

A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock

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Post-translational processes are essential for the generation and dynamics of mammalian circadian rhythms. In particular, phosphorylation of the key circadian protein PER2 precisely controls the period and phase of circadian oscillations. However, the mechanisms underlying that control are poorly understood. Here, we identified in a high-throughput RNAi-based genetic screen casein kinase 2 (CK2) as a PER2-phosphorylating kinase and novel component of the mammalian circadian clock. When CK2 subunits are silenced by RNAi or when CK2 activity is inhibited pharmacologically, circadian rhythms are disrupted. CK2 binds to PER2 in vivo, phosphorylates PER2 specifically at N-terminal residues in vitro, and supports normal nuclear PER2 accumulation. Mutation of CK2 phosphorylation sites decreases PER2 stability and copies CK2 inhibition regarding oscillation dynamics. We propose a new concept of how PER2 phosphorylation and stabilization can set the clock speed in opposite directions, dependent on the phase of action.

[*Keywords*: Circadian clock; casein kinase 2; period 2; RNAi screen; phosphorylation]

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Circadian rhythms in physiology, metabolism, and behavior are ubiquitous from cyanobacteria to humans (for review, see Young and Kay 2001). They are generated by endogenous clocks and entrained by environmental Zeitgebers (e.g., light, temperature, food), providing organisms with an internal representation of external time. Circadian clocks regulate a huge number of physiological processes, and interference with normal circadian rhythms by genetic or environmental perturbations likely has consequences for human health (for review, see Takahashi et al. 2008). The molecular basis of circadian clocks is cell-autonomous. In mammals, the machinery responsible for rhythm generation is present not only in neurons of the suprachiasmatic nucleus (SCN)—the master clock in the hypothalamus—but also in nearly every cell of the periphery, as well as in many standard tissue culture cell lines (Kowalska and Brown 2007).

In recent years, a number of components of the mammalian circadian clock have been identified. However, the intrinsic dynamics of their interaction generating molecular rhythms with a period close to 24 h are poorly

understood. The current model is based on a gene regulatory network with a negative delayed feedback loop as the essential core module (for review, see Lowrey and Takahashi 2004): The transcription factor heterodimer CLOCK/BMAL1 activates the expression of the *Period* (*PER1-3*) and *Cryptochrome* (*CRY1-2*) genes, whose protein products form a complex, accumulate, and, after a delay of several hours, translocate in the nucleus to inhibit the expression of their own genes. Subsequent degradation of PER and CRY proteins leads to a disinhibition of CLOCK/BMAL1, and the cycle can restart. In a second positive feedback loop, *Bmal1* transcription itself is oscillating through the rhythmic inhibition by the nuclear repressor REV-ERB α .

The dynamics of these networks are critically dependent on post-translational processes, with phosphorylation and dephosphorylation probably being most important (Harms et al. 2004; Gallego and Virshup 2007; Vanselow and Kramer 2007). Recently, it has been speculated that rhythmic phosphorylation and dephosphorylation may even comprise the heart of the circadian mechanism (Morrow et al. 2006). In mammals, most clock proteins are phosphorylated in vivo with a circadian variation in the degree of phosphorylation. Nevertheless, little is known about the dynamics of phosphorylation and dephosphorylation, about the number, location, and function of phosphorylation sites, as well as about the kinases and phosphatases involved. In fact, good genetic evidence

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exists only for *casein kinase 1 ϵ* and *1 δ* (CK1 ϵ/δ), which have been shown to phosphorylate and thereby destabilize PER proteins in vivo (Lowrey et al. 2000; Xu et al. 2005).

In a recent study by our laboratory (Vanselow et al. 2006), we found by extensive mass spectrometric screening and subsequent functional analyses that phosphorylation of the key circadian protein PER2 has at least two opposing effects: destabilization of PER2, but also its stabilization. Whereas destabilization of PER2 can be clearly attributed to CK1 ϵ/δ function, it is completely unknown which kinase(s) are required for PER2 stabilization. It is important to note that interfering with PER2 stabilization—e.g., by mutation of the respective phosphorylation sites—substantially changes circadian dynamics. For example, in humans suffering from one form of familial advanced sleep phase syndrome (FASPS), a phosphorylation site of PER2 is mutated, which results in an inhibition of normal PER2 stabilization (Toh et al. 2001; Vanselow et al. 2006). Thus, elucidating the processes that affect PER2 stability would be a major step toward an understanding of the complex, however largely unknown, post-translational control of circadian timing in mammals.

Here, we report a systematic effort to elucidate unknown processes modulating PER2 stability. Initial attempts to chemically cross-link kinases with phosphorylation site-mutated versions of PER2 and a mechanism-based cross-linker (Maly et al. 2004) were not successful. Therefore, we decided to set up an unbiased large-scale RNAi screen with a functional readout, because perturbing the activity of these kinases is predicted to have a strong effect on circadian dynamics. We individually knocked down all known and predicted kinases (as well as phosphatases and F-box proteins as additional regulators of protein stability) and analyzed the effect on oscillation dynamics. We identified casein kinase 2 (CK2) as a new and essential component of the mammalian circadian clock that interacts with and phosphorylates PER2. When CK2 subunits are down-regulated by RNAi or when CK2 activity is pharmacologically inhibited, circadian rhythms are disrupted, probably due to an attenuated nuclear accumulation of PER2. Using a comparative mass spectrometric approach, we found that CK2 phosphorylates N-terminal residues of PER2. Thereby, PER2 is stabilized, which we show is required for normal circadian rhythms. We propose a new concept of how phosphorylation of the same clock protein (PER2) can set the clock speed in opposite directions, dependent on its phase of action.

Results

RNAi-mediated silencing of clock genes phenocopies “classical” mouse genetics

Prerequisites for the success of our large-scale RNAi screen are robustness of circadian oscillation in the cell culture model, efficient RNAi-mediated knockdown, as well as circadian phenotypes similar to those of “classi-

cal” loss-of-function mouse models. We chose human osteosarcoma U-2 OS cells because, firstly, circadian rhythms of these cells can be robustly monitored in 96-well plates via ~7-d live-cell imaging of luciferase activity using a *Bmal1*-promoter fragment driving the expression of luciferase (Supplemental Fig. 1). Secondly, the RNAi library used in this study—a second-generation library of the Hannon-Elledge laboratories—was studied extensively and optimized in U-2 OS cells (Silva et al. 2005; Stegmeier et al. 2005, 2007; Draviam et al. 2007). MicroRNA-based RNAi constructs are lentivirally delivered and thus stably integrated into the genome, resulting in long-lasting gene knockdown.

For proof of concept, we first tested whether silencing known clock components (*Cry1*, *Cry2*, *Bmal1*, and *Clock*) phenocopies the effects observed in mouse knockout studies (Fig. 1; for quantification and statistics of all time series, see Supplemental Table 1). Down-regulating *Cry1* expression shortened the period and decreased the amplitude of the rhythms, whereas silencing *Cry2* lengthened the period, very similar to the behavioral and cellular phenotypes of corresponding mouse mutants (Vitaterna et al. 1999; van der Horst et al. 1999; Liu et al. 2007). Knockdown of *Bmal1* and *Clock* almost disrupted circadian rhythms completely, consistent with the arrhythmic behavioral and molecular phenotypes of *Bmal1*^{-/-} mice (Bunger et al. 2000; Liu et al. 2007) and the arrhythmic phenotype of *Clock*^{-/-} peripheral cells (DeBruyne et al. 2007). Thus, RNAi-mediated gene knockdown is well-suited to detect circadian phenotypes.

RNAi screen reveals many new candidate clock components

For identifying kinases and other components that regulate PER2 stability, 2660 RNAi constructs targeting 695 kinases or putative kinases, 146 phosphatases including regulatory subunits, as well as 64 F-box proteins were individually screened in high-throughput format for effects on circadian dynamics (Fig. 2A). 287 RNAi constructs led to period phenotypes or disrupted circadian rhythms. Due to potential off-target effects of RNAi constructs, we considered only those genes for further analysis for which at least two RNAi constructs resulted in a similar phenotype. Using these criteria, 22 kinases, seven phosphatases/regulatory subunits, and six F-box proteins were classified as candidate clock components (Fig. 2B).

Further support of the significance of our screen came from the fact that among the F-box candidates was *FBXL3*, recently reported to be involved in CRY protein degradation (Busino et al. 2007; Siepka et al. 2007). Its down-regulation lengthened the period up to 5 h (Supplemental Fig. 2), similar to phenotypes of hypomorphic mouse mutations (Godinho et al. 2007; Siepka et al. 2007). Silencing of another F-box gene, *FBXW11* (β -*TRCP2*), also lengthened the circadian period and reduced oscillation amplitude. Simultaneous knockdown of both β -*TRCP2* and its paralog β -*TRCP1*, previously reported to be involved in PER protein degradation (Eide et al. 2005; Shirogane et al. 2005; Reischl et al. 2007), led to a strongly

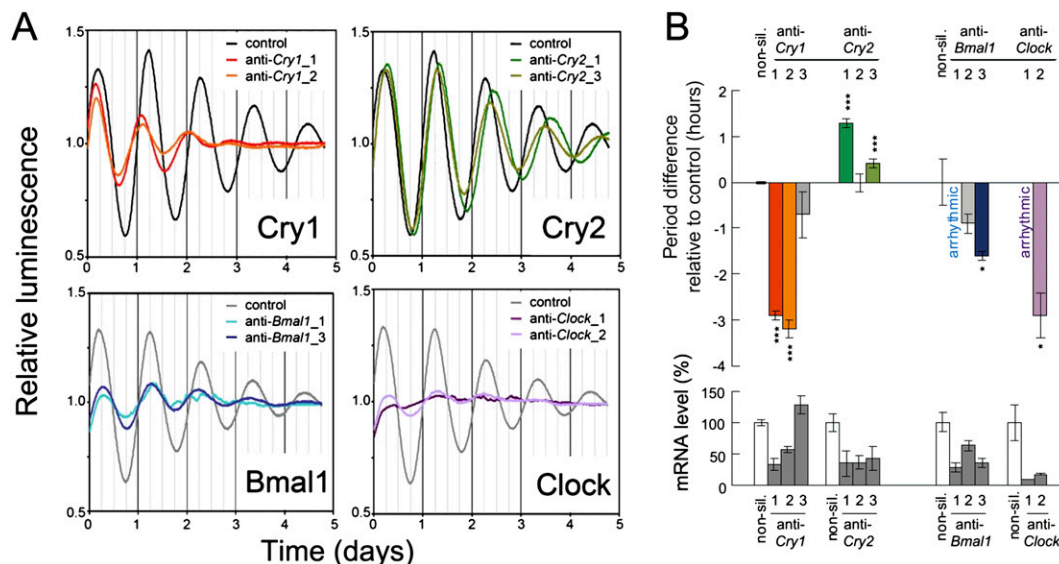


Figure 1. RNAi-based reverse genetics reproduces circadian phenotypes. (A) Human U-2 OS cells harboring a *Bmal1*-luciferase reporter were lentivirally transduced with RNAi constructs targeting the indicated transcripts, synchronized with dexamethasone, and luciferase activity was continuously monitored. Detrended average time series ($n = 2-3$) for two RNAi constructs and a nonsilencing control are given. (B) Quantification of circadian period and silencing efficiency. Error bars, mean \pm standard deviation. (***) $P < 0.001$, (*) $P < 0.01$ (t -test). See also Supplemental Table 1.

reduced amplitude, indicating that both proteins have partly redundant functions (Supplemental Fig. 2).

CK2 is an essential component of the mammalian circadian clock

One result of our screen, *CK2*, especially attracted our attention as a potential candidate for being (one of) the unknown PER2 phosphorylating kinase(s). While a function for *CK2* in the mammalian circadian clock has not yet been reported, *CK2* plays important roles in the circadian systems of *Arabidopsis* (Sugano et al. 1999), *Neurospora* (Yang et al. 2002; Dunlap et al. 2007), and *Drosophila* (for a review, see Allada and Meissner 2005). In *Drosophila*, phosphorylation of PER by CK2 is required for normal nuclear entry dynamics (Lin et al. 2002; Akten et al. 2003; Smith et al. 2008). In mammals, CK2 is an ubiquitously expressed kinase involved in many cellular processes with a large number of substrates (Litchfield 2003). The holoenzyme is a tetramer consisting of two catalytic subunits (α and/or α') and two regulatory β -subunits.

Down-regulation of both catalytic subunits *CK2 α* and *CK2 α'* as well as the regulatory subunit *CK2 β* lengthened circadian periods by a degree that is correlated with the silencing efficiency of the respective RNAi construct (Fig. 3A; Supplemental Fig. 3). Low-amplitude residual rhythmicity was observed when both *CK2* catalytic subunits were targeted (Supplemental Fig. 3).

Overexpression of *CK2 α* and *CK2 α'* had the opposite period effect, leading to a period shortening of ~ 2 h (Fig. 3B). This phenotype was dependent on the kinase activity, since overexpression of kinase-inactive dominant-negative versions of the catalytic subunits (Vilk et al. 1999) resulted in a period lengthening of ~ 1 h (Supplemental Fig. 3).

In addition to its abundance, the activity of CK2 is crucial for normal circadian rhythms, since pharmacological inhibition of CK2 with the specific inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT) (Pagano et al. 2004) dose-dependently lengthened circadian periods in lung explants of *Per2::Luc* knock-in mice (Fig. 3C; Yoo et al. 2004) in U-2 OS cells (Supplemental Fig. 4) as well as in murine NIH3T3 cells (data not shown).

CK2 binds to the clock protein PER2

The rhythmicity and expression levels of clock gene transcripts are differentially affected in *CK2 α /CK2 α' /CK2 β* triple-knockdown U-2 OS cells, as revealed by quantitative RT-PCR analysis (Fig. 4A). *Bmal1* and *Rev-Erba* transcript levels are arrhythmic and low in these cells, while damped rhythms persist for *Per1* and (to a lesser extent) also for *Per2* with a very low expression level and substantially altered phase for *Per1*. These molecular phenotypes suggest that the positive feedback loop is severely damaged upon *CK2* knockdown, and resemble those found in *Per2* mutant mice (Shearman et al. 2000; Oster et al. 2002).

Thus, we hypothesized that CK2 exerts its function in the mammalian clock on PER2. If PER2 is a substrate of CK2, a direct interaction of the two components is predicted. Indeed, coimmunoprecipitation (co-IP) assays with overexpressed and with endogenous proteins revealed that both *CK2 α* and *CK2 β* are in a complex with PER2 in tissue culture cells and in mouse liver (Fig. 4B; Supplemental Fig. 5), suggesting that PER2 may be a substrate for CK2 in vivo.

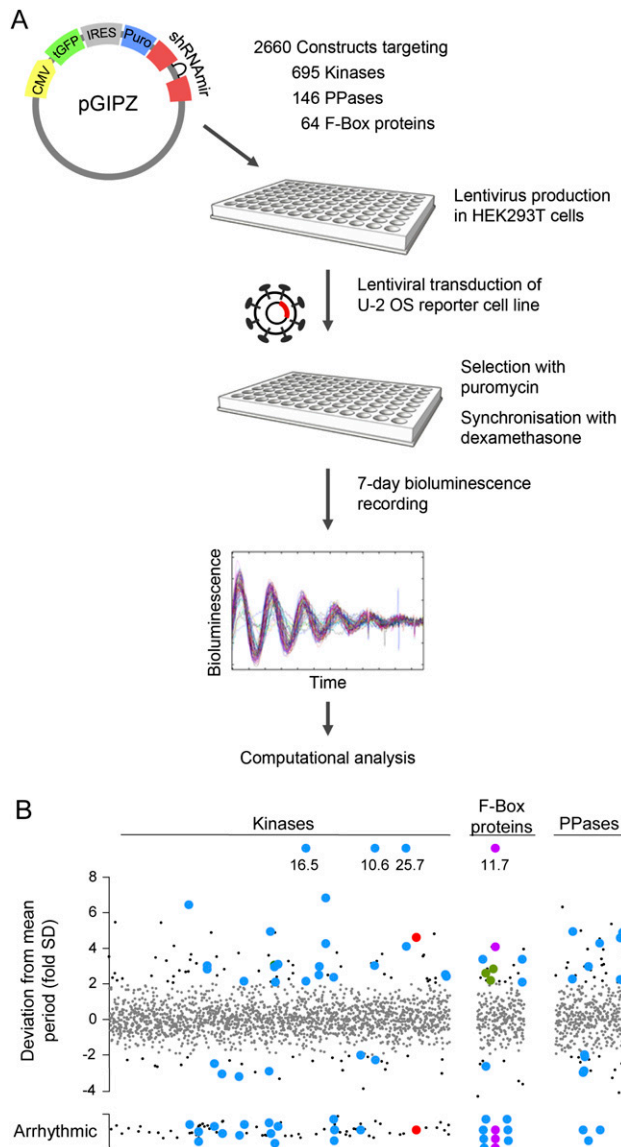


Figure 2. RNAi-based functional screen identifies new candidate clock components. (A) Schematic diagram of the large-scale RNAi-based screen for kinases, phosphatases, and F-box proteins required for normal circadian rhythms. For experimental details, see the Materials and Methods and the Supplemental Material. (B) Results of the screen described in A. Dots represent plate-normalized period values corresponding to an individual RNAi construct. Values within or outside a range of two standard deviations from the mean period are given as small gray or small black symbols, respectively. For hit evaluation details, see the text and the Supplemental Material. Among the 22 kinases, seven phosphatases, and six F-box proteins identified as potential clock genes (blue dots), we found *FBXL3* (purple), β -*TRCP2* (green), and *CK2 α* (red) disrupted circadian rhythmicity in the reporter cell line.

CK2 activity is necessary for PER2 accumulation

According to the current model (Lowrey and Takahashi 2004), PER and CRY proteins accumulate in the nucleus several hours after their synthesis to repress their own

transcription. If CK2-mediated phosphorylation of PER2 is required for protein accumulation, as has been shown in the *Drosophila* circadian system (Lin et al. 2002; Akten et al. 2003; Smith et al. 2008), interference with CK2 activity should alter nuclear accumulation. As an accurate readout for PER2 abundance, we exploited the fact that in (behaviorally normal) *Per2::Luc* knock-in mice (Yoo et al. 2004) endogenous PER2 is represented as a PER2-LUC fusion protein, and thus luciferase activity can be directly correlated with endogenous PER2 levels. We treated primary fibroblasts from *Per2::Luc* mice with the translation inhibitor cycloheximide (CHX) for 15 h to deplete the cells of endogenous PER2. After removal of CHX, nuclear accumulation of newly synthesized PER2 was followed by analyzing PER2-LUC activity in isolated cell nuclei as well as in the cytoplasm. Indeed, when CK2 activity is inhibited, both cytoplasmic and nuclear accumulation of PER2 is significantly attenuated (Fig. 4C). These results support the hypothesis that CK2 phosphorylation of PER2 early in the PER2 life cycle stabilizes PER2 and thus allows for timely cytoplasmic as well as eventually nuclear accumulation.

CK2 phosphorylates PER2 at N-terminal residues

Which residues of PER2 are phosphorylated by CK2? To map those, we used a comparative mass-spectrometric approach, which should (at least to a certain extent) circumvent the intrinsically limited specificity of *in vitro* phosphorylation assays. We compared the phosphorylation profiles of PER2 phosphorylated either by endogenous kinases, as described in our previous study (Vanselow et al. 2006), with those of CK2 holoenzyme, CK1 δ , or GSK-3 β phosphorylation *in vitro* (Table 1). Interestingly, most *in vivo* PER2 phosphorylation sites can be phosphorylated by at least two of the tested kinases *in vitro*. Only the very N-terminal region of PER2 (Ser10 to Thr15) is phosphorylated both *in vivo* and *in vitro* by CK2 but not by other kinases tested (Table 1; Fig. 5A).

Given these data, we hypothesize that this N-terminal region of PER2 may be a target site for CK2 activity. If this N-terminal region is stabilizing PER2 upon phosphorylation, mutation of phosphoacceptor sites by alanine is predicted to destabilize the protein. Indeed, when the four serine and threonine residues of this region (Ser10, Thr12, Ser13, and Thr15) are exchanged by alanine (PER2-mut), the stability of the mutant PER2 is substantially decreased (Fig. 5B), consistent with the hypothesis that CK2-mediated phosphorylation at the PER2 N terminus contributes to the stabilization of the protein.

*Mutation of *in vitro* defined CK2 phosphorylation sites phenocopies CK2 down-regulation*

Next, we tested whether a phosphorylation defect of PER2 at the N terminus might cause the disrupted rhythms and long periods, which we observe in cells with reduced CK2 level or activity. To ensure that potential differences in circadian dynamics are not artifacts due to chromosomal position effects or variation of integration

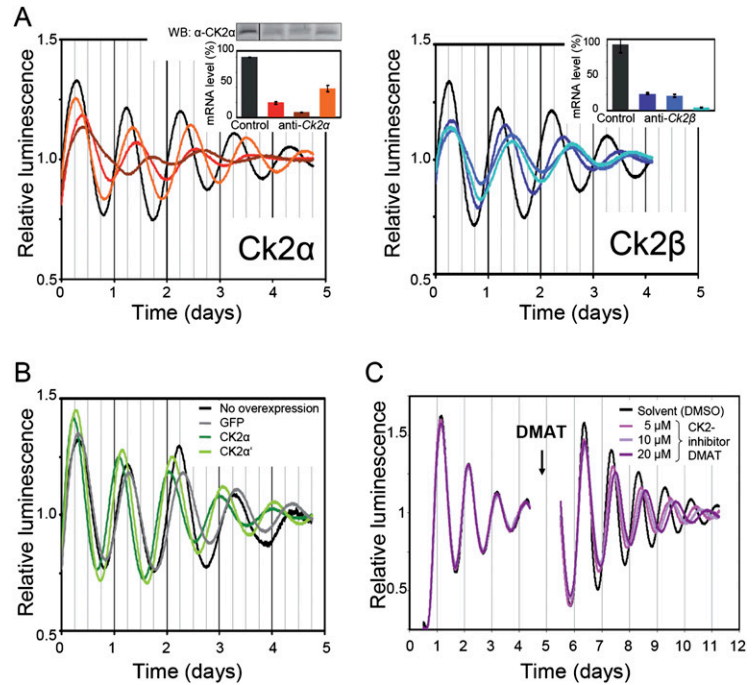


Figure 3. CK2 levels and activity are crucial for normal circadian rhythmicity. (A) Circadian phenotypes of RNAi-mediated knockdown of CK2 α and CK2 β in U-2 OS reporter cells. The experiment was essentially performed as described in Fig. 1A. The insets show the degree of protein or mRNA down-regulation. Error bars, mean \pm SEM ($n = 2-3$). (B) Circadian phenotypes of U-2 OS cells ectopically expressing CK2 α and CK2 α' or GFP as control. Shown are detrended average time series ($n = 2-3$). (C) Lung tissue explanted from *Per2::Luc* knock-in mice (Yoo et al. 2004) was cultured first without and after 5 d in the presence of the specific CK2 inhibitor DMAT.

events, we used our previously described NIH3T3 reporter cells harboring a Flp Recombinase Target (FRT) site, in which either wild-type *Per2* or *Per2-mut* was integrated (Vanselow et al. 2006). Indeed, cells expressing PER2-mut have reduced amplitude and longer periods compared with those expressing PER2 wild type (Fig. 5C). Based on these results, it seems very likely that phosphorylation by CK2 is stabilizing PER2 in vivo, which is required for normal nuclear accumulation and thereby a normal circadian period and amplitude.

Discussion

In this study, we searched for as-yet-unknown factors that ensure that a relatively short-lived clock protein such as PER2 can create rhythms that last an entire 24-h day. We knew from our previous study (Vanselow et al. 2006) that there are likely yet undiscovered PER2-stabilizing kinases, and we also knew (from many studies in various clock models) that the precise fine-tuning of phosphorylation events is of critical importance for circadian dynamics. Therefore, we predicted that systematic down-regulation of all kinases should lead us to the discovery of unknown kinase(s) important for PER2 protein stability and dynamics.

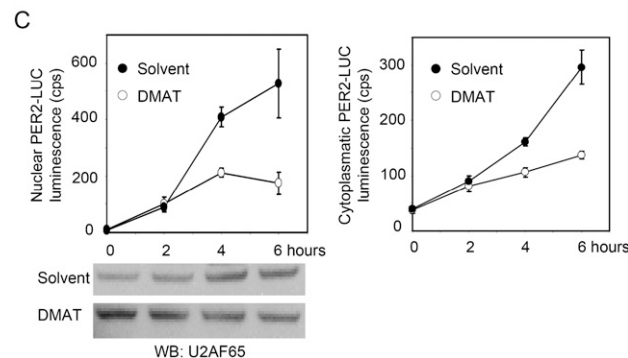
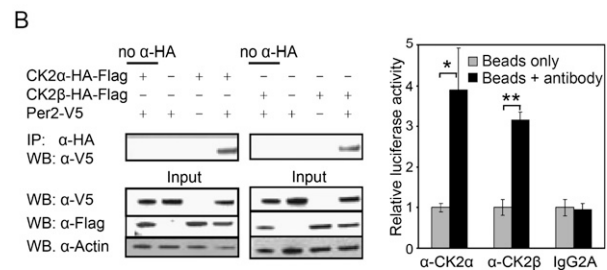
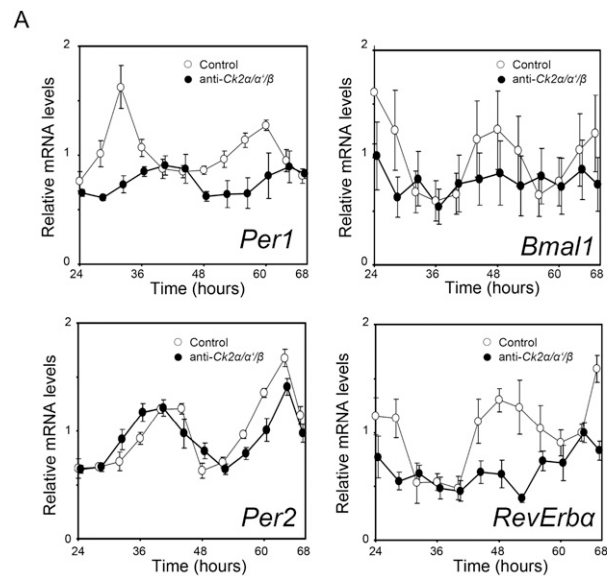
The unbiased RNAi screen in cell culture, which we set up here, has several features that makes it complementary to classic forward genetic screens in mice: (1) high throughput at relatively low costs—we can screen ~ 2000 RNAi constructs per week. (2) The circadian clock can be studied in a human model system. Our results provide direct evidence that the molecular mechanism of the human circadian clock is likely to be very similar to that of mice (Fig. 1). (3) The sensitivity for detecting circadian

phenotypes is predicted to be higher, since loss-of-function genetics results in more severe circadian phenotypes in uncoupled cells compared with behavioral outputs (Liu et al. 2007). (4) Clock components, whose ablation is lethal during development (as is probably the case for CK2 subunits, given the many roles CK2 plays in cellular physiology) (Litchfield 2003), or whose loss-of-function phenotypes are masked by developmental compensation effects, may be uncovered. Therefore, we are currently extending our screen to a genome-wide scale to identify more clock components that are predicted by genetic studies, suggesting as-yet-unknown quantitative trait loci regulating circadian rhythms (Lowrey and Takahashi 2004; Takahashi 2004).

Our RNAi screen led to the discovery of many potential new clock components, most of them kinases. One of them, CK2, especially caught our eyes because of its role for PER regulation in *Drosophila* as well as its importance for the circadian clocks of *Neurospora* (Yang et al. 2002; Dunlap et al. 2007) and *Arabidopsis* (Sugano et al. 1999). In *Drosophila*, PER protein level, phosphorylation, as well as nuclear entry dynamics are substantially altered in hypomorphic mutations of *Drosophila* CK2 subunits displaying long circadian periods (Lin et al. 2002; Akten et al. 2003; Smith et al. 2008). Therefore, we focused our subsequent work on CK2. For most of the other new candidates, we have not yet accumulated enough functional data to unambiguously prove their role within the circadian clockwork. Nevertheless, since RNAi-based perturbations copied “classical” phenotypes from mouse genetics (Fig. 1), it seems reasonable to assume that at least some of the newly discovered clock candidates will turn out to be true clock components. While future studies will nail down the function of these components

within the mammalian clockwork, the sheer number of candidates suggests that the post-translational control of mammalian circadian rhythms is very complex.

Our results clearly demonstrate that normal CK2 levels and activity are essential within the mammalian circadian clockwork. Down-regulation of CK2 catalytic and regulatory subunits either individually or simultaneously leads to long circadian periods or to arrhythmicity, respectively. In contrast, overexpression of CK2 catalytic subunits but not kinase-inactive forms results in shortening of the circadian period. Given the fact that CK2 is involved in many cellular processes with a large number of substrates (Litchfield 2003), we cannot exclude other functions of CK2 within the mammalian clock in addition to those we uncovered in this study. The data



presented here indicate that one function of CK2 is to bind PER2 and to promote its phosphorylation at N-terminal positions. This seems to stabilize PER2 early in its life cycle, which leads to normal nuclear accumulation. Thus, CK2 is most likely a PER2-stabilizing kinase.

How does phosphorylation of PER2 regulate the circadian period, and how is PER2 phosphorylation correlated with stability? Previous studies as well as the present study suggest that the answer to that question is not simple and one-dimensional. In contrast, interference with PER2 phosphorylation can lead to either short or long periods, dependent on which phosphorylation sites are affected. For example, when phosphorylation of Ser662 is inhibited by mutation (as occurs in FASPS patients), the circadian period gets shorter (Jones et al. 1999; Vanselow et al. 2006; Xu et al. 2007), while pharmacologic inhibition of CK1 ϵ/δ activity (Eide et al. 2005; Vanselow et al. 2006; Reischl et al. 2007) lengthens the period. In both cases, PER2 is less phosphorylated, but with opposite effects on circadian period. Since PER2 in FASPS is unstable and CK1 ϵ/δ inhibition stabilizes PER2, one might assume that the circadian period is directly correlated with PER2 stability. This, however, is not the case. As in FASPS, mutation of the N-terminal phospho-acceptor sites also leads to an unstable PER2 (Fig. 5B), but the resulting circadian periods are either short (in FASPS) or long (upon interference with CK2 phosphorylation).

We propose that not PER2 stability, but nuclear localization of the PER/CRY repressor complex, is a major determinant of circadian period (Fig. 6). Our data on the delayed nuclear accumulation upon CK2 inhibition (Fig. 4C) and the premature nuclear clearance of PER2-FASPS (Vanselow et al. 2006) indicate that the circadian period is critically determined by the beginning and the end of nuclear repression. When the beginning of nuclear

Figure 4. PER2 is a target of CK2. (A) Expression levels of clock genes were analyzed in *CK2 α/α' β* triple knockdown cells (or control cells transfected with a nonsilencing RNAi construct) by quantitative RT-PCR. Expression levels of clock genes are given relative to *Gapdh* and normalized to the average expression level of the control cells. Error bars, mean \pm SEM ($n = 3$). Whereas *Per1* ($P < 0.01$) and *Per2* ($P < 0.001$) transcript levels are still significantly rhythmic, *Bmal1* ($P > 0.05$) and *RevErb α* ($P > 0.05$) levels cease to oscillate (CircWave analysis; Oster et al. 2006). (B) (Left) Lysates from HEK293 cells transfected with the indicated constructs were subjected to IP with or without anti-HA antibody and analyzed by Western blotting (WB). (Right) Liver lysate from *Per2::Luc* mice was subjected to IP with or without anti-CK2 α , anti-CK2 β , as well as an idiotypic control antibody (IgG2A). Binding to PER2 was analyzed using luciferase activity of PER2-LUC. Error bars, mean \pm SEM ($n = 3$); (*) $P < 0.05$; (**) $P < 0.01$ (t -test). (C) Primary fibroblasts from *Per2::Luc* mice were treated with CHX for 15 h and subsequently released in medium with or without DMAT. Cell nuclei and cytoplasmic fractions were analyzed for PER2-LUC abundance. Western blots for the nuclear marker protein U2AF65 serve as loading control. Error bars, mean \pm SEM ($n = 3$). Two-way ANOVA revealed significant differences between DMAT treatment and solvent control ($P < 0.01$).

Table 1. Phosphorylated residues of mPER2

Phosphorylated by endogenous kinases ^a	In vitro phosphorylated by		
	CK1δ ^b	CK2	GSK-3β
[S8/S10/T12/S13/T15] ^c	—	[S12/T13] ^d	—
S74	—	—	—
T523	—	—	—
S525, S528, S531	S525, S528, S531	—	—
S538	S538	—	S538
[S544/S545] ^d	[S544/S545] ^d	—	—
S624	—	—	—
S659, S662	—	—	—
[S693/S697] ^d	[S693/S697] ^d	[S693/S697] ^d	—
S763	S763	—	—
S776	S776	—	S776
T959	—	—	—
S971	S971	S971	S971
S980, S981	S980, S981	[S980/S981] ^d	—
[S997/T998] ^d	[S997/T998] ^d	[S997/T998] ^d	[S997/T998] ^d
S1231	—	S1231	S1231

^aPhosphorylation by endogenous kinases in HEK293 cells (Vanselow et al. 2006).

^bPhosphorylation in vitro by CK1δ (Schlosser et al. 2005).

^cTwo of the sites in brackets are phosphorylated.

^dOne of the sites in brackets is phosphorylated.

repression is delayed, because, for example, a signal for nuclear accumulation is missing, the circadian period gets longer. On the other hand, if the end of nuclear repression happens earlier because PER2 disappears earlier from the nucleus, the period gets shorter. In both cases, PER2 stays longer in the cytoplasm, either at a phase when it should *already* be in the nucleus or at a phase when it should *still* be in the nucleus. Since proteasomal degradation of PER2 happens primarily in the cytoplasm (Vanselow et al. 2006; Meng et al. 2008), this phase-dependent shift to a more cytoplasmic localization of PER2 protein would explain its reduced stability in both situations.

Based on our findings, we speculate that CK2-mediated phosphorylation of PER2 provides a signal that allows the timely nuclear accumulation of the repressor complex. While the exact mechanism of the nuclear accumulation dynamics of the PER/CRY repressor complex is elusive, we know that complex formation of PER and CRY proteins leads to nuclear localization and probably thereby to stabilization of both proteins (Kume et al. 1999). However, several questions remain to be investigated: (1) Is a signal required to allow nuclear accumulation of a cytoplasmic complex that is otherwise constantly degraded by CK1ε/δ in the cytoplasm? (2) Does the complex need to reach a concentration threshold or a certain stoichiometry in the cytoplasm to enter the nucleus, while CK1ε/δ-mediated degradation of PER proteins is slowing down this process? (3) Do PER/CRY complexes shuttle between the cytoplasm and nucleus and is a signal needed to allow nuclear retention? (4) Do PER and CRY proteins dissociate before entering the nucleus as has been reported for PER and TIM in *Drosophila* (Meyer et al. 2006)? In principle, CK2-mediated phosphorylation of PER2 could play a role in each of these—not mutually

exclusive—scenarios. Future studies investigating the spatio-temporal pattern of phosphorylation, complex formation, stoichiometry, and subcellular localization are required to elucidate the mechanism of how and when

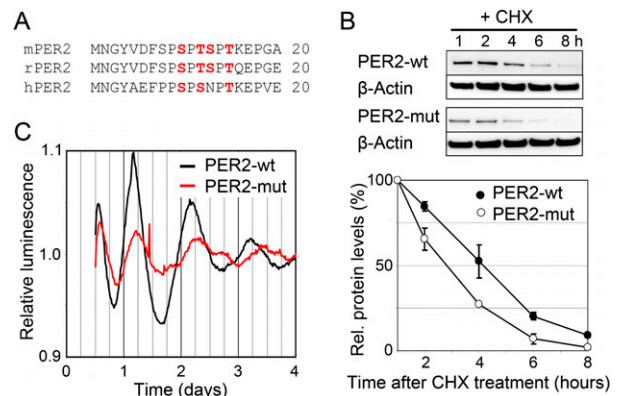


Figure 5. Mutation of N-terminal phosphorylation sites alters PER2 stability as well as circadian dynamics. (A) Two serine and/or threonine residues (red) of the highly conserved N-terminal region of PER2 are phosphorylated in living cells (Vanselow et al. 2006). The mass spectra do not allow a more exact location of the phosphorylated residues in this region. When phosphorylated in vitro, this region is phosphorylated only by CK2, but not by CK1δ or GSK-3β (see Supplemental Table 1). (B) NIH3T3 circadian reporter cells expressing PER2-wt or a mutant version of PER2 (PER2-mut: Ser10, Thr12, Ser13, and Thr15 were exchanged by Ala) from the same chromosomal location (Vanselow et al. 2006) were treated with the protein translation inhibitor CHX. Protein abundance was analyzed by immunoblotting and normalized to β-Actin-levels. Error bars, mean ± SEM (n = 3). (C) NIH3T3 reporter cells expressing either PER2-wt or PER2-mut were synchronized with dexamethasone and circadian oscillations were recorded by luminescence. Shown are detrended average time series (n = 3).

35-mm dishes, and bioluminescence was recorded in light-tight boxes with a photomultiplier tube (Hamamatsu). After 5 d, medium was replaced by medium with or without various concentrations of 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) in 0.2% dimethyl sulfoxide (DMSO) and bioluminescence recording was continued. The concentration of the solvent DMSO was kept constant in all samples. U-2 OS reporter cells were seeded in complete medium into a white 96-well plate. The next day, cells were synchronized by adding 1 μ M dexamethasone for 1 h. After synchronization, the medium was exchanged to complete medium with luciferin without phenol red. DMAT (1 μ M, 5 μ M, or 10 μ M) or DMSO was added. The concentration of the solvent DMSO was kept constant in all samples. Luminescence was monitored in a 96-well plate luminometer Orion II for 7 d.

Co-IP and immunoblotting

Overexpressed epitope-tagged proteins. HEK293 cells were grown to 60% confluence, transfected with equal amounts of DNA (mPer2-V5: pcDNA-DEST40 [Invitrogen]; rCK2 α / β -HA-Flag: VP5 [Clontech]) with FuGene 6 (Roche) according to the manufacturer's protocol. 48 h after transfection, cells were harvested in co-IP buffer (20 mM Tris-HCl at pH 8.0; 140 mM NaCl; 1.5 mM MgCl₂; 1 mM TCEP; 1% Triton-X-100; 10% glycerin) containing protease and phosphatase inhibitor cocktails (Sigma). For each condition, 500 μ g of total protein were incubated with 2 μ g of anti-HA antibody (sc-7392, Santa Cruz Biotechnologies) and equivalent amounts of Protein A-agarose or G PLUS-agarose beads (Santa Cruz Biotechnologies) overnight at 4°C under constant agitation. Beads were washed three times in washing buffer (20 mM Tris-HCl at pH 8.0; 150 mM NaCl; 0.5% Igepal CA-630). Proteins were denatured in SDS-loading buffer (Invitrogen) by boiling for 5 min, separated by SDS-PAGE using 4%–12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose membrane, and incubated with the following primary antibodies according to the manufacturer's instructions: anti-V5 (R960-25, Invitrogen), anti-Flag (F7425, Sigma), and anti- β -Actin (A3853, Sigma). The next day, membranes were probed with HRP-conjugated secondary antibodies (Santa Cruz Biotechnologies), and a chemiluminescence assay was performed using Super Signal West Pico substrate (Pierce) followed by protein detection with a Lumi-Imager (Roche).

Endogenous components. Liver tissue from *Per2::Luc* mice was processed under nondenaturing conditions according to the protocol available at <http://www.abcam.com/technical>. Co-IP was performed as described above using the following antibodies: anti-CK2 α (sc-6479, Santa Cruz Biotechnologies), anti-CK2 β (sc-12793, Santa Cruz Biotechnologies), IgG2A (sc-3878, Santa Cruz Biotechnologies). Bioluminescence recordings were performed by incubation of the bead pellets for 10 sec with 20 μ L of LARI (Promega) using the Beta Scout (PerkinElmer) detection system. Binding of CK2 subunits to PER2 was confirmed independently by co-IP followed by Western blot analysis (see Supplemental Fig. 5). Detection of coimmunoprecipitated PER2-LUC was performed with the anti-LUC antibody (sc-32896, Santa Cruz Biotechnologies). Nonspecific binding of recombinant luciferase generated in HEK293 cells to Protein A-agarose or G PLUS-agarose beads (Santa Cruz Biotechnologies) was excluded previously (data not shown).

Nuclear accumulation assay

Primary fibroblasts from *Per2::Luc* mice were treated with cycloheximide (final concentration 355 μ M) for 15 h. Cell viability was confirmed by visual inspection of the cells as well

as detection of robust circadian rhythmicity after CHX removal (data not shown). Then medium was removed, and cells were washed and treated with either DMAT (20 μ M) or DMSO. At indicated time points, nuclei were isolated by adding 200 μ L of Hypotonic Lysis Buffer. After centrifugation, the supernatant was used as cytoplasmic fraction. The nuclear pellet was washed and resuspended. Nuclei and cytoplasmic fractions were analyzed for PER2-luciferase activity using standard luminometry.

For additional methods, see the Supplemental Material.

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