Title
Modeling of a human circadian mutation yields insights into clock regulation by PER2

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A Phosphorylation Cascade Regulating Circadian Period in Mammals

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One sentence summary: Modeling of a human *Per2* mutation provides direct *in vivo* demonstration that PER2 regulates circadian period in humans and defines a critical role for a phosphorylation cascade downstream of serine 662 in circadian period regulation.

Familial advanced sleep phase syndrome (FASPS) in humans manifests with extreme early sleep onset times and early morning awakening. We previously reported a missense mutation (S662G) in the Period 2 (PER2) protein causing FASPS in one family and resulting in hypophosphorylation *in vitro*. Mice transgenic for S662G recapitulate the short period FASPS phenotype seen in humans. Mice with a transgene encoding an acidic residue at position 662 showed period elongation. These and additional biochemical experiments demonstrate the charge on residue S662 is critical for a phosphorylation cascade of downstream serines that regulates circadian period. This represents direct *in vivo* demonstration that phosphorylation of PER2 regulates circadian period in mammals and defines a critical role for Per2 in circadian period length determination.

Many organisms have an internal timekeeping mechanism that precisely regulates 24-hour rhythms of body function and behavior and synchronizes them to the day-night cycle. Tremendous advances have been made in elucidating the function of clock genes by the combination of forward mutagenesis screening and gene targeting in flies and mice (*1-4*). Recently, two human clock gene mutations were found to cause FASPS (*5, 6*) and normal variants in human clock genes have been associated with human sleep time preference (*7-9*). Although many clock components are conserved across species, genetic, biochemical, and circadian system differences exist.

We previously identified a S662G mutation in hPER2 causing FASPS. Individuals with this mutation are “morning larks” with a 4-6 hour advance of sleep,

temperature, and melatonin rhythms (*6, 10*). This protein was hypophosphorylated by casein kinase (CK) Iε *in vitro*. We hypothesize that phosphorylation of PER2 by CKIε is regulated by a priming phosphorylation event at residue 662. Replacement of S662 with an acidic residue (D or E) was sufficient to restore phosphorylation of PER2 by CKIε *in vitro*.

The serine at position 662 in hPER2 is the first of 5 serines spaced three amino acids apart. This SxxSxxSxxSxxS motif is highly conserved in mammalian PER proteins (*6*). We hypothesized that phosphorylation of S662 could facilitate the phophorylation of serines at amino acid positions 665, 668, 671, and 674 by CKI, thus regulating PER2 protein stability, nuclear translocation, and transcriptional repressor activity (*11-13*). To further test this idea, we carried out *in vitro* phosphorylation reactions with two peptides of the same sequence (encompassing residues 660-674 of PER2) either with or without a phosphate group covalently linked to S662 (Fig. 1A). *In vitro* kinase assays showed that the peptide with a phosphate covalently linked to the first serine is phosphorylated at other residues by CKI. The peptide without a phosphate at S662 is not phosphorylated by CKI (Fig. 1B). These data suggest that S662 is not phosphorylated by CKI and that a phosphate at S662 is required for CKI to phosphorylate other residues in the peptide. A quantitative assay showed that an additional 4 moles of phosphate were incorporated per mole of Pep-2P substrate (Fig. 1C). We then carried out phospho-amino acid analysis and showed that the threonine at position 667 and the tyrosine at position 672 are not targets of CKI (Fig. 1A,D). These results support the hypothesis that the phosphorylation of

S662 plays a critical role in the regulation of PER2 by CKI via a cascade of phosphorylations that requires a priming event at residue 662.

BAC transgenic mice were generated by introducing a point mutation encoding S662G in the hPER2 protein (*14*) (Fig. S1). The resulting BAC construct included the human promoter and a large flanking region (extending 45kb upstream) and was expected to contain all of the cis-acting regulatory elements needed to accurately mimic the steady state transcript levels found in humans. To further test our hypothesis *in vivo*, we also generated S662D BAC transgenic mice. The S662G and S662D transgenic mice were fertile, of normal weight and growth, and had no gross phenotypic or behavioral abnormalities. Copy number analysis showed that the S662G lines (867, 865) and the S662D lines (382, 405) contain 2–3 copies. The S662G (864) and S662D (390) lines contain one copy each of h*Per2* (Fig. S1). The expression levels were examined by semi-quantitative RT-PCR and no obvious differences were found among different lines. We selected lines 864 & 865 (S662G) and lines 382 & 405 (S662D) for further analysis.

Circadian function was assessed by monitoring locomotor activity (*14*). The transgenic mice were on a predominantly C57BL/6J background. Transgenic mice and transgene-negative littermates were individually housed in circadian wheel running cages maintained in a 12 h light/12 h dark (LD12:12) environment for 7 days to establish entrainment. Subsequently, the mice were switched to constant darkness (DD) for 21 days. These mice had grossly normal entrained behavioral rhythms under LD12:12. As expected, S662G mice displayed a short period (τ) in constant darkness (21.60 hr. +/- 0.32 hr., n=20), while the transgene-negative littermates had an average period of 23.70

hr. +/- 0.11 hr. (n=25, $p < 0.001$) (Fig. 2, Table S1). To exclude the possibility that these differences were caused by h*Per2* over-expression, we set up intercrosses between S662G transgenic mice. The resulting mice (with different transgene copy numbers) showed no obvious difference in circadian period when compared with the original S662G mice (21.60 hr. +/- 0.45 hr., n=10) and exhibited sustained circadian rhythms (Table S1). This suggests that increasing h*Per2* copy number has small to no effect on circadian period, at least for mice with transgene copy numbers of 4 or less. All mice showed robust circadian rhythmicity. These results demonstrate that the S662G mutation is responsible for human FASPS. In contrast, the S662D mice showed a significantly longer period (24.26 hr. +/- 0.39 hr., n=27).

To examine the effect of the transgenes without endogenous m*Per2* in the background, we crossed S662G and S662D transgenes onto a homozygous m$Per2^{ldc}$ (*15*) background (-/-, tg+). Comparisons among the different genotypes showed significant differences in each group (Fig. 2, Table S1). Mice homozygous for the m*Per2* null allele in a C57BL/6J background (F4) had a mean period similar to the wild-type (WT) control (23.60 +/- 0.59, n=8). Mice transgenic for S662G on the m$Per2^{ldc}$ background gave a period length of one hour shorter than mice with endogenous m*Per2* (20.67 vs. 21.60, Table S1). Mice transgenic for S662D on the m$Per2^{ldc}$ background showed a period almost half an hour longer than those on a WT background (24.70 vs. 24.26). Thus, we concluded that the changes of circadian period in S662G and S662D mice are due to the charge of amino acid 662.

Closer inspection of the activity of these mice in LD12:12 revealed a significantly earlier onset of activity in the S662G mice when compared with other genotypes (Fig. 3). To quantify this difference, the average activity profiles of mice with the different genotypes were analyzed. The S662G mice (with and without endogenous m*Per2*) showed an onset of activity around CT8 (Fig. 3A,B). This onset was followed by a gradual increase in wheel running activity, which reached a maximum level at CT12 (lights off). In contrast, the maximal activity level of the WT, S662D, S662D/m$Per2^{ldc}$, and m$Per2^{ldc}$ mice was reached immediately after the onset of activity at lights off (CT12) as described previously (Fig. 3) (*15*). Thus, the S662G mice not only exhibited an ~4 hour advance of activity onset, but also showed a gradual increase of activity which peaked at CT12. The offset of activity in the S662G mice was also advanced by ~4hrs (CT20) and declined gradually to the base level at CT24. This phenotype shares a compelling similarity with human FASPS subjects harboring S662G (*10*). We next tested S662G and S662G/m$Per2^{ldc}$ mice in LD10.5:10.5 for 7 days. These mice were able to resume the normal "on-off" activity patterns as observed in other control mice (Fig. 3 E,F) suggesting that early onset of activity on/off time is due to the shortened endogenous period length of S662G mice.

We examined the expression profiles of clock related genes (m/h*Per2*, m*Per3*, m*Cry1*, m*Bmal1*, and m*Clk*) in liver by Northern blot analysis to investigate the molecular mechanism underlying period alterations (*14*) (Fig. 4A). Mouse *Per2* expression peaked at CT9-12 in wild-type mice (*16*) at CT8 in S662G; and at CT14 in S662D. Human *Per2* also reached its highest expression level at CT8 in S662G and at

CT14 in S662D. The fact that the h*Per2* transgene shows circadian expression similar to the endogenous m*Per2* also indicates that our transgene contains all the genomic elements required for transcriptional regulation. While each gene showed its own temporal rhythmic cycle, they all followed the same altered pattern of peak expression time: S662G shifted forward and S662D shifted backward when compared to wild-type. Regardless of changes in the cycling schedule, *Bmal1* and *Clk* expression remain antiphase to *Per* and *Cry* in both S662G and S662D as in WT animals. The RNA oscillations of these clock genes are consistent with previous reports (*16-18*) and the expression levels of each gene were similar for WT, S662G, and S662D.

We next examined the expression pattern of h*Per2* in the SCN by *in situ* hybridization (*14*) at CT0, CT3, CT6, CT9, CT12, and CT15 (Fig. 4B). Mouse *Per2* expression reached highest levels at CT12 in wild-type mouse SCN (*17*). Human *Per2* mRNA showed highest expression at CT3 and declined to background level at CT12 in S662G, while it peaked at CT12 for S662D. The constitutive expression of h*Per2* in the hippocampus and piriform cortex was not detectably different in these mice, indicating that the alteration of h*Per2* in transgenic mice is specific to circadian regulation (*19*).

PER2 protein is phosphorylated rhythmically and, in wild-type mice, is phosphorylated maximally at CT15-18 (*16*). In transgenic mice, hPER2 was predominantly found in the nucleus as described previously for mPER2. To examine the level of phosphorylation of hPER2 protein in transgenic mice, we prepared liver protein extracts at different time points from S662G and S662D transgenic mice on a homozygous null background (*14*). The -/-, S662G+ mice showed a maximum

phosphorylation level at CT12 to CT16, whereas -/-, S662D+ mice maximized at CT20. Moreover, the maximum phosphorylation level for -/-, S662D+ is much higher than for -/-, S662G+ (Fig. 4C, D). Thus, the phosphorylation state of S662 appears to be the gate-keeper of this regulation. These data support the hypothesis that the level of phosphorylation on S662 regulates the circadian period (i.e. increasing phosphorylation correlates with longer periods).

Per2 has been established as an integral component of the rodent circadian clock. It forms one of the regulatory feedback loops with CRY proteins on their own expression. The indispensable role for *Per2* in phase delay responses (*20*) has recently been challenged (*21, 22*). The discovery of the S662G mutation causing human FASPS was the first evidence that *Per2* plays an essential role in the human circadian clock. This single amino acid change was enough to elicit a 4 hour phase advance in entrained humans. We previously showed that this mutation is located in a CKI binding domain of the PER proteins and that the mutated protein is hypophosphorylated by CKI. These proteins are regulated by CKI for their stability, nuclear translocation, and repressor activity (*13, 16, 23*). The abundance and phosphorylation level of these proteins also shows striking temporal regulation (*16*). We therefore contemplated the possibility that the PER2 protein is intricately regulated in the circadian system by phosphorylation of S662. Results of *in vitro* kinase assays with synthetic peptides directly support this notion since the unphosphorylated peptide could not be phosphorylated by CKI *in vitro*. This result also implies that the serine at amino acid 662 is phosphorylated by a kinase other than CKI *in vivo*, therefore adding another layer of regulation. CKIs phosporylate the

serine/threonine at the "S/T-X-X-S/T" motifs. Our phospho-amino acid analysis further supports this prediction with serines being the only amino acids that were phosphorylated in the synthetic peptides. The four serines that follow S662 at these consecutive motifs likely serve as CKI regulatory sites on PER2 as supported by the stoichiometry of phosphate incorporation.

Our S662G mouse showed a τ ~2 hours shorter than wild-type and, with the S662G on a homozygous knock-out background, the period was even shorter (~3 hr). Mice with the S662G transgene all showed a robust phase advance of activity rhythms regardless of the number of wild-type alleles (2 vs. 0). In contrast, the mice with the S662D transgene showed a longer τ (0.7 hr) that was further lengthened on a homozygous knock-out background (1.1 hr). Not surprisingly, the phenotype is more dramatic when there are no wild-type alleles. It is interesting that the homozygous m*Per2*^{ldc} mice that we used as controls remained rhythmic. This is different from the initial report (*15*) and is likely due to the different genetic background onto which it was crossed (C57BL/6J vs. SV129).

Consistent with the phenotype observed in human FASPS, S662G mice reproducibly started their wheel running activity before CT12 (lights off) in LD12:12. This finding is also congruent with the current understanding that *Per2* is important for phase delay in the mammalian clock. Malfunction or mis-regulation of Per2 would lead to the inability to delay the clock properly and, therefore, the phase-advanced activity phenotype.

The clock gene transcripts are expressed rhythmically and their proteins are regulated temporally (expression level and phosphorylation state) (*16*). Of all the clock genes that we examined (including *Per2*, *Per3*, *Cry1*, *Bmal1*, *Clk*), the transcript expression levels are all shifted in the same direction (advanced for S662G and delayed for S662D) when compared to wild-type. Moreover, the phosphorylation level of the PER2 protein is also shifted. This further supports the theory that all the regulatory circuits of circadian clocks are integrally interacting and that the *Per2* feedback loop drives the mammalian circadian clock.

We propose that the homeostasis of cellular *Per2*/PER2 is a critical determinant of circadian period length. Transgenic S662G PER2 is likely hypophosphorylated leading to accelerated accumulation of PER2 (because of decreased targeting for degradation) and/or decreased transcriptional repression activity, thus shortening the period.

Assuming that the promoters of the transgene and endogenous alleles are driving transcription at approximately equal levels, one or two copies of the S662G transgene on a wild-type background would lead to approximately 1/3 or 1/2 of the PER2 protein being mutant. Hypophosphorylation of mutant protein would lead to a τ shorter than wild-type. Eliminating the endogenous *Per2* (by crossing the transgene onto a null background) will lead to a further shortening of τ. Conversely, PER2 from the S662D transgene will be hyperphosphorylated (hence increasing rate of degradation and/or increasing its transcriptional repression activity), thus lengthening the period. These results provide the first direct demonstration that PER2 phosphorylation is an essential determinant of mammalian circadian period length.

Transgenic mice with a single copy each of h*Per2* S662G and wild-type m*Per2* had an average circadian τ that was significantly shorter than the one circadian τ (23.3 hours) so far reported from a heterozygous hPER2 S662G human (*10*). The relative endogenous periods of mice and humans carrying the same circadian mutation have not previously been reported and therefore the reasons for this discrepancy are not known with certainty. Possible explanations include molecular-genetic features unique to transgenic mice and differences in circadian physiology or experimental design. As one example of the latter we note that the one human hPER2 S662G circadian period measurement was obtained from a paradigm (*10*) that is different from the mouse experiments and considered by some to over-estimate τ (*24-26*). More comparisons of human and murine circadian phenotypes under the influence of the same human clock genes are needed to enhance the applicability of murine models to human circadian disorders.

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Figure Legends

Fig. 1. *In vitro* phosphorylation assay of synthetic peptides. (**A**) Phosphorylated (Pep-2P) and unphosphorylated (Pep-2U) peptides were used as substrates for the CKI *in vitro* assay. Another protein (Adenomatous polyposis coli, APC) with a similar SXXS motif was also studied. (**B**) A comparison of initial *in vitro* phosphorylation rates of Pep-2U and Pep-2P. (**C**) Stoichiometric analysis of *in vitro* phosphorylation of Pep-2P and APC2. The stoichiometry of phosphate incorporation is shown for each peptide as a function of time. (**D**) Phospho-amino acid analysis of Pep-2P, performed in duplicate. Both photographic (upper panel) and autoradiographic (lower panel) records are shown. Only the phospho-amino acid standards are at a sufficiently high concentration to be detected by ninhydrin staining in the photographic records. When the records are aligned with the aid of the markers at the bottom, phospho-serine is shown to be the only labeled phospho-amino acid in each case (a-phosphoserine, b-phosphothreonine, c-phosphotyrosine).

Fig. 2. Circadian phenotypes of transgenic mice. Left columns show locomotor activity recordings of mice with the following genotypes (top to bottom: S662G transgene, S662D transgene, WT, S662G/m*Per2*^{ldc}, S662D/m*Per2*^{ldc}). In this and subsequent figures, the alternating light and dark bars indicate the LD cycles. The corresponding period analysis for each activity record is shown in the right columns. Circadian time is plotted on the x-axis. Mice were entrained to LD12:12 for 7 to 10 days before release into DD.

Fig. 3. Activity patterns in LD12:12. For each animal, wheel activity was accumulated in 3 minute bins for ~7 days. Data from mice of each genotype were then averaged and plotted vs. light/dark cycles using Clocklab software (ActiMetrics, Evanston, IL). Shadows indicate standard error of the mean. The slope and commencing time of activity onset in S662G and S662G/m*Per2*^{ldc} mice are significantly different from WT and S662D mice. (**A**) S662G mice show activity phase advance of ~4 hours relative to WT (**B**) S662G/m*Per2*^{ldc} mice show activity phase advance of ~4 hours relative to m*Per2*^{ldc} (**C**) Activity onset is identical for S662D and WT mice and, (**D**) S662D/m*Per2*^{ldc} and m*Per2*^{ldc} mice. The activity onset is at 'lights out' for S662G (**E**) and S662G/m*Per2*^{ldc} (**F**) mice in LD10.5:10.5.

Fig. 4. Effects of the S662G and S662D mutations on transcription of circadian genes. (**A**) Northern blot analyses of circadian gene expression. RNAs were isolated from mouse livers at indicated time points on the second day of constant darkness. Actin mRNA and 18S were monitored as internal controls. (**B**) Circadian rhythm of h*Per2* mRNA expression in the SCN at indicated time points. h*Per2* expression is shown for S662G mice (top) and S662D mice (bottom). (**C**) hPER2 phosphorylation in S662G and S662D transgenic mice. The lower band represents unphosphorylated PER2. The upper band (arrows) is shifted as a result of phosphorylation. (**D**) Electrophoretic mobility shift of PER2 is due to phosphorylation. The liver extract from CT16 was subjected to immunoprecipitation by hPER2 antibody (*14*). Immune complexes were split into three

aliquots and incubated in the absence(-) or presence(+) of protein phosphatase (lamda ppase) and its inhibitor (Na_3VO_4).

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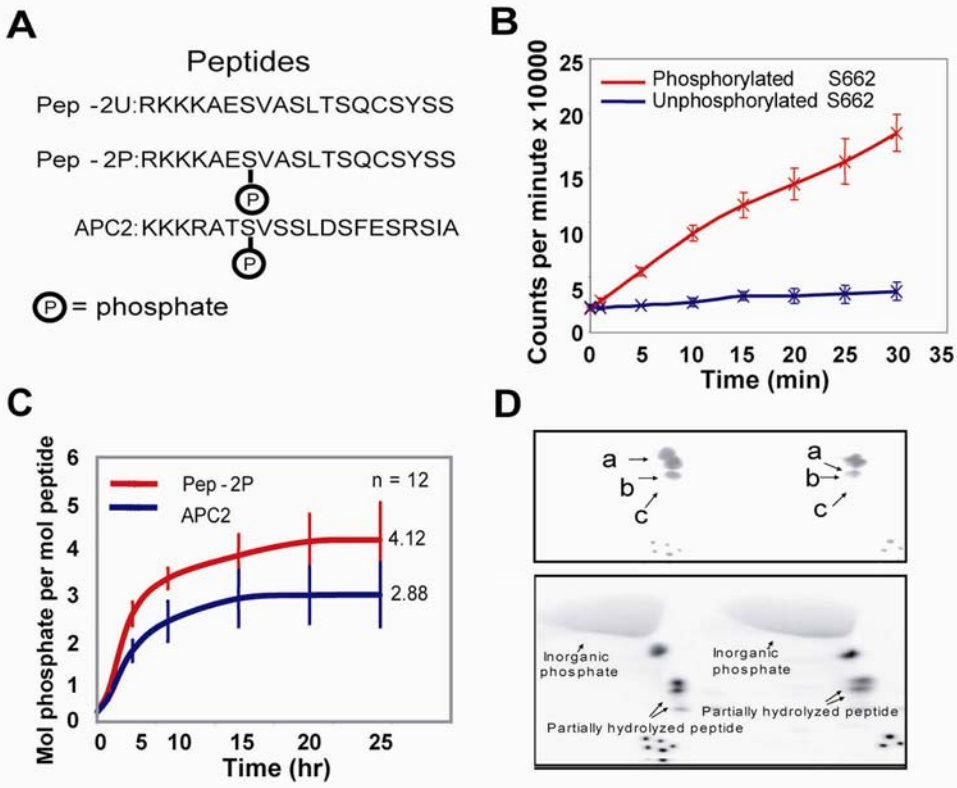
Xu Fig. 1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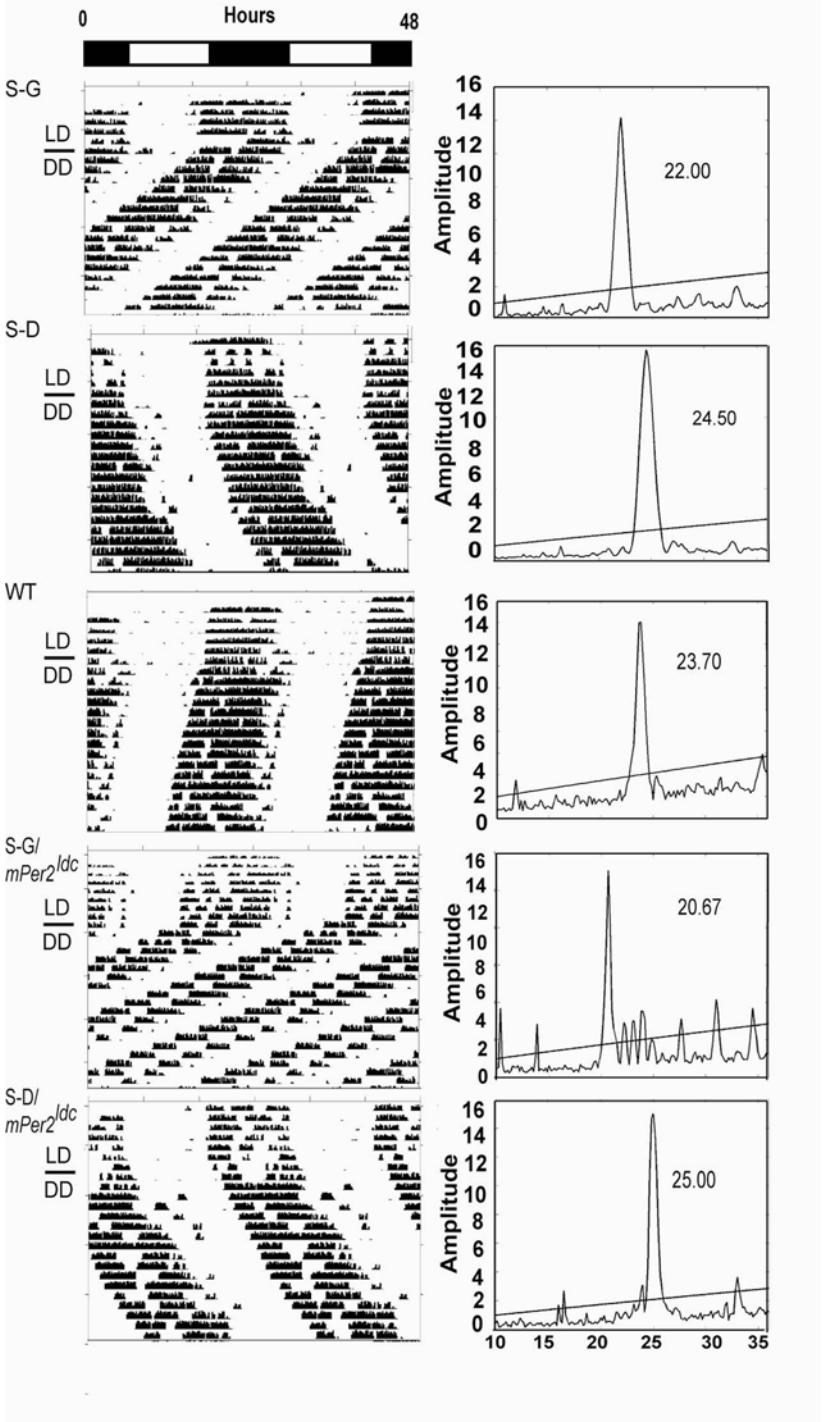
Xu Fig. 2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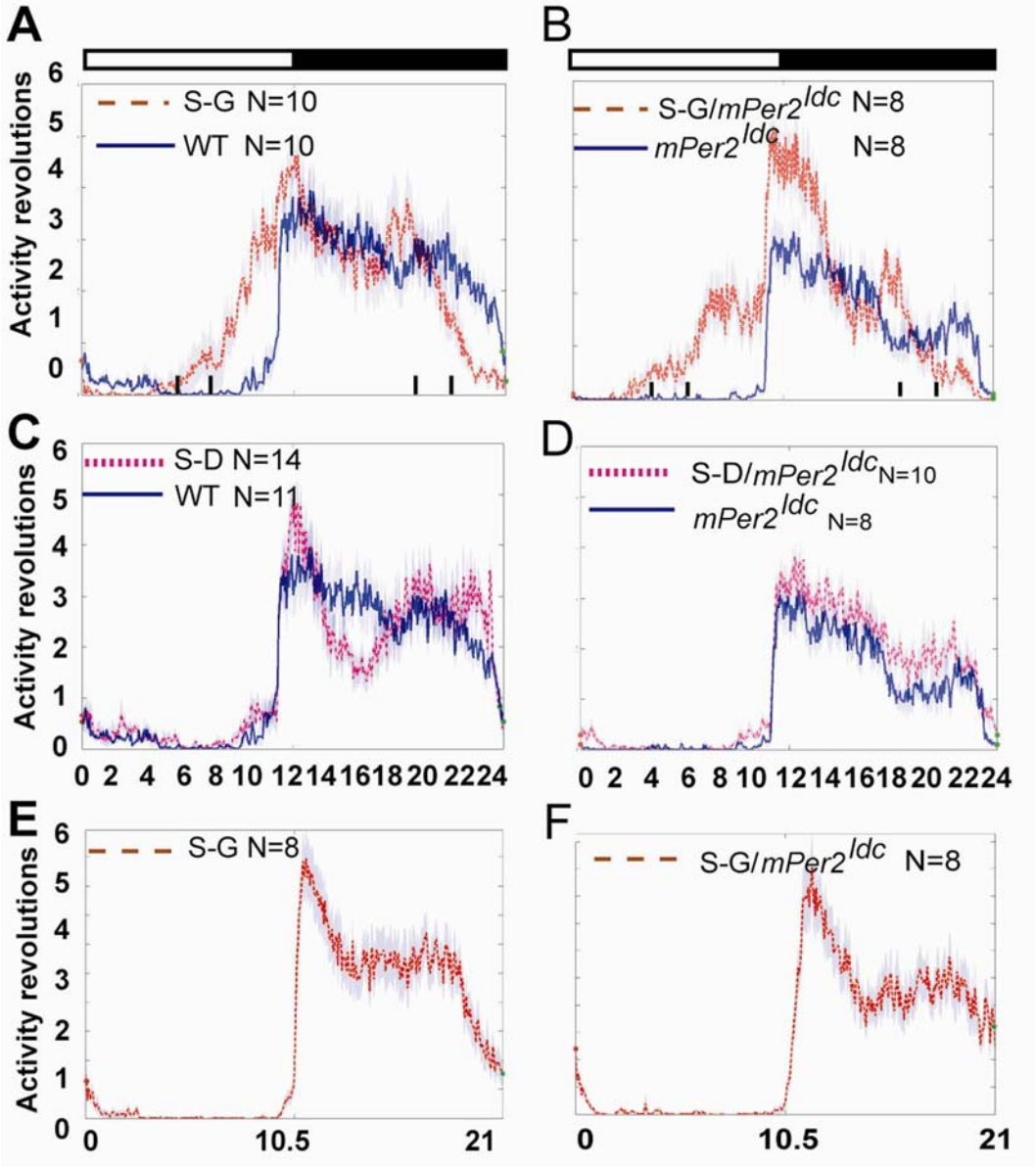
Xu Fig. 3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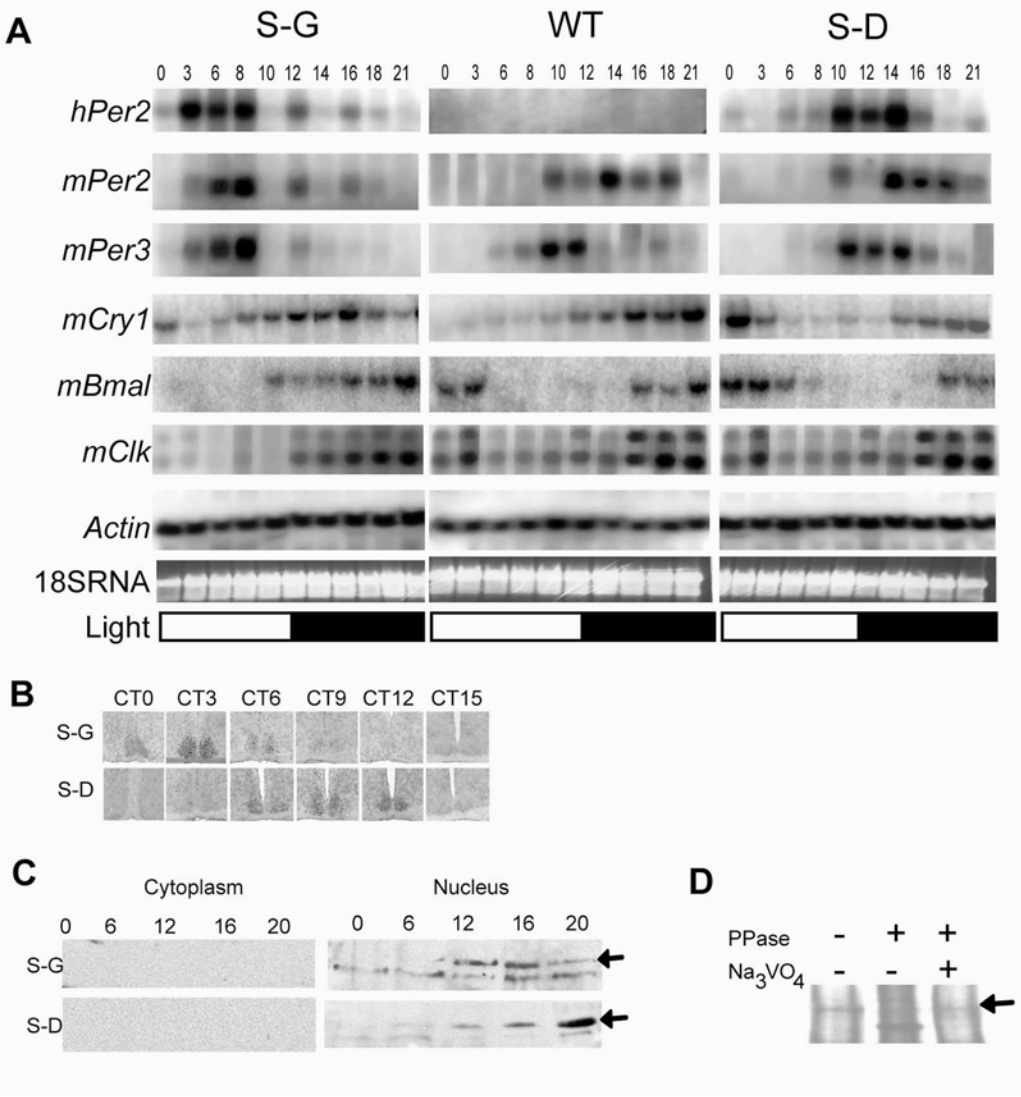
Xu Fig. 4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S-D
C
Cytoplasm
Nucleus
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S-G
S-D
D
PPase - + +
Na3VO4 - - +

Supporting Online Material:

Material and Methods

Generation of S662G and S662D mutant transgenic mice

RP 11-139K17 is a bacterial artificial chromosome (BAC) clone from the RPCI-11 human genomic library which contains the entire *Per2* locus on a 156-kb genomic insert (accession number AQ382142)(Children's Hospital Oakland Research Institute). RP11-139K17 665-S662G mutant BAC and 666-S662D mutant BAC were generated by ET-cloning as described elsewhere with modification {Narayanan, 1999}. BAC transgenic mice were generated by standard method. The transgenic founders were in a C57BL/6XSJL F1 background and were backcrossed to C57/BL6 in successive generations. Founders were determined by PCR using 6-pair primers every 15kb around this gene. Transgenic mice were identified by PCR analysis of tail biopsy DNA with primers 5'CTCTGACCGTAAGGATTTTCTGAT3' and 5'ACTGCAAATACAACTACCGTTTCA3'. The PCR protocol consisted of 3 min at 94°c, 35 cycles of amplification (each consisting of 30s at 94°C, 30s at 57°c and 90s st 72°c), and a final extension phase (10 min at 72°C) to produce a ~500bp fragment. We used F2 and F3 offspring from this backcross for analysis under an IACUC approved protocol.

Behavioral Analysis

Experiments were carried out with 2-4 month old mice and littermate controls were included. Activity was measured and stored on a computer (ClockLab, Actimetrics

Software). Period (τ) was determined by a chi-square periodogram. Activity profile was employed to evaluate onset and offset time.

Northern Blotting

Total RNA was extracted from liver at the indicated time using TRIzol (Invitrogen). Probes for h*Per2* (nt 10-410 of NM022817), m*Per2* (nt 10-620 of AF035830), m*Per3* (nt 1760-2310 of AF050182), m*Cry1* (nt 1090-1640 of AB000777), *Bmal* (nt 670-1470 of AF015203), and *Clock* (nt 1343-1831 of AF000998).

In Situ Hybridization

Animals were sacrificed by cervical dislocation under a 15W safety red light at indicated time points. Coronal brain sections through the SCN were processed for *in situ* hybridization with an antisense h*Per2* cRNA and hybridization steps were performed as described elsewhere {Lee, 2004}.

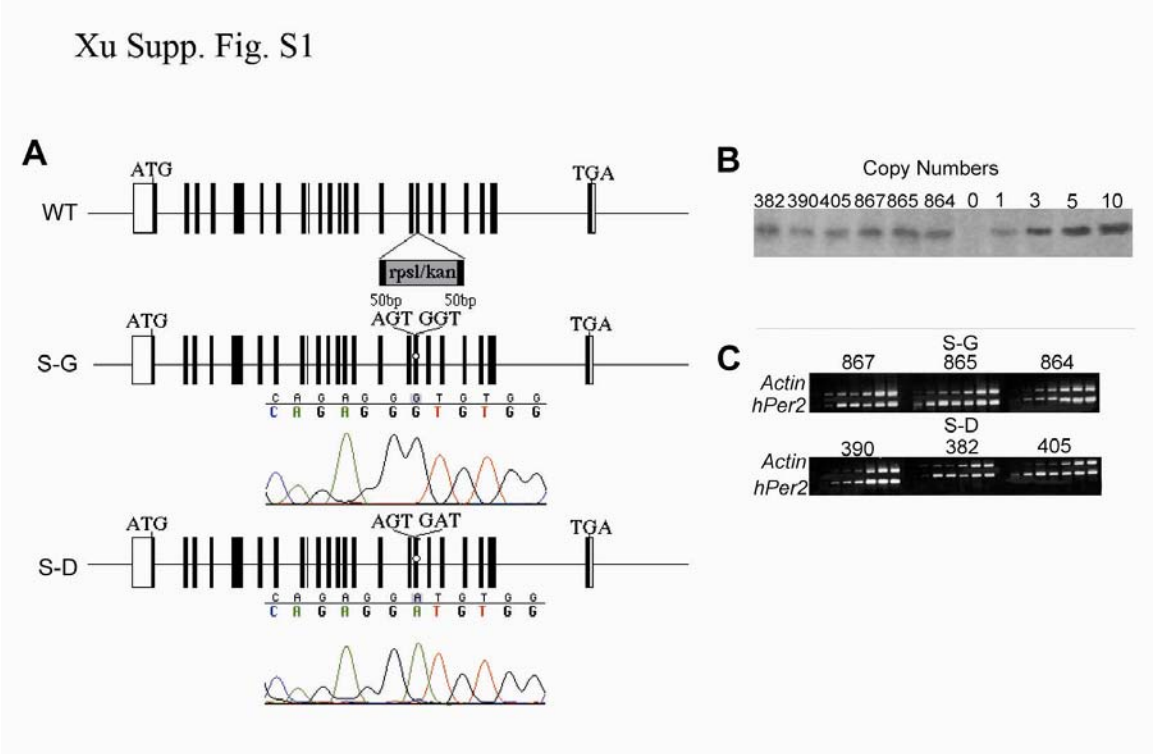
Western Blotting

Liver extracts from designated circadian times were fractionated (Active Motif) and the cytoplasmic and nuclear fractions were used to prepare Western blots {Miyazaki, 2004}. hPER2 antibody (Cosmo Bio, Japan), Vanadate (ACROS ORGANICS), and phosphatase (Promega) were used in these experiments.

Supplementary References:

Nawathean P., Rosbash M, *Mol Cell* **13**, 213-23

Lee C., Weaver D. R., Reppert S. M., *Mol Cell Biol* **24**, 584-94



Supp. Fig. S1. Generation of h*PER2* S662G and S662D transgenic mice. (**A**) Schematic diagram of the RP 11-139K17 BAC clone in the region surrounding the human *PER2* gene (WT), modified S662G (S662G) and S662D (S662D). (**B**) Southern blot analysis of copy numbers in S662G mice lines 864, 865, and 867 and SD mice lines 398, 384, and 405. (**C**) Estimation of *hPER2* expression levels in each transgenic mouse line by comparative RT-PCR amplifications. Serial dilutions of the cDNA were prepared from mice brain tissues and mRNA content in each sample was calibrated with β-actin.

Table S1. Period variation in transgenic mice.

Genotypes	N=	Period (Hours)	SD
S-G	20	21.60	0.32
S-D	27	24.26	0.39
WT	25	23.70	0.11
S-G/*mPer2*^{ldc}	8	20.67	0.09
S-D/*mPer2*^{ldc}	8	24.70	0.35
mPer2^{ldc}	8	23.60	0.59
S-G/S-G	10	21.60	0.45