP*** Denatured whole-cell lysates were prepared using a lysate buffer containing 1% SDS, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 15 mM NEM and 1x protease inhibitors, followed by boiling at 95°C for 8 minutes and sonication. After centrifugation at maximum speed, Flag-SUMO conjugated proteins were immunoprecipitated over night at 4°C. The samples were washed using high salt RIPA buffer (20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 250 mM NaCl, 0.5% NP40, 10% glycerol, 10 mM NEM, 1xprotease inhibitors), low salt RIPA buffer (20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 150 mM NaCl, 0.5% NP40, 10% glycerol, 10 mM NEM, 1xprotease inhibitors), and PBS before being boiled in 2xsample buffer (with 100 mM DTT) and processed for SDS-PAGE followed by immunoblot.

***Immunoblotting (IB)*** Whole-cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.05% SDS, 1 mM PMSF, 1x protease inhibitor, 10 mM NEM). IB analysis was performed after 6-7.5% SDS-PAGE or 4-20% Mini-PROTEAN TGX Precast Protein Gels (BioRad), with overnight incubation with a 1:1000 dilution of primary antibody and followed by a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Jackson ImmunoResearch). Signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Primary antibodies used in this study are listed in Appendix Table S3. Protein concentration was determined by the Bradford assay (Bio-Rad) before loading and verified by α-tubulin level. The optical density was determined using the National Institutes of Health ImageJ program.

***Nano LC-MS/MS Analysis*** In order to identify the sumoylation site(s) of PER2, flag-PER2 which was expressed with and without over-expressed SUMO1(RGG) were purified by SDS-PAGE and followed by in-gel digestion for the mass spectrometric analysis. Briefly, gel slices were cut into 1 mm squares and washed in 50 mM triethylammonium bicarbonate buffer (TEABC) and then TEABC/ACN (25 mM TEABC, 50% acetonitrile). Repeated the washing steps three times. The gel slices were reduced in 20 mM dithiothreitol (DTT) at 56 °C for 1 h, and alkylated in 55 mM iodoacetamide at room temperature for 45 min in dark before trypsin digestion at 37 °C overnight. The enzymatic digestions were quenched through the addition of formic acid (10%) and the tryptic peptides were collected and vacuum-dried prior to mass analysis.

MS data were acquired on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) equipped with Ultimate 3000 RSLC system from Dionex (Dionex Corporation) and nanoelectrospry ion source (New Objective, Inc.). Samples in 0.1% formic acid were injected onto a self-packed precolumn (150 μm I.D. x 30 mm, 5 μm, 200 Å) at the flow rate of 10 μl min-1. Chromatographic separation was performed on a self-packed reversed phase C18 nano-column (75 μm I.D. x 200 mm, 3 μm, 100 Å) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile as mobile phase B operated at the flow rate of 300 nL min-1. The MS/MS were run in top speed mode with 3s cycles; while the dynamic exclusion duration was set to 60 s with a 25 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor ion selection was enabled and 1+ charge state ions were rejected for MS/MS. The MS/MS analyses were carried out with the collision induced dissociation (CID) mode. Full MS survey scans from m/z 300 to 1,600 were acquired at a resolution of 120,000 using EASY-IC as lock mass for internal calibration. The scan range of MS/MS spectra based on CID fragmentation method were from m/z 150 to 1,600. The precursor ion isolation was performed with mass selecting quadrupole, and the isolation window was set to m/z 2.0. The automatic gain control (AGC) target values for full MS survey and MS/MS scans were set to 2e6 and 5e4, respectively. The maximum injection time for all spectra acquisition was 200 ms. For CID, the normalization collision energy (NCE) was set to 30%.

For data analysis, all MS/MS spectra were converted as mgf format from experiment RAW file by msConvert then analyzed by Mascot for MS/MS ion search. The search parameters included the error tolerance of precursor ions and the MS/MS fragment ions in spectra were 10 ppm and 0.6 Da and the enzyme was assigned to be trypsin with the miss cleavage number two. The variable post-translational modifications in search parameter were assigned to include the oxidation of methionine, carbamidomethylation of cysteine, and GlyGly tag of lysine as the sumoylation site.

***Transient reporter assay*** HEK293T cells ($1x10^6$ cells) were transfected with 200 ng

pGL3.PER2.Luc promoter, 50 ng Renilla.Luc (for normalization), 200 ng MYC-PER2$^{WT}$ and GFP-SUMO1 (0, 100, 200 ng) plasmids in a 35-mm plate. Cell extracts were prepared at 24 h after transfection, and the luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

***Bioluminescent recording and data analysis*** CRISPR/CAS edited HEK-293T cells were transfected with PER2.Luc reporter for 24 hours before synchronization with 100 nM dexamethasone [58] for 2 h. Subsequently, the media was changed to recording media (phenol red free DMEM (Gibco), 400 μM D-Luciferin Potassium Salt (Goldbio)) and monitored via the use of a Kronos Dio luminometer (AB-2550) at 37°C. Bioluminescence from each dish was continuously recorded (integrated signal of 60 s with intervals of 9 min). Data were smoothed and detrended with the KronosDio analysis tool. JTK_Cycle analysis (version 3) was employed to test the rhythmicity and amplitude of the signals between circadian time 5 and 31 hours [59,60]. The phase shift was determined by measuring the time difference between the peaks of the PER2 promoter luciferase rhythms between the PER2$^{WT}$ and PER2$^{K736R}$ cells.

***Statistical analysis*** All data are presented as means ± SD. Student's *t*-test was used to identify statistically significant differences. Asterisk (*) indicates statistical significance with $p$-value <0.05 and (**) indicates $p$-value < 0.01.

**Acknowledgements:** This work was supported by Academia Sinica and Ministry of Science and Technology [MOST 105-2628-B-001-008-MY3] and [MOST-108-2311-B-001-005-MY3] to WWHV. We thank the National RNAi Core Facility (Academia Sinica) for RNAi and CRISPR reagents. We also thank Mr. Kuo-Chih Cheng and Dr. Ming-Ta Lee for their assistance and discussion.

**Author contributions**: Conceptualization, L.C.C., Y.L.H. and W.W.H.-V; Methodology, L.C.C., Y.L.H., Y.C.C., P.H.H., W.W.H.-V.; Investigation, L.C.C., T.Y.K.; Writing – Original Draft, L.C.C. and W.W.H.-V.; Writing – Review & Editing, L.C.C., Y.L.H. and W.W.H.-V.; Funding Acquisition, W.W.H.-V.; Supervision, W.W.H.-V.

**Conflict of Interests:** The authors declare that they have no conflict of interest.

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## Figure Legends

**Figure 1. PER2 can be SUMOylated by both SUMO1 and SUMO2.**

**A.** Immunoblotting (IB) assay of endogenous PER2 and SUMO1 at CT1, 5, 13 and 17 using HEK-293T cells treated without or with NEM. Tubulin was used as a loading control.

**B.** Co-IP assay using HEK-293T cells. Cell lysates were immunoprecipitated (IP) with anti-PER2 antibody. Immunoprecipitates and input lysates were analyzed by IB. Arrows indicate the unmodified PER2 and brackets indicated PER2-SUMO1 conjugates.

**C.** Co-IP assay using HEK-293T cells co-transfected with HA-PER2, GFP-SUMOs and FLAG-SENP1. Cell lysates were IP with anti-HA antibody. Immunoprecipitates and input lysates were analyzed by IB. Arrow indicates the PER2-SUMO conjugates.

**D.** Co-IP assay using HEK-293T cells co-transfected with FLAG-PER2 and GFP-SUMO1 or GFP-SUMO2. Cells were treated without or with proteasome inhibitor MG132. Cell lysates were IP with anti-FLAG antibody and analyzed by IB. Arrows indicate the PER2-SUMO conjugates.

**Figure 2. Lysine 736 is important for PER2 SUMOylation and required to maintain correct circadian period; however, modification by SUMO1 and SUMO2 leads to different PER2 protein fate.**

**A.** IB assay of PER2 and SUMOs using HEK-293T cells co-transfected with MYC-PER2 and increasing amount of GFP-SUMOs. Tubulin was used as a loading control.

**B.** Schematic representation of human PER2 protein. Three predicted SUMOylation sites (K87, K736, K1163) are indicated (red). Coactivator LXXLL nuclear receptor recognition motifs (orange), CK1 targeted serine regions (S480, S662, green), the familial advanced sleep phase syndrome (FASPS) mutation site (S662G, black), Per-Arnt-Sim (PAS) domains (brown) and a CRY binding domain (blue) are also indicated.

**C.** The MS/MS spectrum of the tryptic peptide m/z 700.6972 from PER2 with SUMOylated K736. All b and y product ions from the SUMOylated peptide with di-glycine modification on K736 are labelled in the spectrum.

**D, E.** Denatured IP of PER2 and SUMO1 (D) or SUMO2 (E) using HEK-293T cells co-transfected with MYC-PER2 and FLAG-SUMOs. Cell lysates were IP with anti-FLAG antibody and analyzed by IB analysis. Brackets indicate the PER2-SUMO conjugates.

**F.** Co-IP assay of PER2 and β-TrCP using HEK-293T cells transfected with MYC-PER2$^{WT}$ or -PER2$^{K736R}$. Cell lysates were IP with indicated antibodies and analyzed by IB. Normal IgG was used as a control.

**G**. Circadian rhythm of bioluminescence using CRISPR/CAS-edited PER2$^{K736R}$ knock-in or control (PER2$^{WT}$) HEK-293T cells transiently transfected with PER2 luciferase reporter. Data were smoothed and detrended with the KronosDio analysis tool and the rhythmicity (JTK *p*-value) and amplitude (Amp.) were analyzed using JTK_Cycle analysis (version 3). n=3

**H.** Phase shift (Δϕ) was determined from the data presented in G by measuring the time difference between the peaks of the PER2 promoter luciferase rhythms from PER2$^{WT}$ and PER2$^{K736R}$ cells. Data are means ± SD, n=3 (H).

All experiments were performed at least twice with similar results.

**Figure 3. PER2 K736-dependent SUMO1 conjugation is a signal for CK1-mediated phosphorylation and translocation into the nucleus.**

**A.** Fractionation analysis using HEK-293T cells co-transfected with HA-PER2 and GFP-SUMOs. The levels of HA-PER2 in cytoplasmic, soluble nuclear and chromatin-bound fractions were determined using IB. Tubulin and nuclear matrix protein p84 were used as loading controls for cytoplasmic and nuclear proteins, respectively.

**B.** Co-IP assay using HEK-293T cells co-transfected with FLAG-PER2$^{WT}$ and GFP-SUMO1 treated without or with CK1 inhibitor PF670462. Cell lysates were IP with anti-FLAG antibody and analyzed by IB to detect PER2-SUMO conjugates. Normal IgG was used as an IP control. The inhibition efficiency of CK1 inhibitor was determined using IB analysis for PER2 S662-phosphorylation

**C.** Co-IP assay using HEK-293T cells co-transfected with FLAG-PER2$^{WT}$ or -PER2$^{K736R}$. Cell lysates were IP with anti-FLAG antibody and analyzed by IB to detect CK1 and S662-phosphorylated PER2.

**D.** Fractionation analysis using HEK-293T cells co-transfected with MYC-PER2$^{WT}$ or -PER2$^{K736R}$. The levels of MYC-PER2 in cytoplasmic, soluble nuclear and chromatin-bound fractions were determined using IB. Tubulin and nuclear matrix protein p84 were used as loading controls for cytoplasmic and nuclear proteins, respectively.

All experiments were performed at least twice with similar results.

**Figure 4. PER2 SUMO1 conjugation mediated by RanBP2 promotes CK1 phosphorylation of PER2 S662 and CRY1/GAPVD1 interactions required for PER2 nuclear translocation and PER2-mediated transcriptional suppression.**

**A-C.** The depletion efficiency of RanBP2 protein was determined using IB analysis (A). Co-IP assay using HEK-293T cells without or with RanBP2 depletion co-transfected with MYC-PER2 and GFP-SUMOs (B, C). Cell lysates were IP with anti-MYC antibody and analyzed by IB to detect PER2-SUMO1 (C, upper panel) and PER2-SUMO2 (C, lower panel) conjugates indicated by brackets.

**D.** Co-IP assay using HEK-293T cells without or with RanBP2 depletion co-transfected with MYC-PER2 and GFP-SUMO1. Cell lysates were IP with anti-MYC antibody and analyzed by IB to detect CK1 and S662-phosphorylated PER2.

**E, F.** Co-IP assay of PER2 and CRY1 (C) or GAPVD1 (D) using HEK-293T cells transfected with MYC-PER2$^{WT}$ or -PER2$^{K736R}$. Cell lysates were IP with indicated antibodies and analyzed by IB. Normal IgG was used as a control.

All experiments were performed at least twice with similar results.

**G.** Expression of PER2 downstream circadian genes analyzed by real-time quantitative (q)-PCR using HEK-293T cells transfected with control vector, MYC-PER2$^{WT}$ or -PER2$^{K736R}$. GAPDH was used as an internal control. All data points were performed in at least triplicates and all experiments were performed at least twice with similar results. Data are presented as mean ± SD, n=3. *, $p < 0.05$; ***, $p < 0.001$ (Student's t-test)

**H.** Relative fold-change in luciferase activity of the PER2 reporter construct in HEK-293T cells transiently co-transfected with MYC-PER2$^{WT}$ and GFP-SUMO1. Luciferase activity in samples transfected with PER2-Luc and PER2$^{WT}$ only was arbitrarily set at 1. Data are means ± SD, n=3. ***, $p < 0.001$ (Student's t-test). All data points were performed in at least triplicates and all experiments were performed at least three times with similar results.

**Figure 5. Diagram of the proposed mechanism by which SUMOylation regulates PER2 protein stability and transcriptional suppression function.** PER2 K736 is important for PER2 SUMOylation by multiple SUMO isoforms. SUMO2 facilitates PER2 interaction with β-TrCP, ubiquitination and degradation. In contrast, SUMO1 modification does not affect PER2 protein stability, but enhances PER2 interaction with CRY1 and GAPVD1 resulting in an increase in nuclear and chromatin-bound PER2. Such modification is critical for PER2-mediated repression of circadian genes including PER2 itself, REV-ERBα/β and RORγ. The SUMO E3 ligase RanBP2 facilitates, at least in part, PER2-SUMO1 conjugation which is critical for CK1-mediated PER2 S662 phosphorylation. Identification of SUMO E3 ligases involved in SUMO2 modification of PER2

and analysis of whether SUMOylated K736 affects SUMOylation at other K sites is of interest for further experiments.

Figure 1

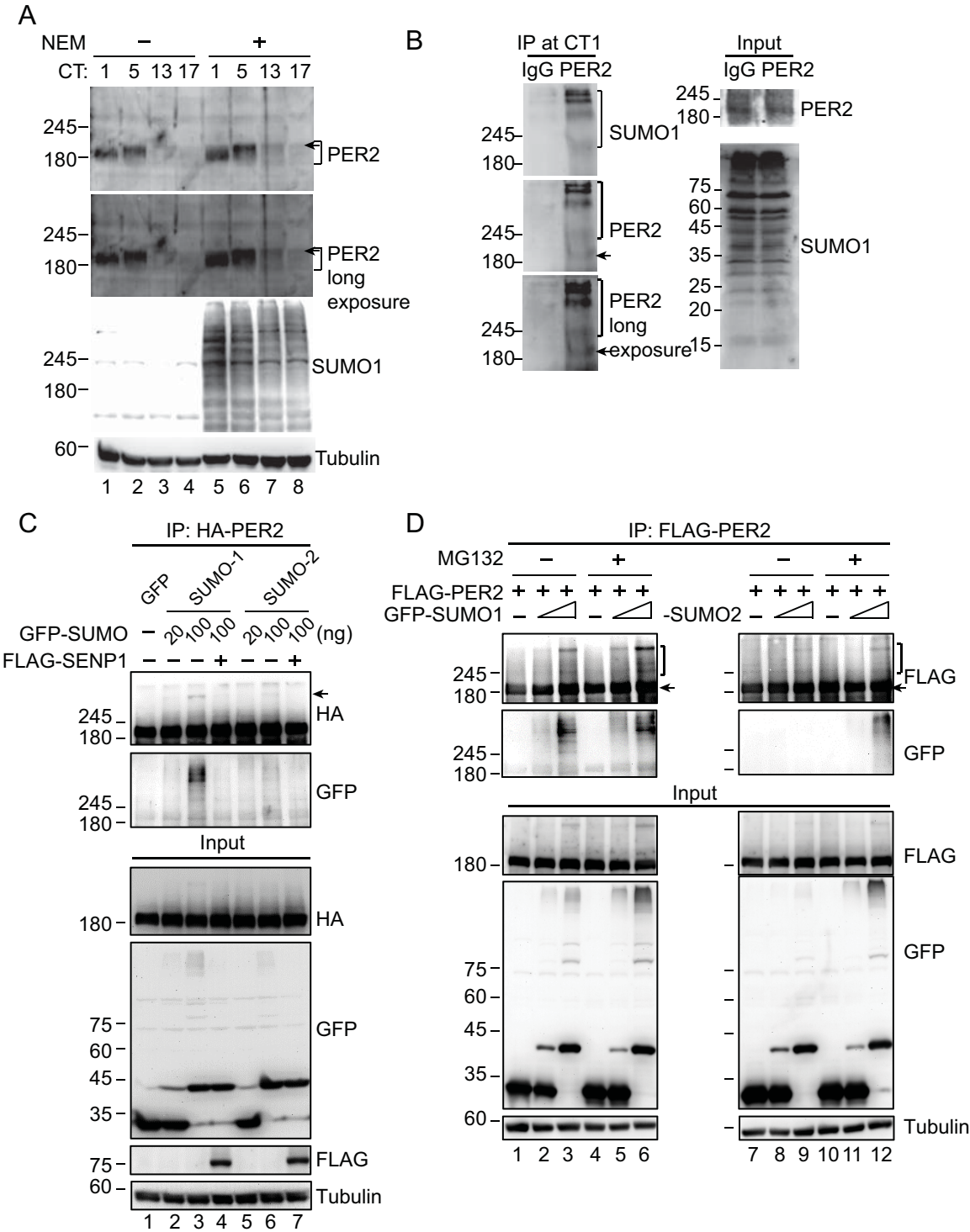
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Figure 2

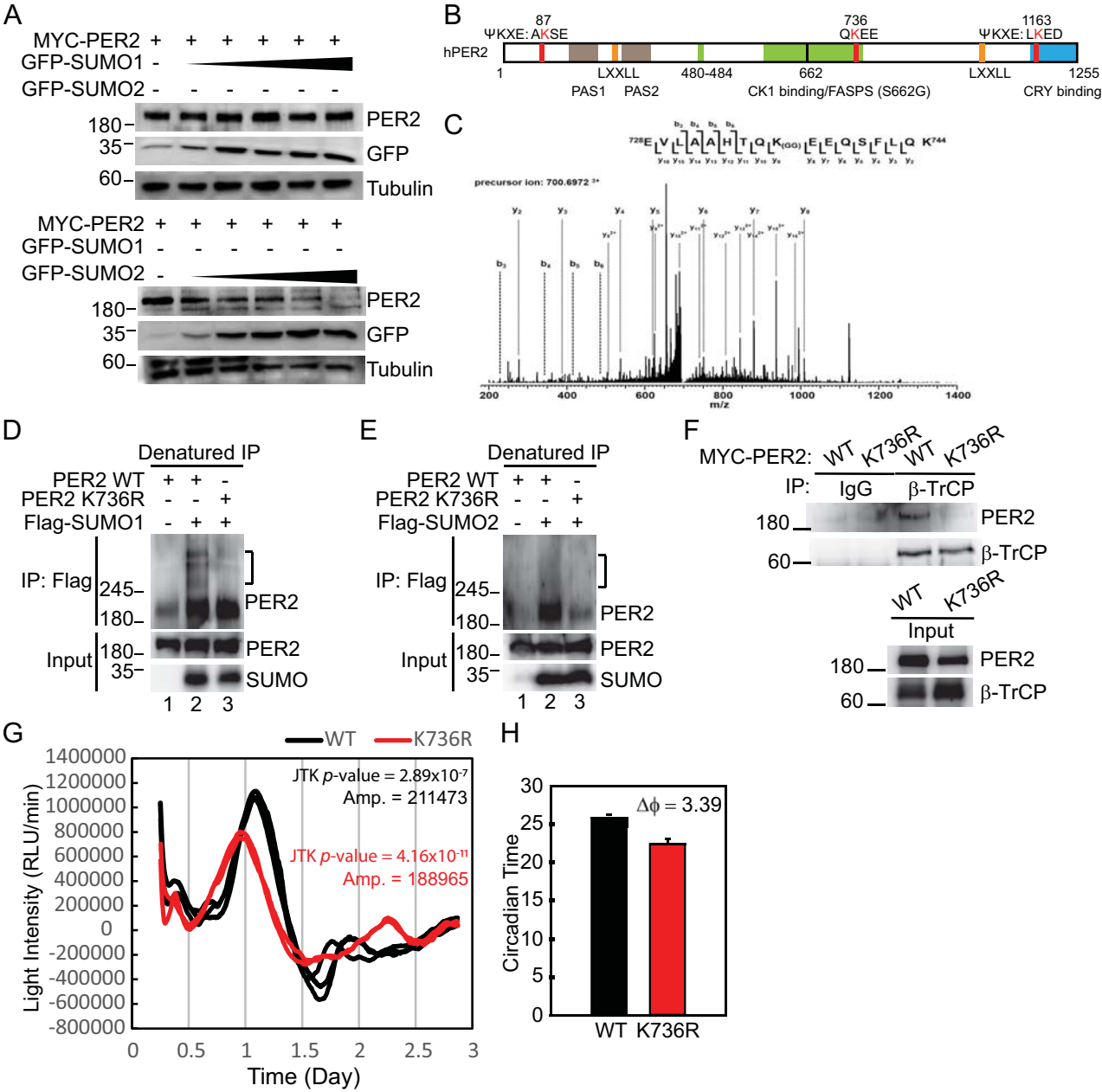
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Figure 3

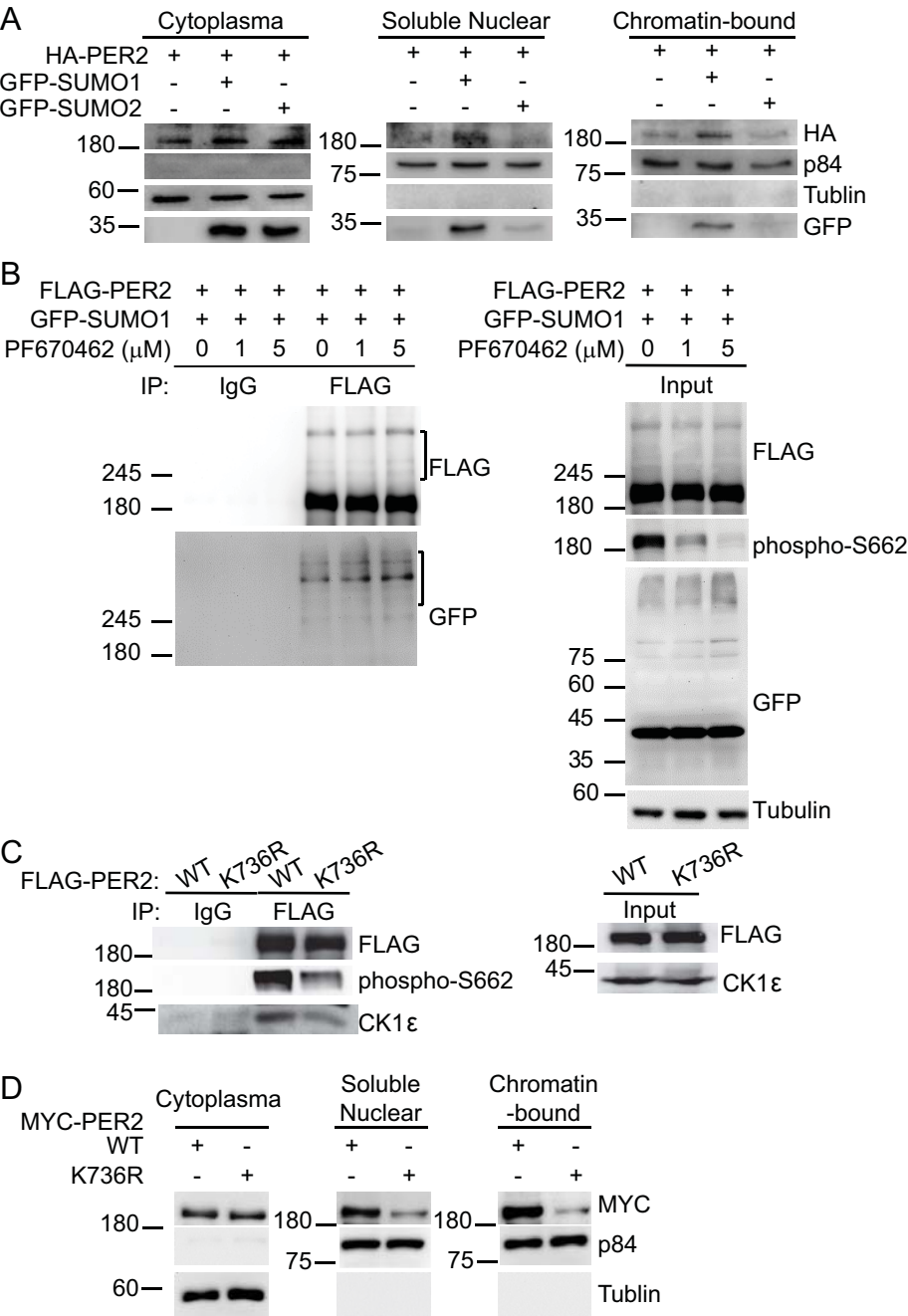
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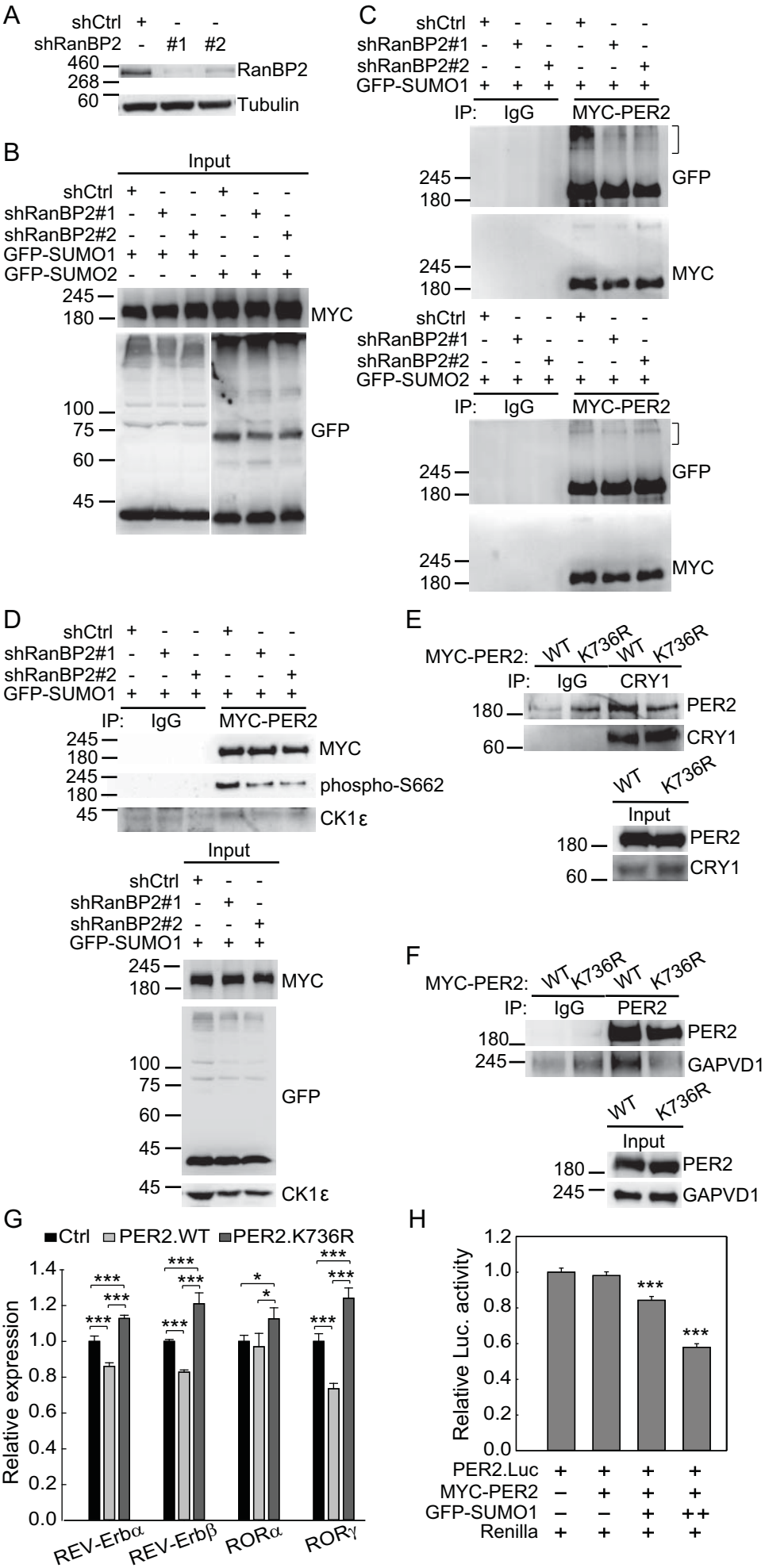
Figure 4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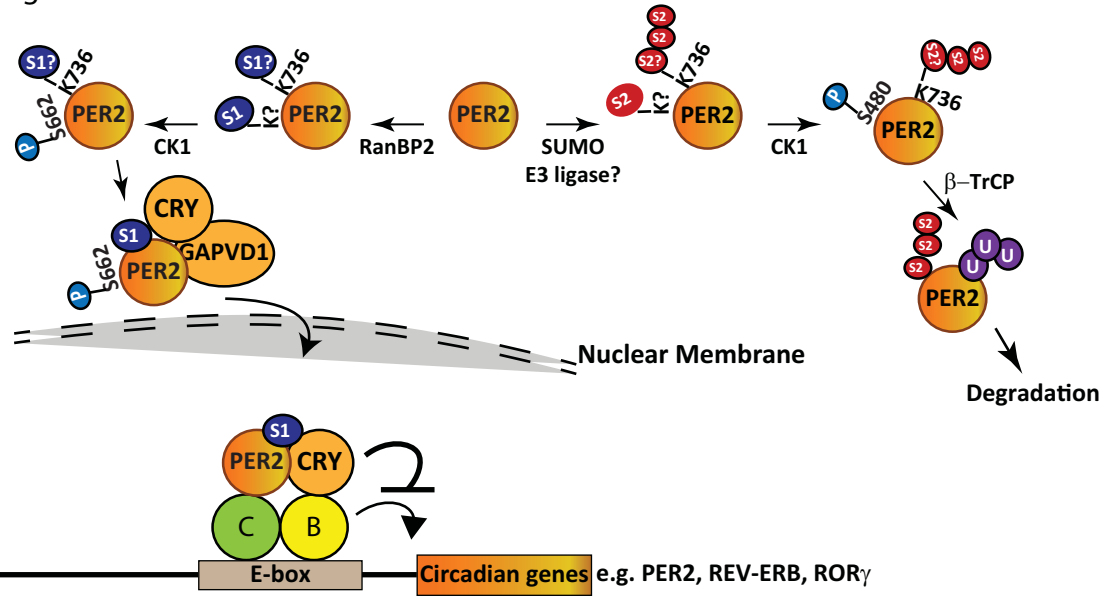
0.2
0.0
REV-Erbα
REV-Erbβ
RORα
RORγ
H
Relative Luc. activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PER2.Luc + + + +
MYC-PER2 – + + +
GFP-SUMO1 – – + ++
Renilla + + + +

Figure 5


S1?
K736
S662
P
PER2
CK1
S1
K?
RanBP2
SUMO
E3 ligase?
S2
S2?
S480
β–TrCP
U
CRY
GAPVD1
Nuclear Membrane
Degradation
C
B
E-box
Circadian genes
e.g. PER2, REV-ERB, RORγ