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RESEARCH ARTICLE

Dynamics of the circadian clock protein PERIOD2 in living cells

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ABSTRACT

In mammals, circadian rhythms are generated by delayed negative feedback, in which period (PER1–PER3) and cryptochrome (CRY1, CRY2) proteins gradually accumulate in the nucleus to suppress the transcription of their own genes. Although the importance of nuclear import and export signals for the subcellular localization of clock proteins is well established, little is known about the dynamics of these processes as well as their importance for the generation of circadian rhythms. We show by pharmacological perturbations of oscillating cells that nuclear import and export are of crucial importance for the circadian period. Live-cell fluorescence microscopy revealed that nuclear import of the key circadian protein PER2 is fast and further accelerated by CRY1. Moreover, PER2 nuclear import is crucially dependent on a specific nuclear-receptor-binding motif in PER2 that also mediates nuclear immobility. Nuclear export, however, is relatively slow, supporting a model of PER2 nuclear accumulation by rapid import, slow export and substantial nuclear degradation.

KEY WORDS: Circadian rhythms, Period2, Nuclear localization

INTRODUCTION

Circadian clocks have evolved in a multitude of organisms, allowing the prediction of and preparation for daily recurring environmental events. In almost all eukaryotes, molecular circadian rhythms are generated by cell-autonomous gene regulatory networks within nearly every cell of an organism. In mammals, period (PER1–PER3) and cryptochrome (CRY1 and CRY2) proteins are integral components of the circadian oscillator in that they inhibit their own expression by repressing the activity of the transcription factor heterodimer CLOCK–BMAL1 (the latter of which is also known as ARNTL) (Buhr and Takahashi, 2013). Importantly, this negative feedback is delayed, allowing the generation of oscillations in PER and CRY transcript and protein levels. In recent years, many details about the mechanisms of transcriptional activation by CLOCK–BMAL1 and inhibition by PER–CRY-containing complexes have been uncovered; surprisingly, however, little is known about the processes that create the delay in transcriptional auto-inhibition, although a precise timing of the negative feedback is crucial for a correct circadian period. Because the localization of PER–CRY-containing protein complexes gradually shifts from cytosolic to nuclear over the course of a circadian cycle, it is believed that appropriately timed nuclear accumulation of PER and CRY proteins contributes to the temporal delay between transcriptional activation and repression, although this has not been directly tested so far.

The shuttling of macromolecules between cytoplasm and nucleus occurs through the nuclear pore with well-characterized nuclear import and export mechanisms involving receptor-based recognition of nuclear localization signals (NLS) or nuclear export signals (NES) on protein cargoes (Ullman et al., 1997). Specialized import and export receptors, such as importin β or heterodimers of importin-α or importin-β and exportin-1 (also known as CRM1) bind to NLS and NES sequences, respectively, and together the complexes move through the nuclear pore. Subsequently, importin and exportin proteins are released from the cargo, which involves the small GTP-binding protein Ran. For many clock proteins (including PERs and CRYs), classical nuclear import and export signals have been described (for a review, see Tamanini et al., 2005). Factors that influence subcellular localization – possibly by modulating the accessibility of NLS and/or NES sequences – are phosphorylation and dephosphorylation, as well as complex formation with other clock proteins. For example, phosphorylation of PER2 by CK2 and GSK3β has been reported to modulate its subcellular localization (Iitaka et al., 2005; Maier et al., 2009). In addition, although PER and CRY proteins can localize to the nucleus even in the absence of one another, binding to CRY promotes nuclear accumulation and stabilization of PER proteins (Kume et al., 1999; Miyazaki et al., 2001; Yagita et al., 2002). Moreover, the CoRNR site of PER2, a binding motif for nuclear receptors, and the promyelotic leukemia protein PML have both been reported to be important for nuclear entry (Albrecht et al., 2007; Miki et al., 2012).

Despite these insights into the domains and factors that contribute to the nucleocytoplasmic distribution of clock proteins, the dynamics of nuclear import and export of negative clock factors are essentially unknown for the mammalian circadian system. In addition, the impact of altering import or export rates on circadian rhythms has not been tested. In two other eukaryotic clock systems, the dynamics of nuclear/cytoplasmic localization seems to be largely distinct: in *Drosophila melanogaster* an interval timer retains the negative clock elements PER and TIM in the cytoplasm for several hours before they dissociate and shuttle to the nucleus (Meyer et al., 2006). The *Neurospora crassa* negative clock protein FRQ, however, is rapidly (within minutes) shuttled between cytoplasm and nucleus, and its nucleocytoplasmic localization is gradually shifted by phosphorylation during the course of a circadian cycle (Diernfellner et al., 2009).

Here, we show that both nuclear import and nuclear export are essential for normal circadian rhythms. Pharmacological inhibition of both pathways lengthens the circadian period in U2-OS reporter cells by up to 4 hours. We use live-cell fluorescence microscopy to analyze the kinetics of nuclear import and export of PER2 as one of the scaffold proteins of the negative feedback complex. Nuclear import of PER2 is fast and further accelerated by CRY1; however,

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PER2 nuclear export is relatively slow. In addition, we analyze the mobility of the PER2-containing complexes within the subcellular compartments and find that the rather low nuclear mobility of PER2 is crucially dependent on a specific nuclear-receptor-binding motif in PER2. This motif is also essential for nuclear import, because its mutation renders PER2 almost completely cytosolic and drastically decreases the nuclear import rate. This suggests that a yet-undiscovered protein – possibly a nuclear receptor – not only targets PER2 to chromatin but also helps to escort it into the nucleus.

RESULTS

Shuttling between the cytoplasm and nucleus is essential for circadian dynamics

To test whether the kinetics of key nuclear import and export pathways are important for circadian rhythm dynamics, we pharmacologically inhibited both the importin-α- and importin-β-mediated nuclear import as well as the CRM1/exportin-1-dependent nuclear export in circadian reporter cell lines. To this end, we treated two different U2-OS reporter cell lines expressing firefly luciferase either from a *Bmal1* promoter fragment (Maier et al., 2009) or from the *Per2* promoter (Liu et al., 2008) with ivermectin, a specific inhibitor of importin α/β-dependent nuclear import (Wagstaff et al., 2012) or leptomycin B, an efficient and selective inhibitor of CRM1/exportin-1-mediated nuclear export (Wolff et al., 1997). Both inhibitors dose-dependently dampened circadian rhythms and lengthened the circadian period by up to 3–4 hours (Fig. 1A,B; supplementary material Fig. S1) without substantially affecting the viability of the cells (supplementary material Fig. S2). This suggests that shuttling between the cytoplasm and nucleus – probably of clock proteins – is essential for maintaining normal circadian periods.

The nuclear import rate of PER2 is accelerated by interaction with CRY1

PER2 is a key circadian clock protein and part of a large multi-protein complex that gradually changes its subcellular distribution towards nuclear localization over the circadian day (for a review, see Albrecht et al., 2007). Although NLS as well as NES of PER2 have been identified and alterations of subcellular localization have been correlated with changes in circadian dynamics (Vanselow et al., 2006; Maier et al., 2009), the rates of nuclear import or export of PER2-containing complexes are unknown. We stably expressed PER2 as a C-terminal fusion protein with the yellow fluorescent protein Venus in U2-OS cells and analyzed the dynamics of nuclear import by fluorescence recovery after photo-bleaching (FRAP). The PER2–Venus fusion protein still bound to known PER2 interaction partners, such as PER1, CRY1 and CKIε (supplementary material Fig. S3A), and was hyperphosphorylated and destabilized when co-expressed with CKIε (supplementary material Fig. S3B), indicating that the Venus fusion did not lead to a misfolding of the PER2 protein. We bleached the nuclear fraction of the fluorescent PER2–Venus fusion protein and measured the recovery of fluorescence for 1 hour (Fig. 2A). To prevent loss of fluorescence due to nuclear export, cells were incubated with the nuclear export inhibitor leptomycin B. In addition, bleaching of fluorescence due to repeated measurement was compensated for by normalizing to control cells. We found that the fluorescence of PER2–Venus reached half of the initial fluorescence after around 30 minutes (Fig. 2A,B), suggesting that the nuclear import of PER2 is fast compared with a circadian time-scale, but rather slow compared with the circadian protein FRQ of *Neurospora crassa*, which – dependent on its phosphorylation state – is imported into the nucleus ~5–10-fold faster (Diernfellner et al., 2009).

To investigate a potential influence of known PER2-interacting proteins on the nuclear import rate of PER2, we stably coexpressed either CRY1 or CK1ε and again measured FRAP of PER2–Venus in the nucleus. Although there was a trend towards a faster PER2 nuclear accumulation upon co-expression of CK1ε, this did not reach significance (supplementary material Fig. S4A); however, CRY1 coexpression significantly accelerated the nuclear import of PER2 with a half recovery time of

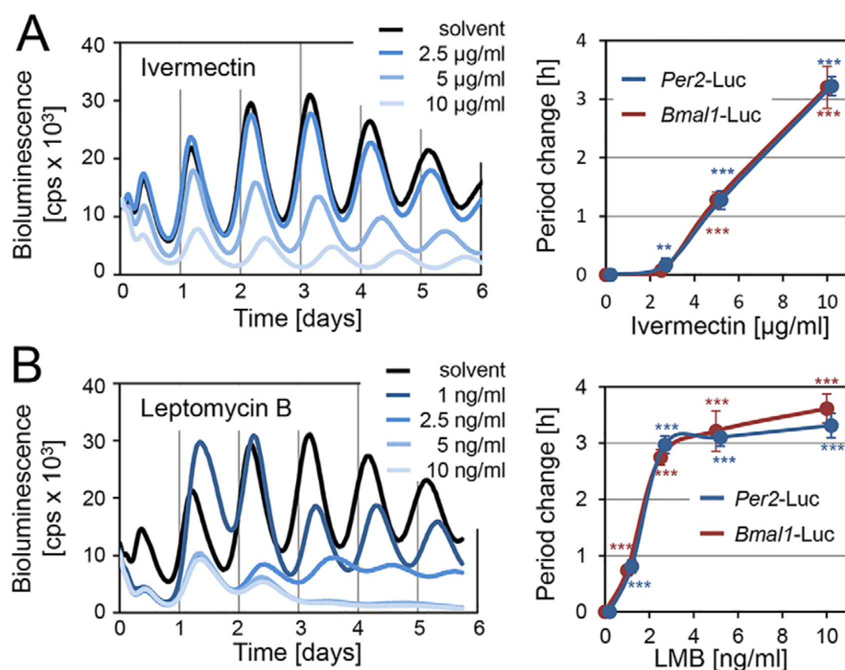

Fig. 1. Nuclear import and export is crucial for circadian period. (A,B) Circadian oscillation dynamics (mean of eight independent time-series) and quantification of period change (mean±s.d.; n=8) of human osteosarcoma cells (U2-OS) harboring a *Per2* (blue) or *Bmal1* (red, see also supplementary material Fig. S1) promoter-driven luciferase reporter construct and treated with the nuclear import inhibitor ivermectin or export inhibitor leptomycin B. One-way ANOVA analysis revealed significant ($P<0.0001$) influence of inhibitor concentration. **$P<0.01$, ***$P<0.0001$ (post-hoc Student's *t*-test, Bonferroni corrected).

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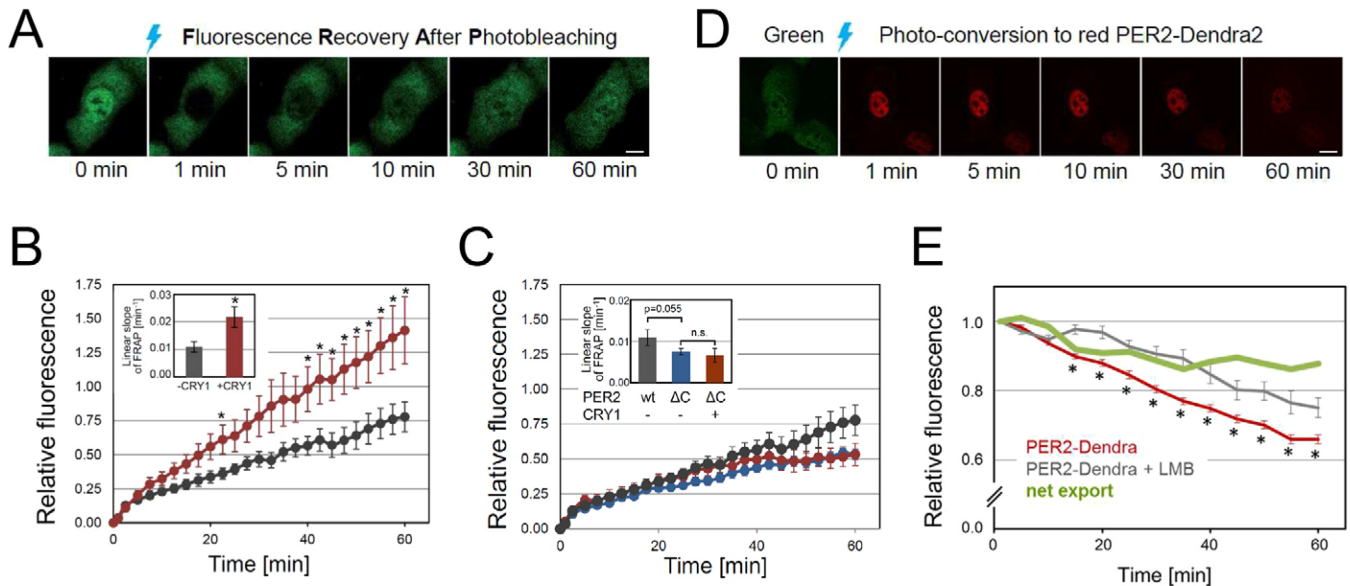

Fig. 2. Nuclear import and export dynamics of PER2. (A) Analysis of PER2–Venus nuclear import in U2-OS cells. Recovery of fluorescence is measured after bleaching the nuclear fraction of PER2–Venus. To avoid loss of fluorescence due to nuclear export, cells were treated with the nuclear export inhibitor leptomycin B (LMB). Scale bar: 10 µm. (B) Quantification of fluorescence recovery of cells described in A. Data show the mean±s.e.m. (seven cells for each condition). Inset shows the slope of linear regression to fluorescence recovery between 2.5 and 60 minutes after photo-bleaching. Coexpression of CRY1 significantly accelerates nuclear import; $*P<0.05$, Student's *t*-test. (C) FRAP analysis as described for A,B, using cells expressing PER2–Venus either as full-length version (wt, wild type) or with a C-terminal truncation (ΔC) that cannot bind to CRY. n.s., non-significant. (D) Analysis of PER2–Dendra2 nuclear export in U2-OS cells. Green nuclear fusion protein was converted to red and pictures were taken over the course of 60 minutes. Scale bar: 10 µm. (E) Quantification of activated PER2–Dendra2 fluorescence net export (green) calculated from PER2–Dendra2 export (red, $n=31$) relative to loss of PER2–Dendra2 fluorescence (gray, $n=14$) due to bleaching and nuclear degradation (from export-inhibited, LMB-treated cells). Data show the mean±s.e.m.; $*P<0.001$ (Student's *t*-test).

~20 minutes (Fig. 2B). By contrast, a truncated version of PER2 lacking the C-terminal 129 amino acids that are required for CRY1 binding (Yagita et al., 2002) showed an increased half recovery time of ~50 minutes after nuclear bleaching, and coexpression of CRY1 did not influence the rate of nuclear import of the truncated PER2 (Fig. 2C). Taken together, these results indicate an important role of PER2–CRY1 complex formation in normal nuclear import dynamics.

Nuclear export of PER2 is slow

For the analysis of PER2 nuclear export rate, we took advantage of the photo-convertible fluorophore Dendra2, which can be switched from green to red fluorescence with a 405-nm laser beam (Fig. 2D) (Gurskaya et al., 2006). We expressed PER2 as a C-terminal fusion protein with Dendra2 in U2-OS cells and, as expected, found it in both cytoplasm and nucleus, as revealed by bright green fluorescence. After conversion of only nuclear PER2–Dendra2 to red fluorescence, we measured the decrease in red fluorescence every 5 minutes over the course of 1 hour (Fig. 2E). We adjusted for loss of fluorescence by other causes than nuclear export (such as protein degradation and bleaching due to repeated excitation) by also measuring the loss of nuclear red fluorescence in cells incubated with the nuclear export inhibitor leptomycin B. Over the course of 1 hour, we found that only ~10% of nuclear PER2–Dendra fluorescence was decreased due to nuclear export, indicating a considerably slower nuclear export of PER2 compared with PER2 nuclear import. We also investigated a potential influence of CRY1 on the PER2–Dendra2 export rates, but did not find significant changes in nuclear export rates upon CRY1 coexpression (supplementary material Fig. S4B), which suggests that CRY1 primarily affects nuclear import rather than export of PER2.

Mobility of PER2-containing complexes in cellular compartments

Nuclear import and export rates of PER2-containing complexes are probably crucial for the timing of transcriptional repression and thus for the circadian period. As shown above, these rates are influenced by the formation of complexes with other proteins, which not only alter the size of the complex but also might support or decelerate transport. To investigate the dynamic properties of PER2-containing complexes within the cellular subcompartments, which probably influence nuclear import and export dynamics, we measured the mobility of PER2–Venus by using FRAP in the cytoplasm and the nucleus. To this end, PER2–Venus molecules were bleached at a confined small area, either within the cytoplasm or the nucleus, and the exchange of bleached molecules with fluorescent molecules from the surrounding area of the same compartment was measured (Fig. 3A). Previously, we have reported that cytoplasmic PER2–Venus is far less mobile than the control protein βGAL–Venus (Kucera et al., 2012), although *Escherichia coli* β-galactosidase is a homotetramer of ~465 kDa, supporting earlier reports that estimate a PER2 complex size of >1 MDa (e.g. Brown et al., 2005). In the nucleus, we found a mobility of PER2–Venus complexes that was significantly lower ($t_{1/2}=8.14\pm1.35$ seconds; $n=7$) than that observed in the cytoplasm ($t_{1/2}=1.73\pm0.13$ seconds; $n=7$) (Fig. 3B,C). Theoretical considerations, as well as the shape of the recovery curves, indicated that such long FRAP half times in the nucleus cannot be explained by free

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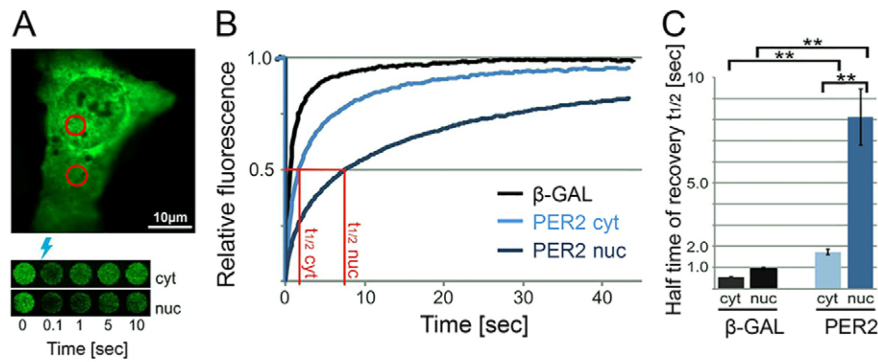

Fig. 3. PER2 is immobile in the nucleus. (A) PER2–Venus expressed in U2-OS cells is localized in both the cytoplasm (cyt) and nucleus (nuc). Representative bleach areas of a FRAP experiment are indicated (red circles). Scale bar: 10 μm. The lower panel shows the recovery of fluorescent molecules in the bleach area during the first 10 seconds. (B) The graph depicts normalized average curves of fluorescence recovery in the cytoplasm (light blue) or nucleus (dark blue) of PER2–Venus in comparison to βGAL (a similarly sized control protein; black). The half time of recovery ($t_{1/2}$) is determined as the time at which normalized fluorescence reaches half of the initial value. (C) Quantification of half time of recovery. Cytoplasmic PER2–Venus ($t_{1/2}$=1.73±0.13 seconds, *n*=7 experiments with ≥10 cells each) is significantly less mobile than the similarly sized control protein βGAL–Venus ($t_{1/2}$=0.56±0.02 seconds, *n*=3 experiments with ≥10 cells each). Remarkably, PER2–Venus exhibits substantially lower mobility in the nucleus ($t_{1/2}$=8.14±1.35 seconds; *n*=7 experiments with ≥10 cells each) than in the cytoplasm. Data show the mean±s.e.m.; ***P*<0.001 (Student's *t*-test).

diffusion of the complexes, but more likely by binding of PER2–Venus to comparably immobile structures such as chromatin.

The nuclear-receptor-binding motif of PER2 is responsible for low nuclear mobility

PER2 does not possess a DNA-binding domain but can bind indirectly to DNA through the transcription factors CLOCK–BMAL1 (Sangoram et al., 1998), as well as the nuclear receptors REV-ERBα (also known as NR1D1) and PPARα (Schmutz et al., 2010). To investigate whether nuclear PER2 complexes are immobilized by chromatin binding, we analyzed the nuclear and cytosolic mobility of a PER2 variant in which the nuclear receptor recognition motif ^{306}LXXLL^{310} was mutated (Schmutz et al., 2010). In FRAP experiments, we found that this PER2 mutant ($PER2_{AXXAA}$–Venus) was now equally mobile in the nucleus ($t_{1/2}$=1.21±0.14 seconds; *n*=3) and in the cytoplasm ($t_{1/2}$ =1.14±0.06 seconds; *n*=3), suggesting that nuclear receptor binding contributes to the immobilization of PER2 on chromatin (Fig. 4A,B). The altered mobility of $PER2_{AXXAA}$–Venus in the FRAP experiments is likely not due to misfolding, which would lead to a non-functional protein; we found that $PER2_{AXXAA}$ still showed an inhibitory effect on CLOCK–BMAL1-mediated transactivation (supplementary material Fig. S3C), although to a lesser extent, probably owing to increased cytosolic localization (see below). In addition, $PER2_{AXXAA}$–Venus still bound to known PER2 interaction partners, such as PER1, CRY1 and CKIε (supplementary material Fig. S3A), and was hyper-phosphorylated and destabilized when coexpressed with CKIε (supplementary material Fig. S3B), indicating that the increased nuclear mobility of $PER2_{AXXAA}$ is a specific effect due to the lack of binding to nuclear receptors such as REV-ERBα and PPARα that can mediate binding to chromatin.

The LXXL-motif of PER2 is required for nuclear import

To test whether decreased binding to chromatin leads to loss of nuclear retention of PER2 and hence redistribution into the cytoplasm, we compared the steady-state subcellular localization of wild-type PER2–Venus and $PER2_{AXXAA}$–Venus in >100 U2-OS cells. We found that indeed $PER2_{AXXAA}$–Venus had an almost exclusive cytosolic localization (Fig. 4C), which could be either due to a decreased nuclear import rate or to an increased nuclear export because of an impaired nuclear retention. To discriminate between these possibilities, we analyzed the nuclear import dynamics of $PER2_{AXXAA}$–Venus compared with those of wild-type PER2–Venus. Because in most cells nuclear $PER2_{AXXAA}$–Dendra2 was undetectable, the nuclear export rate could not reliably be measured for the mutant PER2. When we analyzed the nuclear import rate for $PER2_{AXXAA}$–Venus by treating the cells with the nuclear export inhibitor leptomycin B and measuring the accumulation of fluorescence in the nucleus over the course of 1 hour, we found – in contrast to our expectations – a substantially slower nuclear import of the mutant protein compared with that of wild-type PER2 (Fig. 4D). This implies that the LXXLL motif of PER2 is not only required for nuclear receptor binding and thereby probably for nuclear immobility, but is also modulating the nuclear import rate, likely by mediating the interaction with a yet-unknown protein.

DISCUSSION

The timing of nuclear localization of circadian clock proteins – especially of those that exert negative feedback function – is of essential importance for circadian dynamics, because the delay in negative feedback determines the circadian period. In mammals, it was unclear whether PER and CRY proteins enter the nucleus only at a specific phase in the circadian cycle (like a switch) or whether they always shuttle between cytoplasm and nucleus and gradually shift their subcellular distribution towards nuclear over the course of a circadian cycle (for a conceptual review, see Tataroğlu and Schafmeier, 2010). Precedent for both scenarios exists in other circadian model organisms: in *Drosophila*, studies in S2 cells suggest that PER–TIM dimers form early in the cycle, accumulate in the cytoplasm and, after ~6 hours, rather abruptly dissociate and enter the nucleus (Meyer et al., 2006). In *Neurospora*, however, FRQ rapidly shuttles between cytoplasm and nucleus and changes from mainly nuclear as hypophosphorylated protein to mainly cytosolic when hyperphosphorylated (Diernfellner et al., 2009). Previous studies with mammalian clock proteins analyzing steady state localization using various truncated or mutant proteins were seminal in identifying domains and motifs important for

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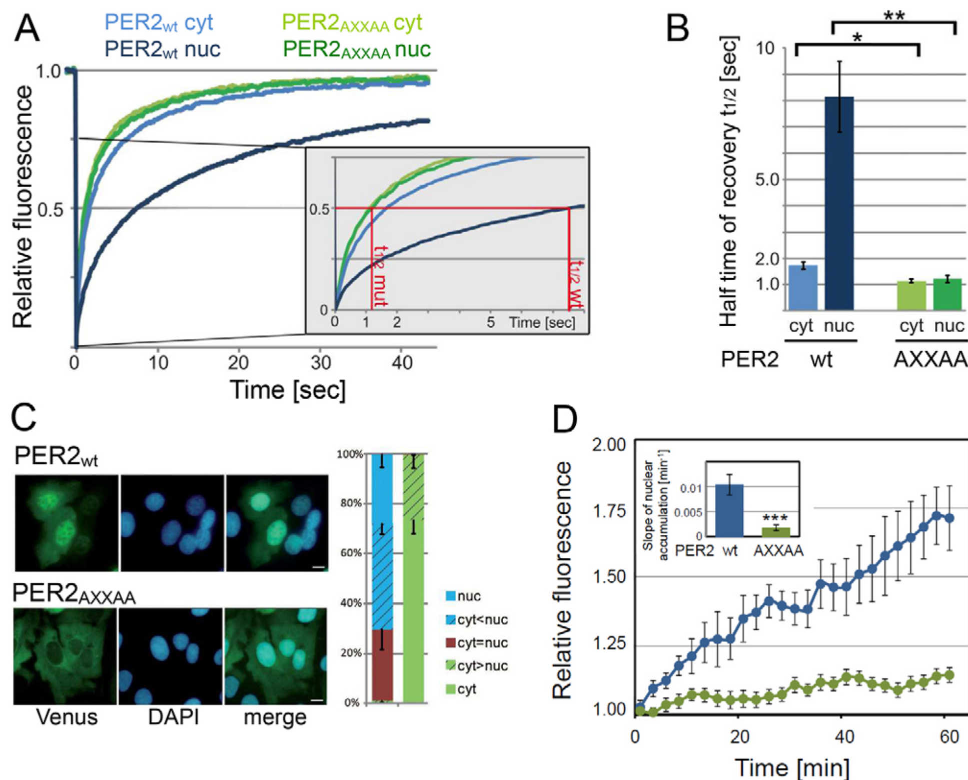

Fig. 4. The nuclear-receptor-binding motif of PER2 is responsible for low nuclear mobility and nuclear import. (A) Normalized average curves of fluorescence recovery of wild-type ($PER2_{wt}$) or nuclear-receptor-binding-motif-mutated ($PER2_{AXXAA}$) PER2–Venus either in the cytoplasm (cyt; light blue and light green) or nucleus (nuc; dark blue and dark green). Data from the first 8 seconds of recovery (boxed area) is enlarged in the inset. (B) Quantification of half time of recovery as in Fig. 3C. Mutated PER2 shows equal mobility in the cytoplasm ($t_{1/2}$=1.14±0.06 seconds; n=3 experiments with ≥10 cells each) and the nucleus ($t_{1/2}$=1.21±0.14 seconds; n=3 experiments with ≥10 cells each). The reduced mobility of wild-type PER2 in the nucleus ($t_{1/2}$=8.14±1.35 seconds; n=7 experiments with ≥10 cells each) is abolished in the mutant. Data show the mean±s.e.m.; *P<0.05, **P<0.001 (Student's t-test). (C) Analysis and quantification of steady-state subcellular localization of Venus-tagged wild-type PER2 and $PER2_{AXXAA}$ in U2-OS cells. Left, Venus; middle, DAPI; right, overlay. Whereas wild-type PER2 is distributed in both compartments with a stronger appearance in the nucleus, $PER2_{AXXAA}$ localizes almost exclusively to the cytoplasm. Scale bars: 10 µm. The graph shows quantification of the steady-state distribution from three independent experiments with ≥100 cells. Left bar, wild-type PER2; right bar, $PER2_{AXXAA}$. Data show the mean±s.e.m. (D) Dynamic measurement of nuclear import. U2-OS cells (n=7 cells for each PER2 version) expressing Venus-tagged wild-type PER2 and $PER2_{AXXAA}$ were treated with the nuclear export inhibitor leptomycin B and analyzed for the accumulation of nuclear fluorescence. Inset, slope of linear regression to fluorescence recovery between 1 and 61 minutes after photo-bleaching. Data show the mean±s.e.m.; ***P<0.001 (Student's t-test).

localization, but could not analyze the dynamics of subcellular localization.

Using live-cell fluorescence microscopy experiments, we show that PER2 – an important scaffold for building the negative feedback complex in mammals (Brown et al., 2005) – shuttles between cytoplasm and nucleus with an import kinetics 5–10-fold slower than that of *Neurospora* FRQ but still relatively fast compared to a circadian timescale. CRY1 accelerates the import rate of PER2 (i.e. unlike in *Drosophila*, CRY1–PER2 dimers do not accumulate in the cytoplasm for hours and then rapidly switch to the nucleus). The nuclear export, however, is relatively slow, with only ~10% of nuclear PER2 being exported within 1 hour. Thus, nuclear degradation (see also Miyazaki et al., 2004) seems to be more important for nuclear clearance than nuclear export. In addition, CRY1 has no detectable influence on the export rate of PER2, which is in contrast to previous models suggesting that CRY proteins mainly act as inhibitors of nuclear export (Yagita et al., 2002). Apart from CRY1, we show that the nuclear-receptor-binding motif ^{306}LXXLL^{310} of PER2 is crucial for nuclear localization (see also Albrecht et al., 2007). In addition, this motif drastically reduces intra-nuclear mobility of PER2, suggesting that it keeps PER2 bound to chromatin. Therefore, upon mutation of this motif, we expected mainly effects on nuclear export rate. However, we found that nuclear import rate was drastically reduced. This suggests that binding to nuclear receptors in the cytoplasm (or to other yet unknown proteins) is essential for nuclear import of PER2. Further studies are required to unravel the identity of these proteins.

Our experiments with ectopically expressed PER2–Venus fusion protein can of course not capture any circadian-phase-dependent effect on subcellular localization dynamics. Because we used unsynchronized U2-OS cells, which likely would anyway not be rhythmic owing to overexpression of PER2 (Wallach et al., 2013), our dynamic parameters might be considered as values representing the repression phase. It is very likely, however, that circadian-phase-specific events, such as phosphorylation and binding to other proteins, considerably modulate nuclear import and export rates (e.g. by masking or unmasking localization signals) as well as nuclear and cytosolic degradation rates. The fact that we see lengthening of the circadian period by inhibiting both the classical import pathway as well as the export pathway also argues for circadian phase-specific dominance of one or the other

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pathway on circadian period. It is rather intuitive that blocking the nuclear import leads to delayed nuclear accumulation of repressors and hence to long periods. A long period upon inhibiting the nuclear export might be explained by preventing nuclear clearance of repressors at the end of the circadian day, which would prolong the repression phase. To investigate such questions further, cellular models with fluorescently tagged circadian proteins controlled by endogenous promoters are needed, allowing phase-specific investigations of circadian kinetic parameters. Our study is a valuable first step towards a more quantitative description of circadian cell biology.

MATERIALS AND METHODS

Bioluminescence recording

U2-OS cells (human, ATCC HTB-96) stably expressing firefly luciferase from a *Bmal1* promoter fragment (Maier et al., 2009) or *Per2* promoter fragment (Liu et al., 2008) were seeded onto a white 96-well plate (20×10^4 cells/well). After 24 hours, cells were synchronized with dexamethasone (1 µM) for 30 minutes, washed with PBS and cultured in 150 µl of Phenol-Red-free DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 250 µM D-luciferin (Biothema, Darmstadt, Germany). Bioluminescence recordings were performed at 35–37℃ in 96-well plate luminometers (TopCount, PerkinElmer, Rodgau, Germany). Pharmacological compounds [ivermectin (Sigma-Aldrich, Munich, Germany) or leptomycin B (AppliChem, Darmstadt, Germany)] were added after synchronization. Data were analyzed using ChronoStar software as described previously (Maier et al., 2009).

Expression vectors

Coding sequences (CDS) of genes of interest were shuttled into pLenti6 Gateway® Destination vector (Invitrogen, Darmstadt, Germany) and lentiviral particles were produced as described previously (Maier et al., 2009). For the expression of Venus- or Dendra2-fusion proteins, either full-length PER2 CDS (wild type) or CDS comprising only the first 1127 amino acids (PER2ΔC) were shuttled into a pLenti6 vector with the CDS of the fluorophore at the C-terminus (Kucera et al., 2012). To transduce U2-OS cells, they were incubated with lentiviral supernatant (half final volume) and 8 µg/ml protamin sulfate (Sigma-Aldrich, Munich, Germany) and selected with blasticidin (Invitrogen, Darmstadt, Germany) from the second day.

Nuclear import analysis

Confocal microscopy of live cells was performed with a FluoView 1000 microscope (Olympus, Tokyo, Japan) with a ×60 (1.35 numerical aperture) oil-immersion objective in a climate chamber at 37℃ under 5% CO_2. For analysis of nuclear import, nuclear export was inhibited with leptomycin B (10 ng/ml) 1 hour before the start of the experiment. Dynamics of nuclear import were measured by bleaching nuclear fluorescence of cells expressing Venus-tagged versions of PER2. Recovery of fluorescence was observed by taking pictures every 2.5 minutes. Mean nuclear and cytoplasmic fluorescence was calculated and mean background fluorescence was subtracted. Initial nuclear fluorescence was set to 1.0 and the bleached fraction was set to 100% (McNally, 2008). Nuclear recovery was normalized to changes in cytoplasmic fluorescence to compensate for overall bleaching due to repeated measurements. For analysis of nuclear import rates in cells expressing $PER2_{AXXAA}$–Venus with nearly exclusive cytoplasmic localization, the accumulation of fluorescence in the nucleus of cells treated with leptomycin B (10 ng/ml) was calculated as described above.

Nuclear export analysis

Nuclear export dynamics were analyzed by using the expression of Dendra2-tagged versions of PER2. Dendra2 is a photo-convertible fluorophore that can be switched from green to red fluorescence by activation with a laser beam at 405 nm (Gurskaya et al., 2006). Fluorescent molecules were converted only in the nucleus, and pictures were taken every 5 minutes. Initial fluorescence was set to 1.0 and the decrease of mean fluorescence in the nucleus – due to nuclear export, degradation and bleaching – was measured. To calculate nuclear export only, these values were normalized to the decrease that was measured in leptomycin-B-treated cells, where decreasing fluorescence should be only due to degradation and bleaching.

Mobility analysis

Fluorescence recovery after photobleaching (FRAP) was performed as described previously (Kucera et al., 2012). Briefly, fluorescent molecules were bleached with a strong laser beam at 515 nm within a confined area in the cell (0.1 seconds; 15 μm^2). Exchange of bleached molecules with surrounding fluorescent molecules was measured at low laser intensity every 0.12 seconds for 45 seconds. For normalization, background values were subtracted and the overall decrease of fluorescence was normalized to a reference area. For each independent experiment, at least ten cells were measured, initial fluorescence was set to 1.0, total bleached molecules were set to 100% and an average recovery curve of all cells was calculated (McNally, 2008). To compare the mobility of different fusion proteins, the halftime of recovery ($t_{1/2}$) was determined as the time at which 50% of bleached molecules are exchanged. At least three independent experiments were conducted for each construct.

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Competing interests

The authors declare no competing interests.

Author contributions

R.Ö., S.K., B.K. and A.K. designed and performed the experiments; T.K. and A.H. provided technical and conceptual support; R.Ö., S.K., B.K., T.K., A.H. and A.K. analyzed the data; A.K., R.Ö. and S.K. wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.156612/-/DC1

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