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

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Abstract

Posttranslational modification (PTM) of core circadian clock proteins, including Period2 (PER2), is required for proper circadian regulation. 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 different effects on PER2 turnover and subcellular localization. SUMO2 conjugation facilitated PER2 degradation. In contrast, SUMO1 conjugation, mediated by E3 SUMO-protein ligase RanBP2, enhanced CK1-mediated $PER2^{S662}$ phosphorylation and increased PER2 nuclear accumulation, chromatin association and transcriptional suppressor function. PER2 K736 was critical for both SUMO1- and SUMO2-conjugation. A $PER2^{K736R}$ mutation was sufficient to alter circadian periodicity, decrease PER2 nuclear entry and abolish PER2-mediated transcriptional suppression. Together, our data revealed differential effects of SUMO1 versus SUMO2 conjugation on PER2 function and demonstrated the effect of PER2 SUMOylation on the circadian regulatory system and circadian periodicity.

Introduction

The circadian clock controls many rhythmic physiological processes such as hormonal oscillation, metabolism and immune function that are essential to maintain homeostasis (Dunlap, 1999; Stephan & Zucker, 1972). 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 (Hughes et al., 2009; Shearman et al., 2000). 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 (Chiou et al., 2016). 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 (Bugge et al., 2012; Liu et al., 2008; Preitner et al., 2002).

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 (Gallego & Virshup, 2007; Hirano, Fu, & Ptacek, 2016; C. Lee, Etchegaray, Cagampang, Loudon, & Reppert, 2001; Partch, Green, & Takahashi, 2014; Reischl & Kramer, 2011; Stojkovic, Wing, & Cermakian, 2014). Among the known PER2 PTMs, phosphorylation has been most intensively studied (C. Lee et al., 2001). Two important serine phosphorylation sites (S662, S480) have been identified. Phosphorylation at S662 affects PER2 subcellular localization and therefore influences transcription of PER2 itself and its downstream genes (Vanselow et al., 2006; Y. Xu et al., 2007). 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 (Toh et al., 2001; Y. Xu et al., 2007). Phosphorylation at S480 facilitates PER2-β-TrCP interaction leading to PER2 proteasomal degradation (β-TrCP phosphodegron) (Eide et al., 2005). Recent research has suggested that casein kinase 1 (CK1) is responsible for PER2 phosphorylation at both S662 and S480 (Narasimamurthy et al., 2018); 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 (Kaasik et al., 2013). Acetylation of PER2 at lysine (K) residues protects PER2 from ubiquitination; whereas deacetylation of PER2 by SIRT1 leads to degradation (Asher et al., 2008). 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 (Gareau & Lima, 2010; Gill, 2005). 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 (Hunter & Sun, 2008). 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 (Matic et al., 2008). 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 (Evdokimov, Sharma, Lockett, Lualdi, & Kuehn, 2008). 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 (Saitoh, Pu, & Dasso, 1997), antagonizes ubiquitination (Desterro, Rodriguez, & Hay, 1998) and modulates protein-protein interaction (Gill, 2004). In contrast, SUMO2/3 is thought to mainly participate in cellular response to stress (Saitoh & Hinchey, 2000) and can facilitate targeted protein ubiquitin-mediated degradation (Tatham et al., 2008). 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) (Gong et al., 2014). 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

PER2 can be SUMOylated

To test whether PER2 is SUMOylated, immunoprecipitation (IP) was performed using HEK-293T cells at circadian time (CT) 1 hour after serum shock, an approach to synchronize and induce circadian gene expression in culture (Balsalobre, Damiola, & Schibler, 1998). The results confirmed that PER2 was SUMO-conjugated (Fig 1A). PER2-SUMOylation was further confirmed by detection of SUMO-conjugated PER2 in HEK-293T cells ectopically expressing tagged-PER2 and SUMO1 or

SUMO2 (Fig 1B, lane 3 and 6). Importantly, co-expression of SUMO1 or SUMO2 with SENP1, a SUMO protease, eliminated the PER2 band shift (Fig 1B, 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 1B, 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 1C, lane 12 vs. lane 9). Also, PER2 protein levels decreased in a dose-dependent manner upon SUMO2 expression (Fig 1D). 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 S1A). 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 S1B, lane 5, 6 vs. lane 2, 3). In contrast, SUMO1 did not affect PER2 protein stability (Fig 1D).

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 ψKxD/E (where ψ is a large hydrophobic residue) (J. Xu et al., 2008). 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 (Zhao et al., 2014) (Fig 2A). An additional SUMOylation motif, QKEE (Hendriks et al., 2017), was also identified at K736 by manual inspection of the PER2 sequence. PER2 K736 and K1163 are conserved between human and mouse (Fig S2).

Figure 1

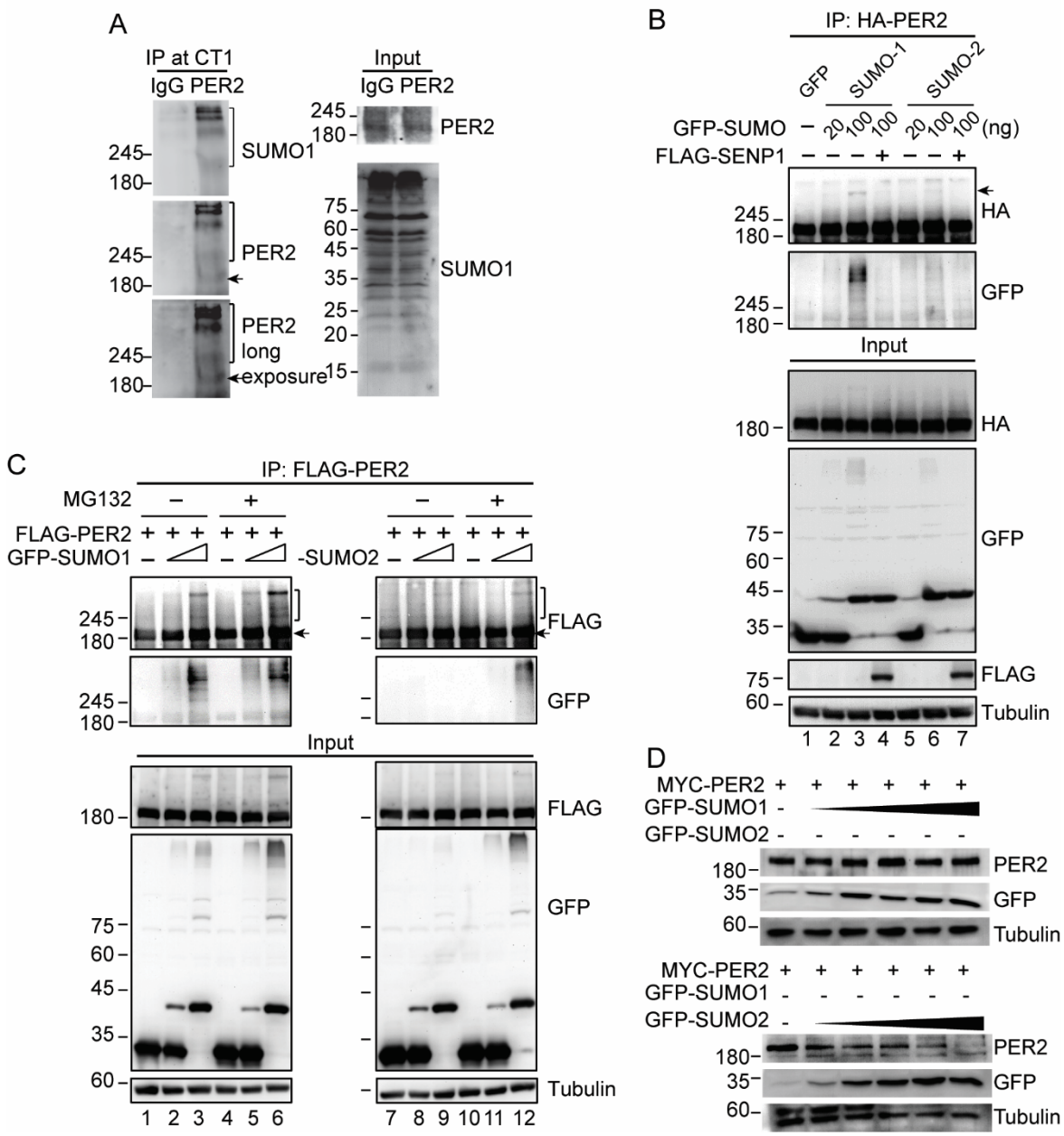

Figure 1. PER2 can be SUMOylated by both SUMO1 and SUMO2.

A. Co-IP assay using HEK-293T cells at one hour after serum shock (CT1). 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.

B. 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.

C. 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.

D. 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.

Figure S1

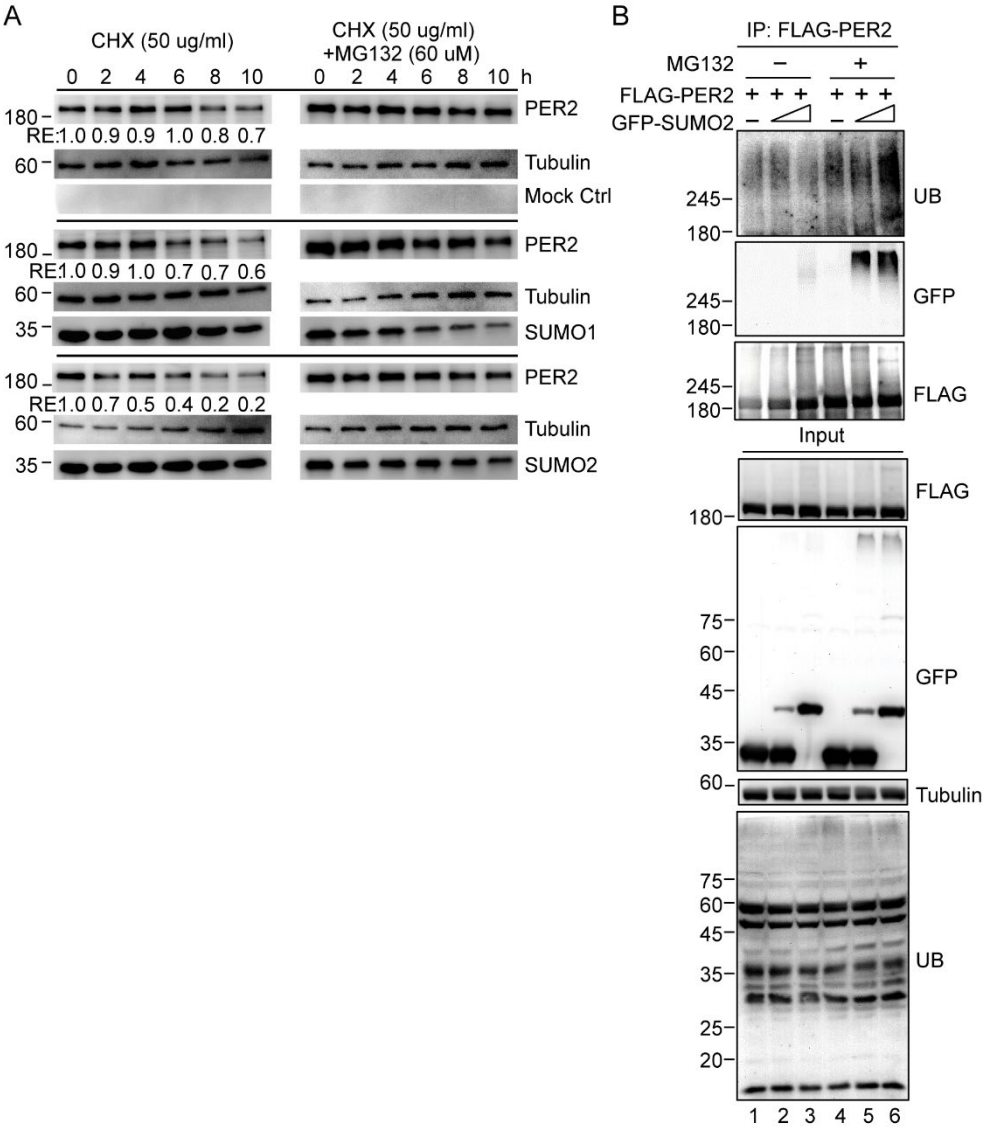

Figure S1. SUMO1 and SUMO2 conjugation lead to different PER2 protein fate

A. Time course assay using cycloheximide (CHX) treated HEK-293T cells co-transfected with MYC-PER2 and GFP-SUMOs. Cells were treated without or with MG132. The levels of PER2 and SUMOs were determined using IB analysis. Tubulin was used as a loading control. RE: relative expression.

B. Co-IP assay using HEK-293T cells co-transfected with FLAG-PER2 and GFP-SUMO2. Cells were treated without or with proteasome inhibitor MG132. Cell lysates were IP with anti-FLAG antibody and analyzed by IB to detect PER2-SUMO and -UB conjugates. Arrow indicates the PER2-SUMO conjugates.

Figure S2. Human and mouse PER2 protein sequence alignment. Putative lysine sites for SUMOylation (K87, K736, K1163) are highlighted in red.

Figure S2

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SP|O15055|PER2_HUMAN MNGYAEFPPSPSNPTKEPVEPQPSQVPLQEDVDMSSGSSGHETNENCSTGRDSQGSDCDD 60
SP|O54943|PER2_MOUSE MNGYVDFSPSPTSPTKEPGAPQPTQAVLQEDVDMSSGSSG---NENCSTGRDSQGSDCDD 57
                     ****.:* ***:.*****  ***:*. *************   *****************
                                                87
SP|O15055|PER2_HUMAN SGKELGMLVEPPDARQSP-DTFSLMMAKSEHNPSTSGCSSDQSSKVDTHKELIKTLKELK 119
SP|O54943|PER2_MOUSE NGKELRMLVESSNTHPSPDDAFRLMMTEAEHNPSTSGCSSEQSAKADAHKELIRTLKELK 117
                     .**** ****  ::: ** *:* ***:::***********:**:*.*:*****:******
SP|O15055|PER2_HUMAN VHLPADKKAKGKASTLATLKYALRSVKQVKANEEYYQLLMSSEGHPCGADVPSYTVEEME 179
SP|O54943|PER2_MOUSE VHLPADKKAKGKASTLATLKYALRSVKQVKANEEYYQLLMSSESQPCSVDVPSYSMEQVE 177

******************************************.:**..*****::*::*
SP|O15055|PER2_HUMAN SVTSEHIVKNADMFAVAVSLVSGKILYISDQVASIFHCKRDAFSDAKFVEFLAPHDVGVF 239
SP|O54943|PER2_MOUSE GITSEYIVKNADMFAVAVSLVSGKILYISNQVASIFHCKKDAFSDAKFVEFLAPHDVSVF 237
                     .:***:************************:*********:*****************.**
SP|O15055|PER2_HUMAN HSFTSPYKLPLWSMCSGADSFTQECMEEKSFFCRVSVRKSHENEIRYHPFRMTPYLVKVR 299
SP|O54943|PER2_MOUSE HSYTTPYKLPPWSVCSGLDSFTQECMEEKSFFCRVSVGKHHENEIRYQPFRMTPYLVKVQ 297
                       **:*:***** **:*** ******************* * *******:***********:
SP|O15055|PER2_HUMAN DQQGAESQLCCLLLAERVHSGYEAPRIPPEKRIFTTTHTPNCLFQDVDERAVPLLGYLPQ 359
SP|O54943|PER2_MOUSE EQQGAESQLCCLLLAERVHSGYEAPRIPPEKRIFTTTHTPNCLFQAVDERAVPLLGYLPQ 357
                     :******************************************* ***************
SP|O15055|PER2_HUMAN DLIETPVLVQLHPSDRPLMLAIHKKILQSGGQPFDYSPIRFRARNGEYITLDTSWSSFIN 419
SP|O54943|PER2_MOUSE DLIETPVLVQLHPSDRPLMLAIHKKILQAGGQPFDYSPIRFRTRNGEYITLDTSWSSFIN 417
                     ****************************:**************:******************
SP|O15055|PER2_HUMAN PWSRKISFIIGRHKVRVGPLNEDVFAAHPCTEEKALHPSIQELTEQIHRLLLQPVPHSGS 479
SP|O54943|PER2_MOUSE PWSRKISFIIGRHKVRVGPLNEDVFAAPPCPEEKTPHPSVQELTEQIHRLLMQPVPHSGS 477
                     *************************** ** ***: ***:***********:********
SP|O15055|PER2_HUMAN SGYGSLGSNGSHEHLMSQTSSSDSNGHEDSRRRRAEICKNGNKTKNRSHYSHESGEQKKK 539
SP|O54943|PER2_MOUSE SGYGSLGSNGSHEHLMSQTSSSDSNGQEESHRRRSGIFKTSGKIQTKSHVSHESGGQKEA 537
                     ***************************:*:*:***: * *...* :.:** ***** **:
SP|O15055|PER2_HUMAN SVTEMQTNPPAEKKAVPAMEKDSLGV-----SFPEELACKNQPTCSYQQISCLDSVIRYL 594
SP|O54943|PER2_MOUSE SVAEMQSSPPAQVKAVTTIERDSSGASLPKASFPEELAYKNQPPCSYQQISCLDSVIRYL 597
                     **:***:.***: *** ::*:** *.     ******* **** ****************
SP|O15055|PER2_HUMAN ESCNEAATLKRKCEFPANVPALRSSDKRKATVSPGPHAGEAEPPSRVNSRTGVGTHLTSL 654
SP|O54943|PER2_MOUSE ESCSEAATLKRKCEFPANIPS------RKATVSPGLHSGEAARPSKVTSHTEVSAHLSSL 651
                     ***.***************:*:      ******** *:***  **:*.*:* *.:**:**
SP|O15055|PER2_HUMAN ALPGKAESVASLTSQCSYSSTIVHVGDKKPQPELEMVEDAASGPESLDCLAGPALACGLS 714
SP|O54943|PER2_MOUSE TLPGKAESVVSLTSQCSYSSTIVHVGDKKPQPELETVEDMASGPESLDGAA-----GGLS 706
                     :********.************************** *** ********  *      ***
                                                736
SP|O15055|PER2_HUMAN QEKEPFKKLGLTKEVLAAHTQKEEQSFLQKFKEIRKLSIFQSHCHYYLQERSKGQPSERT 774
SP|O54943|PER2_MOUSE QEKGPLQKLGLTKEVLAAHTQKEEQGFLQRFREVSRLSALQAHCQNYLQERSRAQASD-- 764
                     *** *::*******************.***:*:*: :** :*:**: ******:.* *:
SP|O15055|PER2_HUMAN APGLRNTSGIDSPWKKTGKNRKLKSKRVKPRDSSESTGSGGPVSARPPLVGLNATAWSPS 834
SP|O54943|PER2_MOUSE -RGLRNTSGLESSWKKTGKNRKLKSKRVKTRDSSESTGSGGPVSHRPPLMGLNATAWSPS 823
                     *******::* **************** ************** ****:**********
SP|O15055|PER2_HUMAN DTSQSSCPAVPFPAPVPAAYSLPVFPAP------GTVAAPPAPPHASFTVPAVPVDLQHQ 888
SP|O54943|PER2_MOUSE DTSQSSCPSAPFPTAVP-AYPLPVFQAPGIVSTPGTVVAPPAATHTGFTMPVVPMGTQPE 882
                     ********:.***: ** ** **** **      ***.****  *:.**:*.**:. * :
SP|O15055|PER2_HUMAN FAVQPPPFPAPLAPVMAFMLPSYSFPSGTPNLPQAFFPSQPQFPSHPTLTSEMASASQPE 948
SP|O54943|PER2_MOUSE FAVQPLPFAAPLAPVMAFMLPSYPFPPATPNLPQAFLPSQPHFPAHPTLASEITPASQAE 942
                     ***** ** ************** ** .********:****:**:****:**:: *** *
SP|O15055|PER2_HUMAN FPSRTSIPRQPCACPATRATPPSAMGRASPPLFQSRSSSPLQLNLLQLEEAPEGGTGAMG 1008
SP|O54943|PER2_MOUSE FPSRTSTLRQPCACPVTPPAGTVALGRASPPLFQSRGSSPLQLNLLQLEEAPEGSTGAAG 1002
                       ******  *******.*  :   *:***********.*****************.*** *
SP|O15055|PER2_HUMAN TTGATETAAVGADCKPGTSRDQQPKAPLTRDEPSDTQNSDALSTSSGLLNLLLNEDLCSA 1068
SP|O54943|PER2_MOUSE TLGTTGTAASGLDCTSGTSRDRQPKAPPTCNEPSDTQNSDAISTSSDLLNLLLGEDLCSA 1062
                     * *:* *** * **. *****:***** * :***********:****.******.******
SP|O15055|PER2_HUMAN SGSA--------ASESLGSGSLGCDASPSGAGSSDTSHTSKYFGSIDSSENNHKAKMNTG 1120
SP|O54943|PER2_MOUSE TGSALSRSGASATSDSLGSSSLGFGTSQSGAGSSDTSHTSKYFGSIDSSENNHKAKMIPD 1122
                     :***        :*:****.*** .:* ******************************  .
                                                          1163
SP|O15055|PER2_HUMAN MEESEHFIKCVLQDPIWLLMADADSSVMMTYQLPSRNLEAVLKEDREKLKLLQKLQPRFT 1180
SP|O54943|PER2_MOUSE TEESEQFIKYVLQDPIWLLMANTDDSIMMTYQLPSRDLQAVLKEDQEKLKLLQRSQPRFT 1182
                       ****:*** ***********::*.*:*********:*:******:*******: *****
SP|O15055|PER2_HUMAN ESQKQELREVHQWMQTGGLPAAIDVAECVYCENKEKGNICIPYEEDIPSLGLSEVSDTKE 1240
SP|O54943|PER2_MOUSE EGQRRELREVHPWVHTGGLPTAIDVTGCVYCESEEKGNICLPYEEDSPSPGLCDTSEAKE 1242
                     *.*::****** *::*****:****: *****.:******:***** ** **.:.*::**
SP|O15055|PER2_HUMAN DENGSPLNHRIEEQT 1255
SP|O54943|PER2_MOUSE EEGEQLTGPRIEAQT 1257
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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 S3A). 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 S3B). 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 2B). In addition, denatured-IP assays confirmed that the PER2 K736R mutation significantly decreased both SUMO1 and SUMO2 conjugation (Fig 2C, 2D). Moreover, the PER2 K736R mutation eliminated the band shift (Fig. 2E, lane 4 vs. lane 3) that was at the same position as the eliminated shift resulting from co-expression of SUMO1 with SENP1 (Fig 2E, lane 2 vs. lane 3). This further confirmed that PER2 could be SUMOylated at K736. 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 (Eide et al., 2005; Reischl et al., 2007). 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 S4). This endogenous PER2 K736R mutant had disrupted rhythmic oscillation at different CT after serum shock compared to the rhythmic oscillation of PER2 WT (Fig. 2G). PER2 WT protein level was high at CT5 and CT21 but low at CT13 and CT17. In contrast, PER2 K736R gradually decreased during

Figure S3

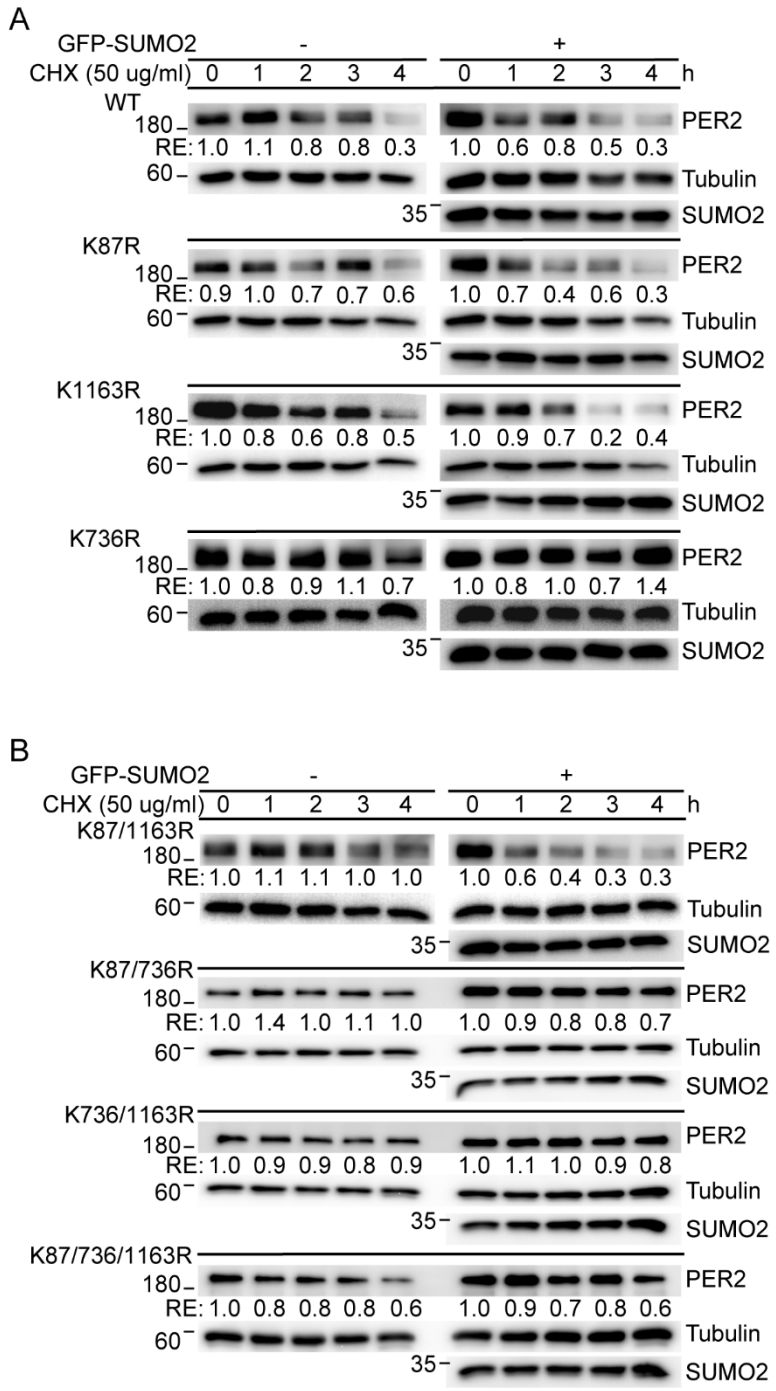

Figure S3. Lysine residue K736 is a critical site for PER2 SUMOylation

A. Time course assay using CHX treated HEK-293T cells co-transfected with K-to-R single-site mutated MYC-tagged PER2 with or without GFP-tagged SUMO2. The levels of PER2 and SUMO2 were determined using immunoblotting. Tubulin was used as a loading controls. RE: relative expression.

B. Time course assays using CHX treated HEK-293T cells co-transfected with K-to-R double- or triple-site mutated MYC-tagged PER2 with or without GFP-tagged SUMO2. The levels of PER2 and SUMO2 were determined using immunoblotting. Tubulin was used as a loading controls. RE: relative expression.

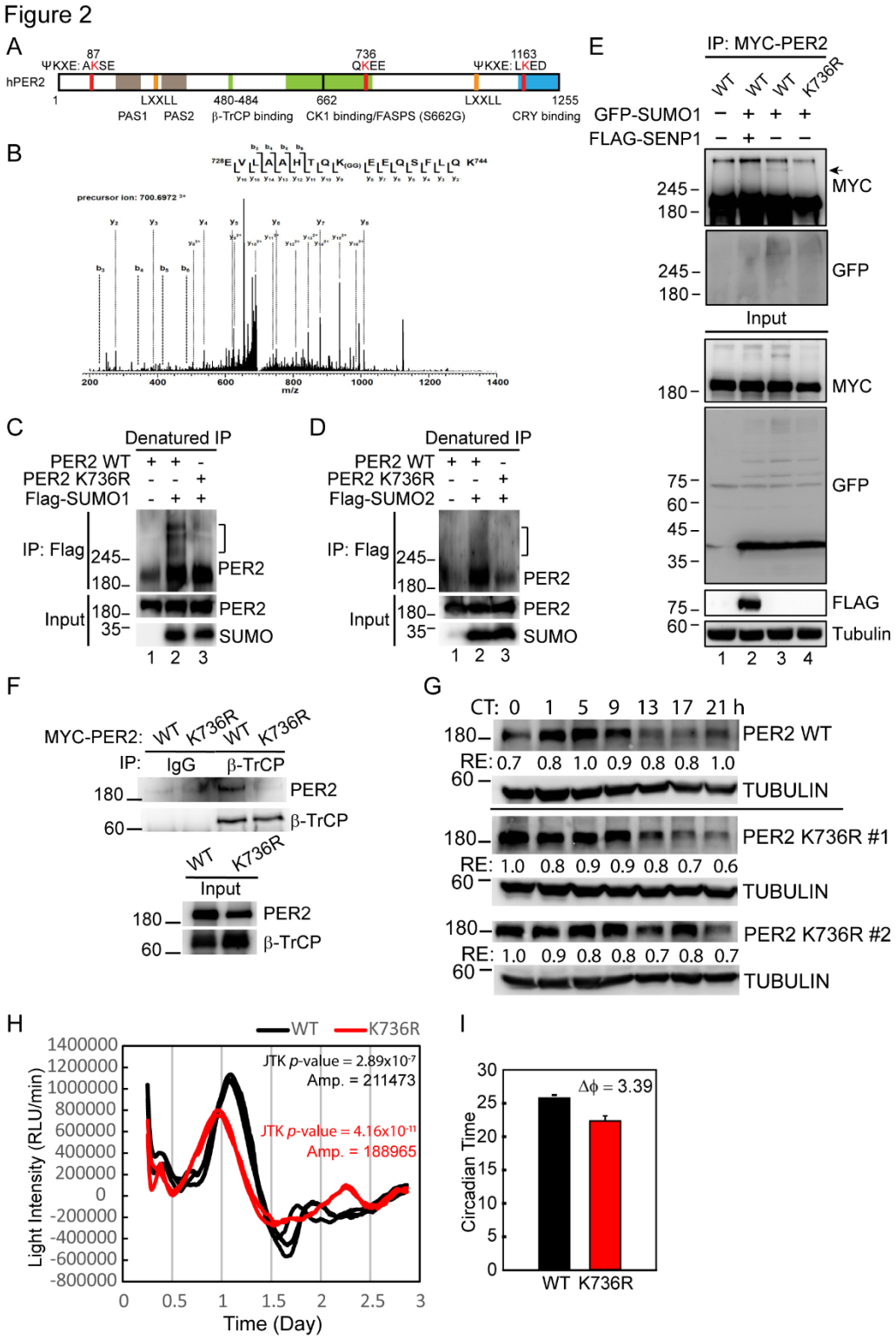

Figure 2. Lysine 736 is important for PER2 SUMOylation and required to maintain correct circadian period; however, modification by SUMO1 and SUMO2 leads to different PER2 protein fate.

A. 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), β-TrCP binding, Per-Arnt-Sim (PAS) domains (brown) and a CRY binding domain (blue) are also indicated.

B. 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.

C, D. 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.

E. Co-IP assay using HEK-293T cells co-transfected with MYC-PER2, GFP-SUMOs and FLAG-SENP1. Cell lysates were IP with anti-MYC antibody. Immunoprecipitates and input lysates were analyzed by IB. Arrow indicates 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. Time course assay using synchronized HEK-293T cells with or without CRISPR/CAS-edited PER2^{K736R} knock-in. After 50% serum shock, total protein was extracted at the indicated circadian time (CT). The levels of endogenous PER2 and SUMOs were determined using IB analysis. Tubulin was used as a loading control. RE: relative expression.

H. 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

I. Phase shift ($\Delta\phi$) 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.

All experiments were performed at least twice with similar results.

Figure S4

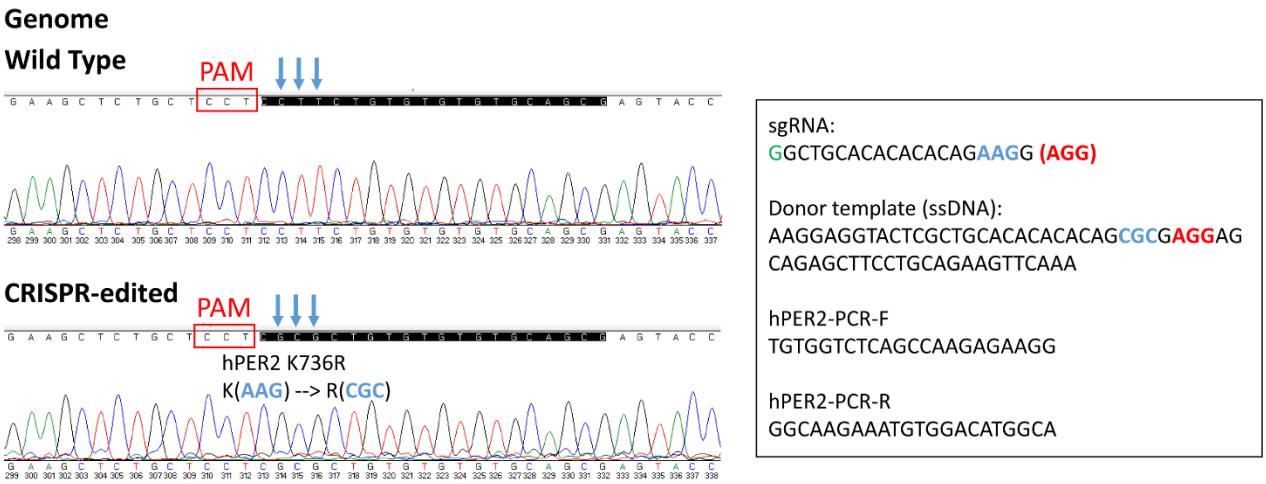

Figure S4. Generation of PER2K736R knock-in mutant HEK-293T cells. The WT and K736R PER2 sequences, 5 -mismatched G of PER2 sgRNA, donor template and PCR primers are shown.

the time course and did not oscillate. 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 2H, 2I). 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 destabilize 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 (Vanselow et al., 2006; Y. Xu et al., 2007). 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 (Eide et al., 2005; Hirota et al., 2010; H. M. Lee et al., 2011; Shanware et al., 2011; Tsuchiya et al., 2009). Two functional CK1 targeted phosphorylation sites in human PER2, S480 (S478 in mPer2) and S662 (S659 in mPer2), have been identified (Eide et al., 2005; Vanselow et al., 2006). Comparing our data with previous results, it was intriguing to note that PER2 K736-SUMO1 conjugation and S662 phosphorylation both led to increased PER2 nuclear retention (Fig 3A) (Vanselow et al., 2006). Conversely, K736-SUMO2 conjugation and S480 phosphorylation both promoted PER2 ubiquitination and proteasomal degradation (Fig 1D, S1A, S1B) (Eide et al., 2005; Reischl et al., 2007). Inhibition of CK1 using the CK1δ/ε inhibitor PF670462 led to reduced PER2 S662 phosphorylation but did not affect PER2 SUMOylation (Fig 3B), indicating that PER2 SUMOylation does not depend

Figure 3

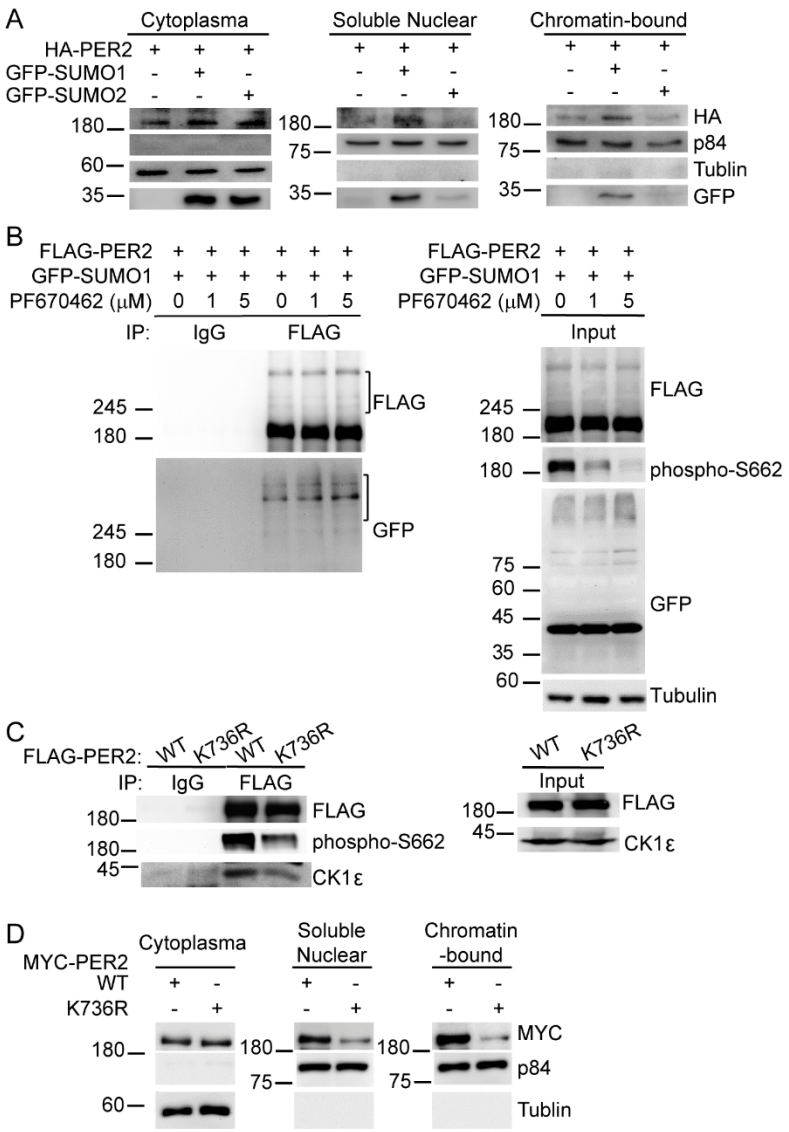

Figure 3. PER2 K736-dependent SUMO1 conjugation is a signal for CK1-mediated phosphorylation and translocation into the nucleus.

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.

upon PER2 phosphorylation. However, $PER2^{K736R}$ had reduced S662-phosphorylation and 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 can 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 interaction 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 (Melchior, 2000; Muller, Hoege, Pyrowolakis, & Jentsch, 2001). 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 to mediate SUMO1 versus SUMO2 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 (Gareau & Lima, 2010; Pichler, Gast, Seeler, Dejean, & Melchior, 2002), 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 (Chaves et al., 2006; K. Miyazaki, Mesaki, & Ishida, 2001; Ollinger et al., 2014; Vanselow et al., 2006) and interaction of the

PER2/CRY/CKI complex with GAPVD1, a Rab GTPase guanine nucleotide exchange factor involved in endocytosis and cytoplasmic trafficking (Aryal et al., 2017). 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 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 (Akashi & Takumi, 2005; Preitner et al., 2002). 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 results further indicate that PER2 modification by SUMO1 is critical for its transcriptional suppressor function in regulating circadian genes.

Discussion

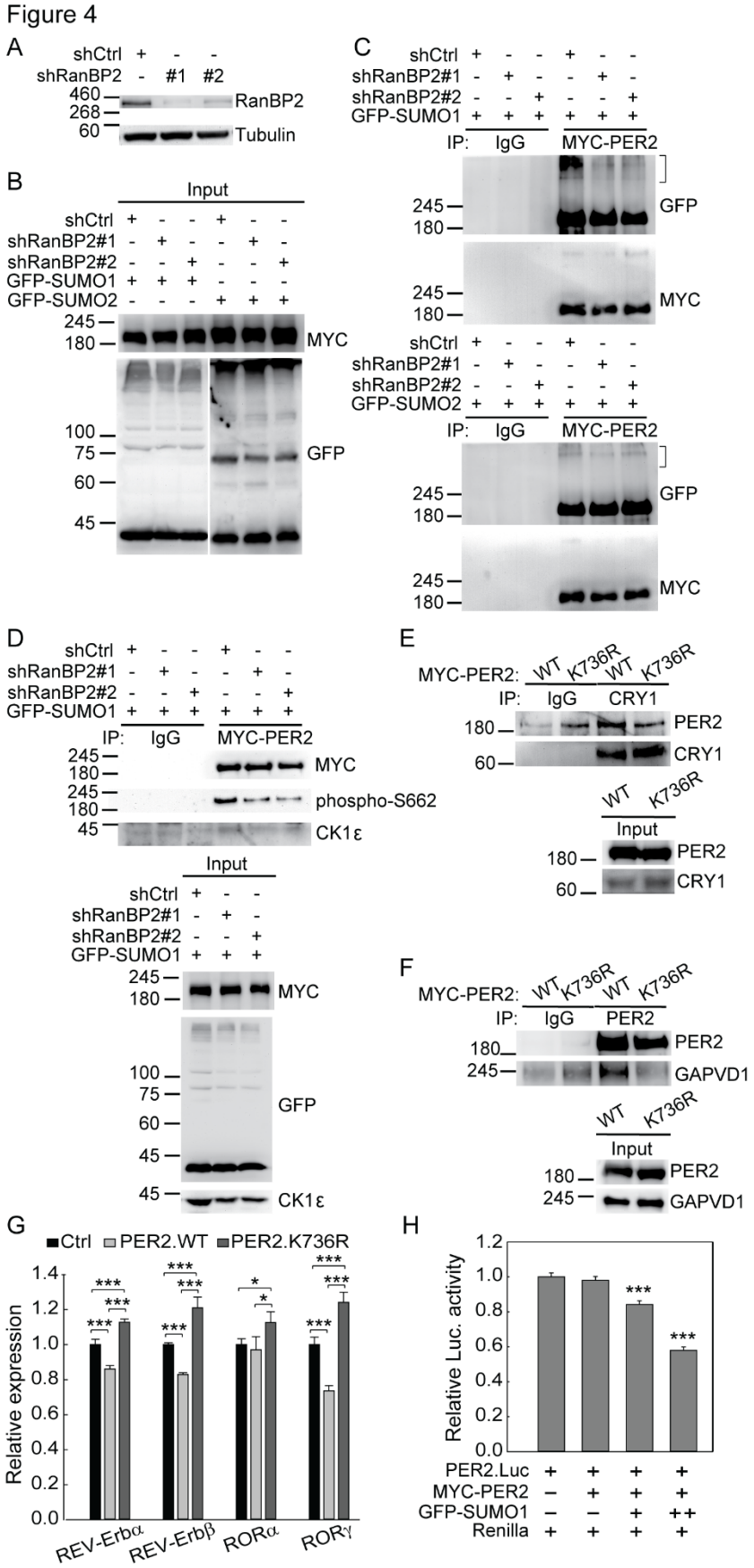

Figure 4. PER2 SUMO1 conjugation mediated by RanBP2 promotes CK1 phosphorylation of PER2 S662 and CRY1/GAPVD1 interactions required for PER2 nuclear translocation and PER2-mediated transcriptional suppression.

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.

Even for a relatively well studied protein like PER2, interaction and cross regulation between PTMs that control protein function can be complex and not well understood. PER2 has a number of well-known and critically important PTMs, particularly the CK1δ/ε-regulated phosphoswitch between two competing phosphorylation sites, S662 and S480, which determines PER2 stability (Fustin et al., 2018; Narasimamurthy et al., 2018; Zhou, Kim, Eng, Forger, & Virshup, 2015) and ubiquitination (Eide et al., 2005). Regulation of PER2 protein stability and subcellular localization is the key process determining the period of circadian clock. However, the signals that influence the priming to determine which site to be phosphorylated remain an important question in the field.

Our data indicate that, compared to other well-established PER2 PTMs, SUMOylation has an equally large role in determining PER2 function and in maintaining correct circadian periodicity. SUMOylation acts upstream of PER2 S662 phosphorylation which is clinically important in FASPS. Consistent with previous findings that S662 phosphorylation mainly promotes PER2 protein nuclear retention and stabilization (Vanselow et al., 2006), our data demonstrated that RanBP2-mediated PER2-SUMO1 conjugation is critical for CK1-mediated PER2 phosphorylation at S662 to promote PER2 nuclear entry. We also found that PER2-SUMO2 conjugation, in line with S480-phosphorylation (the β–TrCP-phosphodegron) mediated PER2 degradation (Eide et al., 2005), facilitated PER2 ubiquitination and degradation. Taken together, our data indicate that PER2 SUMOylation can be a primary PTM that controls downstream PTMs (phosphorylation and ubiquitination) to determine PER2 function.

Recent evidence indicates that many SUMO targets can be modified by both SUMO1 and SUMO2/3 (Becker et al., 2013). 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 (Gong et al., 2014). Another possibility is that SUMO1 and SUMO2/3 may have different conjugation site preferences (Becker et al., 2013; Impens, Radoshevich, Cossart, & Ribet, 2014). In the case of PER2, K736 is important for both SUMO1 and

SUMO2 conjugation (Fig 2C-2F). 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. In addition to its circadian role, PER2 exhibits tumor suppressive functions as a transcriptional suppressor in many cancers (Fu, Pelicano, Liu, Huang, & Lee, 2002; Hwang-Verslues et al., 2013; Papagiannakopoulos et al., 2016). Suppression of PER2 at both gene expression and protein levels facilitates tumor growth, invasion and cancer malignancy (Chen et al., 2005; Hwang-Verslues et al., 2013; Winter, Bosnoyan-Collins, Pinnaduwage, & Andrulis, 2007; Yu et al., 2018). Since PER2 protein level oscillates and PER2 shuttles between nucleus and cytoplasm, its protein stability and subcellular localization can also determine its tumor suppression function. Importantly, SUMO pathways are often dysregulated in cancers (Seeler & Dejean, 2017). Such dysregulation could alter PER2-SUMO conjugation and subsequently inhibit PER2 tumor suppression function by either facilitating its degradation or inhibiting its nuclear entry. Thus, SUMO1 versus SUMO2 conjugation forms a critical crossroads controlling the PER2 fate and influences PER2 activities which extend beyond core circadian regulation. 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 and health more broadly.

Materials and Methods

Figure 5

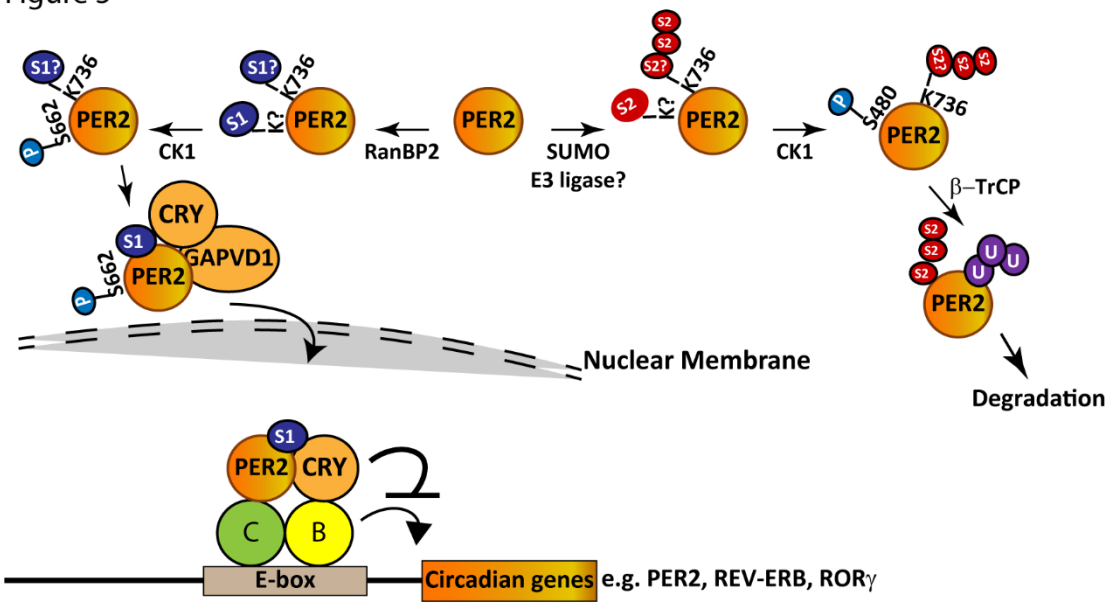

Figure 5. Diagram of the proposed mechanism by which SUMOylation regulates PER2 protein stability and transcriptional suppression function. PER2 K736 is important for PER2 SUMOylation by multiple SUMO isoforms. SUMO2 facilitates PER2 interaction with β-TrCP, ubiquitination and degradation. In contrast, 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.

Plasmids and reagents pcDNA3.1Myc-hPER2 plasmid was generously provided by Dr. Randal Tibbetts (University of Wisconsin, USA) (Shanware et al., 2011). 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, Santa Clara, CA) with primer sets listed in Table S1. Depending on the experiments, these cDNAs were sub-cloned into various tagged vectors including pcDNA3.0.HA (Thermo Fisher Scientific, Waltham, MA), 3XFLAG-CMV-7.1-2 (Sigma-Aldrich, St. Louis, MO) and pcDNA3.1.Myc.HisA/pcDNA3.1.Myc.HisC (Thermo Fisher Scientific) for transient expression in mammalian cells. pGL3.PER2.Luc promoter (-948~+424) was generated as previously described (Yu et al., 2018). 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, Madison, Wisconsin). Transient transfection was done by Mirus TransIT-LT1 Reagent (Mirus Bio, Madison, WI) 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 (Minneapolis, MN).

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 (Thermo Fisher Scientific) 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

1 mutation.

2 ***RNA isolation, reverse transcription and real-time (q)PCR*** Total RNA from cell culture was
3 isolated using Trizol reagent or RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed with
4 Quant iNova Reverse Transcription kit (Qiagen). qPCR was performed using Quant iNova SYBR
5 Green PCR kit (Qiagen) and primer sets for gene expression and analyzed on a Applied Biosystems
6 7300 Real-Time PCR System (Thermo Fisher Scientific). Primers used in this study were listed in
7 Table S2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal
8 control for mRNA expression. Expression levels were calculated according to the relative ΔCt
9 method.

10 ***Co-immunoprecipitation (Co-IP)*** Whole-cell lysates were prepared using a TNE lysis buffer (10
11 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 10 μM MG132, 1 mM PMSF, 10
12 mM NEM and 1x protease inhibitors) followed by sonication and centrifugation at 12,000 × g at 4°C.
13 1 ml of the crude whole-cell extract was incubated with 1-2 μg of antibody as indicated or control IgG
14 antibodies at 4°C overnight. Then, 50 μl prewashed protein A/G agarose was added to the mixture
15 and incubated at 4°C for 4 h with gentle agitation. After extensive washing with diluted NP-40 lysis
16 buffer (0.1-0.5% NP-40), PER2 interacting proteins were eluted with SDS buffer and analyzed by
17 immunoblot. Antibodies and agarose used for PER2 IP were listed in Table S3.

18 ***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, Hercules, CA), 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, West Grove, PA). Signals were detected using Millipore Immobilon Western Chemiluminescent HRP Substrate (Merk Darmstadt, Germany). Primary antibodies used in this study are listed in 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 equipped with Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific) and nanoelectrospry ion source (New Objective, Inc., Woburn, MA). 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 (Balsalobre et al., 2000) for 2 h. Subsequently, the media was changed to recording media (Gibco phenol red free DMEM (Thermo Fisher Scientific), 400 μM D-Luciferin Potassium Salt (Goldbio, St Louis MO) and monitored via the use of a Kronos Dio luminometer (AB-2550, ATTO, Tokyo, Japan) 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 (Hughes, Hogenesch, & Kornacker, 2010; M. Miyazaki et al., 2011). 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.

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Competing interests: The authors declare that they have no conflict of interest.

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.

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Table S1. Primers for site-directed mutagenesis

K87R	5 -GATGGGTTGTGTTCAGATCTTGCCATCATCAGGCTAAA-3 3'-CTACCCAACACAAGTCTAGAACGGTAGTAGTCCGATTT-5'
K736R	5 - CTCGCTGCACACACACAGAGGGAGGAGCAG -3 3'-GAGCGACGTGTGTGTGTCTCCCTCCTCGTC-5'
K1163R	5 -TCTCTGTCCTCCCTCAAAACCGCTTCTAAATTTCGG-3 3'-AGAGACAGGAGGGAGTTTTGGCGAAGATTTAAAGCC-5'

Table S2. Primer sets for qPCR

GADPH	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG
PER2	Forward	GGATGCCCGCCAGAGTCCAGAT
	Reverse	TGTCCACTTTCGAAGACTGGTCGC
REV-Erbα	Forward	GACATGACGACCCTGGACTC
	Reverse	GCTGCCATTGGAGTTGTCAC
REV-Erbβ	Forward	GTTACCTGTGCAACACTGGAGG
	Reverse	ACTGCTGGAGTGAAGCTCATCG
RORα	Forward	CACCAGCATCAGGCTTCTTTCC
	Reverse	GTATTGGCAGGTTTCCAGATGCG
RORγ	Forward	CAGCGCTCCAACATCTTCT
	Reverse	CCACATCTCCCACATGGACT

Table S3. Antibodies

Antibody	Catalog number	Dilution	Company
CRY1	SC-101006	1:1000	Santa Cruze
CK1ε	12448	1:1000	Cell signaling
GFP	2555S	1:1000	Cell signaling
GAPex5 (GAPVD1)	A302-116A	1:1000	Bethyl
PER2(H90)	SC-25363	1:1000	Santa Cruze
PER2 (phosphor S662)	Ab206377	1:1000	Abcam
RanBP2	Ab64276	1:1000	Abcam
SUMO1	PA5-34765	1:1000	Invitrogen
SUMO2	PA5-11373	1:1000	Thermo
β-TrCP	4394S	1:1000	Cell signaling
UB	ab7780	1:1000	Abcam
HA.11 epitope Tag	901503	1:1000	BioLegend
FLAG-M2	F3165	1:1000	Sigma
Myc-tag	ab9106	1:1000	Abcam
alpha tubulin	GTX628802	1:10000	GeneTex
Nuclear Matrix Protein p84	GTX70220	1:1000	GeneTex

For Co-IP assays, 1-2 ug per IP was used.