EMBO reports

concept

Of switches and hourglasses: regulation of subcellular traffic in circadian clocks by phosphorylation

Özgür Tataroğlu & Tobias Schafmeier[+]

University of Heidelberg Biochemistry Centre, Heidelberg, Germany

Investigation of the phosphorylation of circadian clock proteins has shown that this modification contributes to circadian timing in all model organisms. Phosphorylation alters the stability, transcriptional activity and subcellular localization of clock proteins during the course of a day, such that time-of-day-specific phosphorylation encodes information for measuring time and is crucial for the establishment of an approximately 24-h period. One main feature of molecular timekeeping is the daytime-specific nuclear accumulation of clock proteins, which can be regulated by phosphorylation. Here, we discuss increasing knowledge of how subcellular shuttling is regulated in circadian clocks, on the basis of recent observations in *Neurospora crassa* showing that clock proteins undergo maturation through sequential phosphorylation. In this model organism, clock proteins are regulated by the phosphorylation-dependent modulation of rapid shuttling cycles that alter their subcellular localization in a time-of-day-specific manner.

Keywords: circadian clocks; phosphorylation; nucleo-cytoplasmic shuttling

EMBO *reports* (2010) **11,** 927–935. doi:10.1038/embor.2010.174

See Glossary for abbreviations used in this article.

Introduction

The central oscillating unit of eukaryotic circadian clocks consists of transcriptional and translational feedback loops that control—directly or indirectly—the rhythmic expression of many genes (McDonald & Rosbash, 2001; Oishi *et al*, 2003; Schaffer *et al*, 2001; Smith *et al*, 2010). Although the specific components of different circadian clocks are not homologous, the basic mechanisms that control them are similar in a range of organisms from filamentous fungi to vertebrates. In general, circadian clocks function through the activation and inactivation of regulatory elements. Activation of these elements and the negative-feedback loops that subsequently inhibit their transcription or function results in the oscillation of RNA and protein levels over a period of approximately 24 h. The same principle is followed across species: the action of a heterodimeric transcription factor complex harbouring PAS-domain-containing subunits activates the transcription of genes encoding one or more negative regulatory elements. The synthesized proteins enter the nucleus and—after a species-specific delay—inhibit the activity of the transcription factor complex, repressing their own synthesis sometime during the late subjective afternoon (Fig 1). Over the course of the day the negative elements are degraded and the transcription factor complex can be activated again, restarting the cycle. In recent years, the molecular mechanisms of *Drosophila*, mammalian and fungal circadian clocks have been analysed extensively, which has substantially developed our knowledge of the mechanisms underlying the core feedback loops (for a review see Brunner & Kaldi, 2008; Dibner *et al*, 2010; Dunlap & Loros, 2006; Hardin, 2005; Ko & Takahashi, 2006; Nitabach & Taghert, 2008; Reppert & Weaver, 2001; Reppert & Weaver, 2002).

Biochemical analyses of clock components have shown that circadian feedback loops are regulated not only by transcription and translation, but also post-translationally. Post-translational modifications of clock components are crucial for maintaining the pace of the circadian clock (Brunner & Schafmeier, 2006; Gallego & Virshup, 2007; Lee *et al*, 2001; Mehra *et al*, 2009a). It has been demonstrated in cyanobacteria that a circadian oscillation of phosphorylation of the clock protein KaiC occurs *in vitro* in the absence of transcription and translation. This suggests that, at least in prokaryotes, the post-translational modification of clock proteins alone can maintain an approximately 24-h period of the core clock (Nakajima *et al*, 2005; Tomita *et al*, 2005). In eukaryotes, circadian rhythms have also been observed to be independent of transcription (Woolum, 1991), suggesting that circadian clocks that are regulated by rhythmic protein modification—rather than transcriptional-feedback loops—might also have evolved in higher organisms. Support for this comes from the observation that some circadian rhythms in the filamentous fungus *Neurospora crassa* persist in the absence of a central clock component, indicating that they are generated by multiple oscillators (Jolma *et al*, 2010).

The daytime-specific phosphorylation cycles of the negative elements in eukaryotic circadian clocks indicate that the phosphorylation of clock components is crucial for the timing of an approximately 24-h period. The first evidence of the post-translational modification of a clock component came from the *Drosophila* clock protein PERIOD (PER), which is progressively phosphorylated in a circadian manner and eventually degraded to close the feedback loop (Edery *et al*, 1994; Price *et al*, 1998). It has since been shown that almost all clock components are

University of Heidelberg Biochemistry Centre, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany
[+]Corresponding author. Tel: +49 6221 54 47 78; Fax: +49 6221 54 47 69;
E-mail: tobias.schafmeier@bzh.uni-heidelberg.de

Received 15 July 2010; accepted 21 October 2010; published online 5 November 2010

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

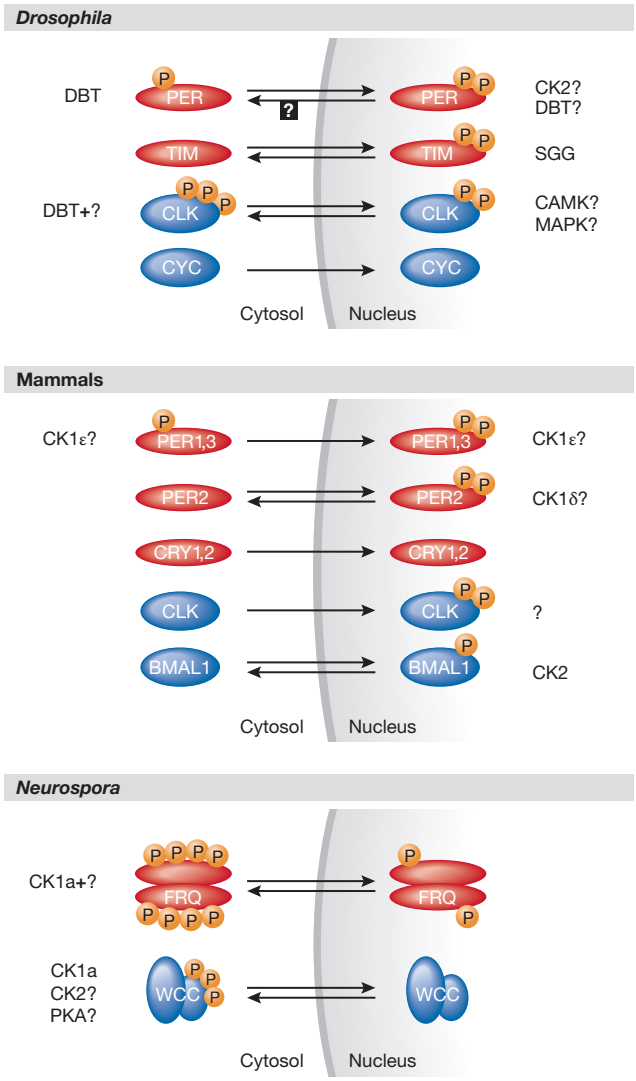

Fig 1 | Regulation of the subcellular localization of clock proteins by phosphorylation. A summary of the subcellular transport of fly, mammalian and fungal clock proteins, as regulated by phosphoryation. The kinases that are thought to be involved in modifying localization are shown. Negative and positive elements are shown in red and blue, respectively. Arrows: nuclear import and export. Question mark: unknown or suspected kinase. BMAL, brain and muscle ARNT-like protein; CYC, CYCLE; CK, casein kinase; CRY, CRYPTOCHROME; DBT, DOUBLETIME; P, phosphate; PER, PERIOD; PKA, protein kinase A; TIM, TIMELESS; WCC, white collar complex.

phospho-proteins and that phosphorylation often occurs in a phase-specific manner (Bae & Edery, 2006; Garceau *et al*, 1997; He *et al*, 2005; Lee *et al*, 1998; Liu *et al*, 2000; Schafmeier *et al*, 2005; Schwerdtfeger & Linden, 2000; Yu *et al*, 2006). In the *Neurospora* clock, for example, phosphorylation events have been shown to regulate the degradation of the negative element FREQUENCY (FRQ) and the activity of the transcription factor complex WCC (Liu *et al*, 2000; Schafmeier *et al*, 2005).

Several laboratories have reported that the subcellular localization of clock proteins is regulated by phosphorylation in flies and mammals. Research focusing on the *Neurospora* clock has shown that phosphorylation events modulate not only the subcellular distribution, but also the import and export kinetics of WCC and FRQ, which shuttle between the cytoplasm and nucleus in minutes (Cha *et al*, 2008; Diernfellner *et al*, 2009; Hong *et al*, 2008; Schafmeier *et al*, 2008). These phosphorylation events seem to function in an hourglass pattern—the steady accumulation of modulating signals—rather than as binary switches. Clock proteins therefore undergo maturation and daytime-specific functional regulation (Baker *et al*, 2009; Diernfellner *et al*, 2009; Schafmeier *et al*, 2008; Tang *et al*, 2009).

Phosphorylation regulates clock protein localization

The accuracy of the length of the circadian period depends, at least partly, on strict regulation of the import of clock components into the nucleus in a phase-dependent manner. Putative or functional NLSs or NESs have been identified in several clock proteins (Kwon *et al*, 2006; Luo *et al*, 1998; Maurer *et al*, 2009; Miyazaki *et al*, 2001; Saez & Young, 1996; Tamaru *et al*, 2003; Vielhaber *et al*, 2001; Yagita *et al*, 2000), and changes in the subcellular localization of clock components can be regulated by phosphorylation (Fig 1).

The negative element of the clock typically accumulates in the cytoplasm for several hours after its synthesis. In order to repress its own transcription, it must translocate into the nucleus at a certain time of day, allowing the clock to move forward. The positive element of the clock—a transcription factor—has to enter the nucleus to promote the synthesis of the negative element. The clearance of the positive element from the nucleus might also be essential to close the feedback loop and prevent the synthesis of the negative element, as has been observed in *Drosophila* and *Neurospora* (Hong *et al*, 2008; Hung *et al*, 2009; Kim & Edery, 2006). Transcription factor clearance might also be a delay mechanism for the clock, as its absence from the nucleus maintains transcriptional repression. In *Neurospora*, the export of the transcription factor WCC from the nucleus is required for its eventual reactivation (Schafmeier *et al*, 2008). We now consider the effect of phosphorylation on the subcellular localization and activity of clock proteins in different systems.

Drosophila

In *Drosophila*, the negative element of the circadian core oscillator is a heterodimer of the proteins PER and TIMELESS (TIM), in which PER is the main repressor of the clock. The synthesis of PER begins in the subjective morning, but it is quickly degraded unless it binds to and is stabilized by TIM in the cytosol. In the subjective evening, PER and TIM, together with the casein kinase 1ε homologue DBT, translocate to the nucleus, where they inhibit the heterodimeric transcription factor complex CLOCK–CYCLE (CLK–CYC) by removing it from the chromatin. Eventually, PER is phosphorylated, ubiquitinated and degraded and the negative feedback is released (Hardin, 2005; Yu & Hardin, 2006). The phosphorylation of PER is controlled by the kinases DBT, CK-2 and PP-2A (Blau, 2008), whereas the F-box protein SLIMB seems to be responsible for its ubiquitination, which is phosphorylation dependent (Chiu *et al*, 2008; Ko *et al*, 2002).

DBT sets the speed of the circadian clock by controlling the phosphorylation of the amino-terminal region of PER, leading to its eventual degradation (Chiu *et al*, 2008; Price *et al*, 1998). DBT is also required for the transcriptional repression of the CLK–CYC complex by the PER–TIM complex, although CLK–CYC is not a direct target of DBT (Kivimae *et al*, 2008; Nawathean & Rosbash, 2004).

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

In addition to its role in PER degradation, DBT-dependent phosphorylation is thought to have a positive regulatory effect on its localization and function. In fact, of the more than 250 putative phosphorylation sites in the PER protein, many have no effect on the stability of the protein. Furthermore, PER phosphorylation seems to occur in clusters, suggesting that the phosphorylation has different effects in different regions (Chiu *et al*, 2008).

Several studies have indicated that phosphorylation promotes the nuclear entry of the PER–TIM–DBT repressor complex. It has been shown that the phosphorylation of TIM by the glycogen synthase kinase 3 (GSK3) orthologue Shaggy is a prerequisite for nuclear import of the repressor complex, supporting the idea that phosphorylation is important for the regulation of nuclear translocation (Martinek *et al*, 2001). Similarly, the dbt^{S} fly mutant—which has reduced DBT kinase activity—exhibits delayed nuclear translocation of PER in photoreceptor cells (Bao *et al*, 2001) and the knockdown of DBT in cultured S2 cells by RNAi results in impaired nuclear import of PER (Nawathean & Rosbash, 2004). However, DBT-dependent phosphorylation has a negative role in the control of PER nuclear import in flies (Cyran *et al*, 2005), indicating that its role in PER nuclear import remains to be defined.

As the activity of CK-2 in the cytoplasm modulates the timing of PER nuclear import in *Drosophila* neurons, and because PER is phosphorylated *in vitro* by CK-2, it seems likely that CK-2-dependent phosphorylation of PER has a role in its nuclear localization (Akten *et al*, 2003; Lin *et al*, 2002). Michael Rosbash's group has identified a conserved motif in the PER protein that is required for nuclear translocation and facilitates the DBT- and CK-2-dependent phosphorylation of PER (Nawathean *et al*, 2007). Interestingly, this region was identified as a kinase binding domain (Kim *et al*, 2007). However, the researchers were unable to demonstrate its role in the nuclear localization of clock proteins in the latter study.

It was previously thought that PER and TIM enter the nucleus as a complex. However, experiments in fly neurons have shown that PER and TIM are able to enter the nucleus separately (Shafer *et al*, 2002), although PER must be stabilized by reduced levels of DBT for this to happen (Cyran *et al*, 2005). These observations have been supported by FRET analysis of cultured S2 cells overexpressing PER and TIM. This showed that the PER–TIM dimer dissociates before both proteins enter the nucleus separately (Meyer *et al*, 2006).

With regard to the positive elements in the *Drosophila* clock, daytime-specific alterations in the phosphorylation state of the CLK protein regulate the stability and activity of the CLK–CYC transcription factor. Kinases that might modulate CLK–CYC activity include MAPK and CAMKII (Weber *et al*, 2006), whereas the inactivation and degradation of CLK depends on the presence of PER/DBT in the nucleus (Kim & Edery, 2006; Yu *et al*, 2006). Studies using cultured insect cells have indicated that hypophosphorylated CLK and CYC interact in the cytoplasm, resulting in the intermediate phosphorylation of CLK—supported by CYC—and the nuclear translocation of CLK and CYC (Maurer *et al*, 2009). By contrast, the nuclear export of the CLK–CYC complex is accompanied by hyperphosphorylation and subsequent degradation of CLK (Hung *et al*, 2009). This suggests that phosphorylation regulates both the activity and subcellular localization of the CLK–CYC heterodimer.

CLK seems to enter and leave the nucleus once a day. In one of the above studies, it was found to undergo sequential phosphorylation at particular sites with different functions in subcellular shuttling (Hung *et al*, 2009). The expression of PER leads to the cytoplasmic accumulation of CLK (Kim & Edery, 2006). This observation could now be explained by the inactivating hyperphosphorylation of CLK, mediated by the PER–TIM–DBT complex.

Several studies have investigated the nuclear import of clock proteins, but little is known about their shuttling dynamics in *Drosophila*. Some clock proteins contain an NES and inhibiting their export from the nucleus affects circadian rhythmicity, indicating a role for shuttling in circadian clock regulation in flies (Ashmore *et al*, 2003; Hung *et al*, 2009; Nawathean & Rosbash, 2004). Both positive and negative roles for phosphorylation in the nuclear localization of clock proteins have been proposed in recent years. Findings have been inconsistent, perhaps due to differences in the experimental procedures—such as the use of fly neurons or cultured insect cells. It is also possible that phosphorylation at certain sites could stimulate nuclear accumulation of clock proteins, whereas others could inhibit it.

Glossary

ARNT	aryl hydrocarbon receptor nuclear translocator
BMAL	brain and muscle ARNT-like protein
CaMK	calcium/calmodulin-dependent kinase
CK	casein kinase
CLK/CLOCK	circadian locomotor output cycles kaput
CYC	cycle
DBT	DOUBLETIME
FRET	Förster resonance energy transfer
FRH	frequency interacting RNA helicase
GSK	glycogen synthase kinase
MAPK	mitogen-activating protein kinase
NES	nuclear export signal
NF-κB	nuclear factor κB
NLS	nuclear localization signal
PAS	PER–ARNT–SIM domain
PKA/C	protein kinase A/C
PP	protein phosphatase
SIM	single-minded
SLIMB	supernumerary limbs
WCC	white collar complex

Mammals

Mammalian circadian clocks have mainly been studied in mice, rats and hamsters, as well as in other animal and human cell lines. In the circadian system of mammals, the negative elements of the core feedback loop are PER1, 2 and 3 and CRYPTOCHROME (CRY)1 and 2. The positive elements are the transcription factors CLOCK and BMAL1. These components of the clock are the orthologues or functional equivalents of the *Drosophila* circadian clock proteins. However, the regulation of the mammalian system might be more complex, due to the partial redundancy of the three PER and two CRY isoforms. In contrast to the *Drosophila* system, the main repressors of the mammalian clock are the CRY isoforms—the regulation of which is poorly understood—rather than the PER isoforms (Yu & Hardin, 2006).

Mammalian PER proteins are substrates of CK-1δ and ε, which regulate their stability and subcellular localization (Akashi *et al*, 2002; Lee *et al*, 2009). Despite the observation that the CK-1δ and ε kinases show a degree of functional redundancy, the disruption of CK-1δ has a more severe effect on the abundance of PER and its phosphorylation than CK-1ε, indicating a dominant role for the δ isoform in clock regulation (Etchegaray *et al*, 2009).

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Similarly to *Drosophila*, nuclear accumulation of PERs is delayed by approximately 6 h with respect to the appearance of maximal transcript levels, indicating that a similar timing mechanism for PER nuclear entry functions in mammals. The regulation of PER nuclear localization has been investigated in murine and human cell cultures. The data suggest a role for CK-1-dependent phosphorylation not only in the degradation of PER proteins, but also in the regulation of their subcellular localization (Miyazaki *et al*, 2004; Takano *et al*, 2004). Serine residues in a region including amino acids 653–663 are phosphorylated by CK-1ε, which leads to nuclear translocation of PER1 (Takano *et al*, 2004). At the end of a circadian period PER1 is degraded. This seems to happen in the nucleus, as blocking the export of PER1 does not influence its turnover in human WI-38 cells (Miyazaki *et al*, 2004). As has been the case in fly studies, analyses of the regulation of the subcellular localization of clock proteins in mammals have yielded some inconsistent observations. An inhibitory role for CK-1-dependent phosphorylation in nuclear localization of PER1 has been proposed by the Virshup lab (Vielhaber *et al*, 2001).

Defective PER2 phosphorylation can lead to a shortened circadian period and has been implicated in familial advanced sleep phase syndrome (FASPS). In one type of FASPS, the defect has been mapped to a change from a serine to a glycine at position 662 in the human PER2 protein (Toh *et al*, 2001). Interestingly, the FASPS PER2 protein is turned over more rapidly than the wild type, indicating that phosphorylation of Ser 662 stabilizes PER2. Faster turnover is apparently caused by the rapid clearance of PER2 from the nucleus (Vanselow *et al*, 2006), which indicates that phosphorylation of Ser 662 has a role in the nuclear localization of mammalian PER2. By generating transgenic mice that express the FASPS PER2 protein, the human phenotype could be reproduced (Xu *et al*, 2007). However, the authors suggested that the role of PER2 Ser 662 is in the enhancement of transcriptional repression rather than the promotion of nuclear localization. The function of this protein remains to be understood.

PER and CRY proteins seem to simultaneously enter the nuclei of suprachiasmatic nucleus cells, in which the master clock is located in vertebrates (Field *et al*, 2000; Tamanini *et al*, 2005), suggesting that dimerization is a prerequisite for nuclear translocation of the PER/CRY complex. However, PER2 has been detected in the nuclei of neurons obtained from *Cry1/2*-deficient mice (Shearman *et al*, 2000), whereas in Cos7 and embryonic fibroblast cell lines, murine PER2 shuttles between the nucleus and the cytoplasm in the absence of CRY proteins, but accumulates in the nucleus when CRYs are coexpressed. These findings have led to the suggestion that CRYs might sequester PERs to the nucleus, where they stabilize each other. This might be a prerequisite for efficient inhibition of the CLOCK/BMAL1 complex (Yagita *et al*, 2002). A common mechanism seems to function in flies and mammals: a negative-acting element undergoes cycles of synthesis and degradation unless it is stabilized by its interaction partner at a certain time of the day. Such stabilization results in nuclear translocation of the proteins and the formation of a functional inhibitory complex.

The subcellular distribution of CLOCK and BMAL1 is also regulated in a circadian manner (Kondratov *et al*, 2003; Tamaru *et al*, 2003). Dimerization induces the phosphorylation and nuclear translocation of CLOCK, eventually leading to its degradation (Kondratov *et al*, 2003). This is similar to the *Drosophila* system, in which CYC promotes the phosphorylation and nuclear import of CLK (Maurer *et al*, 2009). It has recently been shown in a heterokaryon assay using NIH 3T3 and HeLa cells that BMAL1 shuttles between the cytosol and the nucleus in a Crm 1 dependent manner when it is transcriptionally active. Functional NLS and NES domains have also been identified. Shuttling seems to be required for the nuclear accumulation of CLOCK and for CLOCK/BMAL1 transactivation, which is accompanied by the rapid degradation of both transcription factors in the nucleus (Kwon *et al*, 2006). A recent study revealed that a conserved serine residue in BMAL1 (Ser 90) is rhythmically phosphorylated by the kinase CK-2. In cultured cells in which CK-2α was silenced or Ser 90 of BMAL1 was mutated, the nuclear accumulation of BMAL1 was reduced (Tamaru *et al*, 2009). The nuclear translocation of CLOCK/BMAL1 in the mammalian clock therefore seems to depend on site-specific phosphorylation events.

Neurospora

The components of the *Neurospora* clock are not homologous to those proteins found in flies and mammals, but the principle of the feedback loops is conserved. As it has the simplest loop, the *Neurospora* clock is the best understood circadian oscillator. The negative element FRQ represses its own transcription by mediating phosphorylation-dependent inactivation of the transcription factor WCC (Sancar *et al*, 2009; Schafmeier *et al*, 2005). However, FRQ also has a positive function with regard to WCC, as it is required to support its accumulation (Cheng *et al*, 2001; Lee *et al*, 2000; Schafmeier *et al*, 2006).

FRQ forms part of a high-molecular-weight complex that includes CK-1a and FRH, both of which are required for negative and positive feedback (Cheng *et al*, 2005; Gorl *et al*, 2001; Guo *et al*, 2009; He *et al*, 2006; Hong *et al*, 2008; Querfurth *et al*, 2007). It has been shown that phosphorylation events modulate the stability and function of FRQ (Gorl *et al*, 2001; Jolma *et al*, 2006; Liu *et al*, 2000; Schafmeier *et al*, 2006) and that FRQ has many putative phosphorylation sites. Several kinases and phosphatases have therefore been implicated in the control of its phosphorylation status (Cha *et al*, 2008; Gorl *et al*, 2001; Mehra *et al*, 2009b; Pregueiro *et al*, 2006; Yang *et al*, 2003; 2002; 2001; 2004).

The majority of FRQ is located in the cytoplasm, even though it contains an NLS and nuclear import is required for the proper function of the protein (Luo *et al*, 1998; Schafmeier *et al*, 2005). However, when FRQ expression is driven by an inducible promoter, the newly synthesized, hypophosphorylated FRQ rapidly accumulates in the nucleus (Schafmeier *et al*, 2006). This is essentially the same as the situation in the cell after the onset of FRQ expression in the late subjective morning. On further induction, FRQ is progressively hyperphosphorylated and accumulates in the cytosol. The FRQ populations apparently have different functions: the hypophosphorylated nuclear FRQ inactivates WCC, whereas the hyperphosphorylated cytosolic FRQ is mainly required to support WC-1 expression. Therefore, the positive and negative functions of FRQ are separated both spatially and temporally (Schafmeier *et al*, 2006). We have recently shown that both functions arise from the same mechanism, in which FRQ-dependent phosphorylation prevents the WCC binding to DNA. Keeping the WCC inactive protects it from degradation (Schafmeier *et al*, 2008).

Recent findings have indicated that subcellular localization of FRQ is directly regulated by phosphorylation (Diernfellner *et al*, 2009). FRQ undergoes rapid shuttling cycles between the cytosol and the nucleus; the import kinetics of FRQ slow down when the protein is phosphorylated and import eventually stops when FRQ is hyperphosphorylated. This is consistent with the earlier finding that hypophosphorylated FRQ rapidly accumulates in the nucleus

 ©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

(Fig 2; Diernfellner *et al*, 2009; Schafmeier *et al*, 2006). As a result, distribution of the newly synthesized FRQ shifts from being predominantly nuclear to predominantly cytosolic over the course of a day, correlating with its functional maturation (Schafmeier *et al*, 2006). A recent study which used live-cell imaging of *Neurospora* cells expressing FRQ-mCherry found that, under physiological conditions, FRQ undergoes a second round of nuclear accumulation during a circadian cycle. This indicates that the regulation of FRQ nuclear import is not yet fully understood (Castro-Longoria *et al*, 2010).

WCC is composed of the subunits WC-1 and WC-2, which interact through their PAS-domains (Talora *et al*, 1999). WC-1 is not stably expressed in the absence of its interacting partner WC-2 and is therefore considered to be the rate-limiting factor of the complex (Cheng *et al*, 2002). By contrast, the WC-2 subunit is expressed in excess and is stable in the absence of WC-1 (Cheng *et al*, 2001; Schwerdtfeger & Linden, 2000). Negative feedback occurs by FRQ-mediated CK-1a and CK-2-dependent phosphorylation of WCC (He *et al*, 2006; Schafmeier *et al*, 2005).

WC-1 is regulated by PKA and PKC-dependent phosphorylation (Franchi *et al*, 2005; Huang *et al*, 2007). As it is a transcription factor, the nuclear accumulation of WCC is required for it to function properly and both WCC subunits contain a putative NLS (Ballario *et al*, 1998). The nuclear localization of WC-1 and WC-2 has been confirmed by cell fractionation (Schafmeier *et al*, 2005; Schwerdtfeger & Linden, 2000), although fluorescence microscopy of *Neurospora* cells expressing GFP-tagged WC-1 or WC-2 showed a larger cytoplasmic pool of WCC in living cells than had been expected from biochemical data. Furthermore, when the nuclei of these strains were photobleached by a laser pulse, fluorescence recovered within a few minutes, indicating that a rapid exchange of the nuclear WCC pool occurs (Schafmeier *et al*, 2008).

In steady-state conditions, nuclear WCC is hyperphosphorylated, whereas the phosphorylation status of the cytosolic WCC fraction is low (Schafmeier *et al*, 2005). Two protein phosphatases—PP-2A and PP-4—have recently been shown to dephosphorylate WCC. This counteracts the CK-1a/-2-dependent phosphorylations of the WCC and leads to its reactivation (Cha *et al*, 2008; Schafmeier *et al*, 2008; Schafmeier *et al*, 2005). At least in the case of PP-2A, dephosphorylation occurs exclusively in the cytoplasm. When it is compromised, the cytoplasmic accumulation of hyperphosphorylated WCC occurs (Schafmeier *et al*, 2008).

Taken together, these findings suggest that WCC is phosphorylated by CK-1a and -2 in the nucleus and dephosphorylated by PP-2A—and probably also PP-4—immediately after its export. Subcellular shuttling must therefore be accompanied by rapid cycles of phosphorylation and dephosphorylation. Although the cytosolic WCC is mainly hypophosphorylated in steady-state conditions, hyperphosphorylated WCC tends to accumulate in the cytoplasm at certain points in the circadian period (Cha *et al*, 2008; Schafmeier *et al*, 2008). The accumulation of WCC in the cytosol depends on FRQ (Hong *et al*, 2008) and can be explained by FRQ-dependent phosphorylation of WCC. This dominates PP-2A-dependent dephosphorylation when cytoplasmic FRQ levels are high. FRQ therefore regulates transcriptional repression and accumulation of WCC by the same CK-1a/-2-dependent phosphorylation mechanism. Recent findings about the phosphorylation-dependent regulation of WCC are summarized in Fig 3.

The subcellular localization of FRQ might be regulated in a similar way. Rapid cycles of nucleo-cytoplasmic shuttling of FRQ are

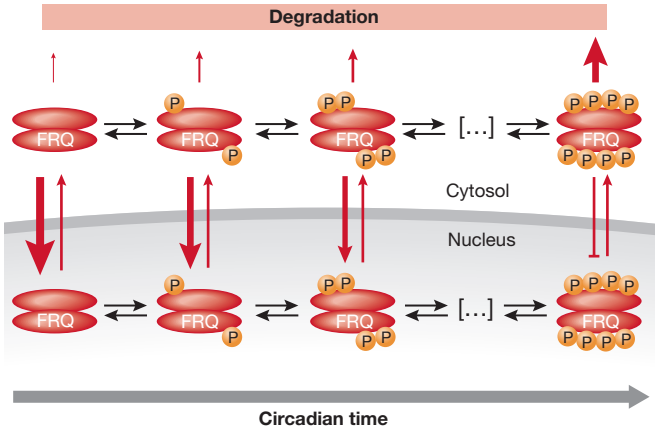

Fig 2 | The phosphorylation-dependent maturation of FRQ determines its subcellular localization and stability. Newly synthesized FRQ is progressively phosphorylated in an hourglass-like manner at multiple sites over the course of a circadian cycle. The higher the phosphorylation state of FRQ, the lower the nuclear import kinetics. Hyperphosphorylation also targets FRQ for degradation (Diernfellner *et al*, 2009). FRQ, FREQUENCY; P, phosphate.

modulated by phosphorylation in a circadian manner. This can explain the seemingly contradictory functions of FRQ, by means of temporal and spatial separation. As the progressive phosphorylation of FRQ results in slower import kinetics, FRQ accumulates in the cytosol in the subjective afternoon and keeps WCC in a phosphorylated state, leading to the stabilization and retention of WCC in the cytoplasm.

Hourglasses compared with phospho-switches

The *Neurospora* clock protein FRQ is phosphorylated at a minimum of 85 sites (Baker *et al*, 2009; Tang *et al*, 2009). Although certain phosphorylation sites have been linked to specific functions—including Ser 513 and Ser 885/887 (Liu *et al*, 2000; Schafmeier *et al*, 2006)—it does not seem likely that each phosphorylation event has a particular role in the regulation of FRQ.

Proteomic analysis has shown that FRQ phosphorylation occurs in clusters (Tang *et al*, 2009), suggesting that the phosphorylation of certain regions affects the charge in these areas. This might result in substantial conformational alterations, such as partial unfolding or loss of interaction between distinct regions (Diernfellner *et al*, 2009; C Querfurth, A Diernfellner & M Brunner, unpublished data; Tang *et al*, 2009). Eventually, clustered hyperphosphorylation could facilitate or prevent the binding of FRQ to interaction partners such as CK-1, thereby altering the characteristics of the FRQ complex in a circadian manner (Baker *et al*, 2009; C Querfurth, A Diernfellner & M Brunner, unpublished data). Experiments in which the phosphorylation sites in FRQ were mutated have found opposing effects on circadian period length—some lengthening it and some shortening it—which is similar to the effects of distinct PER2 phosphorylations in mammals (Tang *et al*, 2009; Vanselow *et al*, 2006).

In order to act as a timing mechanism—to ensure that FRQ is not simply hyperphosphorylated and degraded—FRQ must also be dephosphorylated by one or more protein phosphatases. PP-1, PP-2A and PP-4 are candidates for counteracting the phosphorylation of FRQ (Cha *et al*, 2008; Yang *et al*, 2004). One might speculate that

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

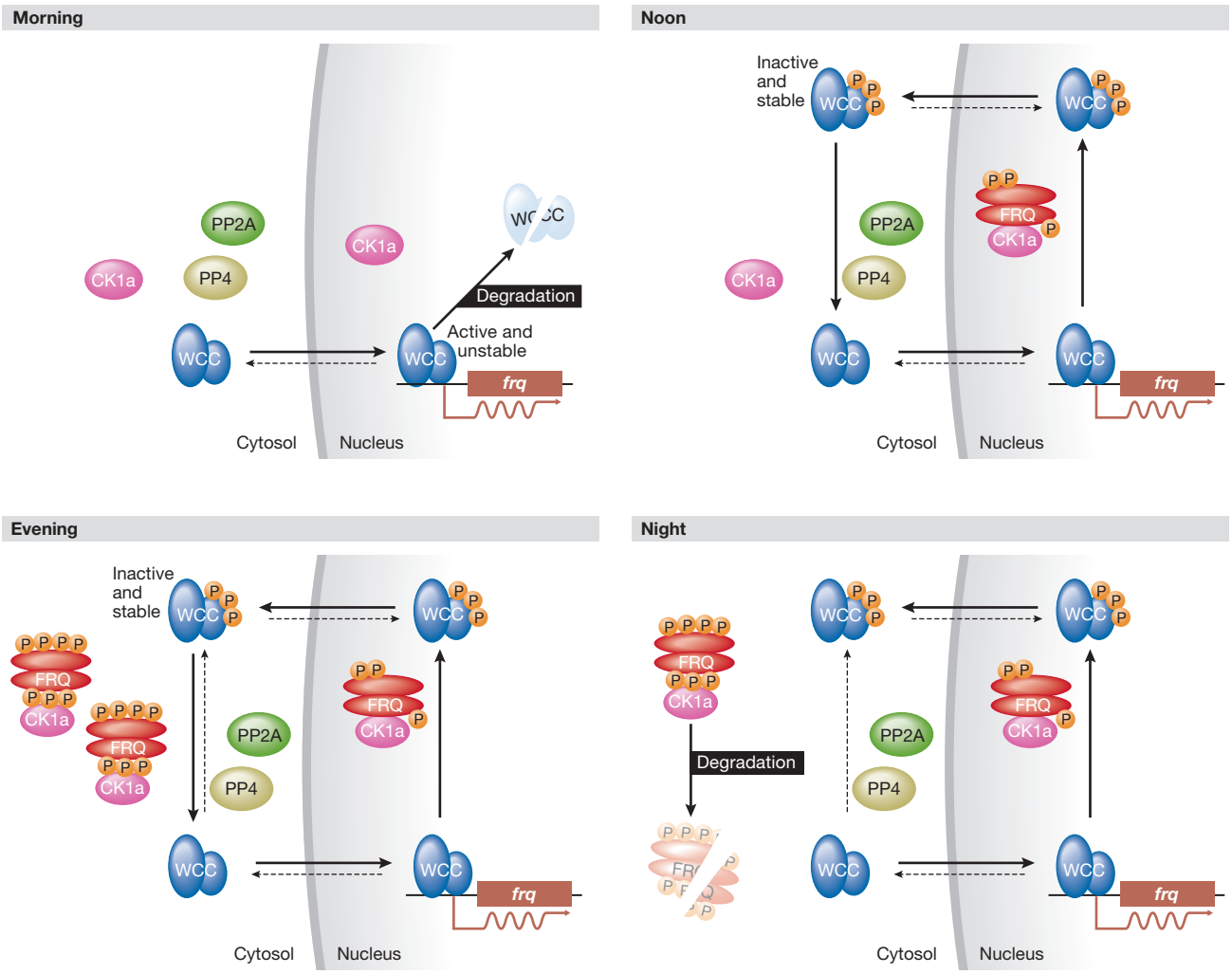

Fig 3 | Modulation of WCC shuttling and activity in the course of a circadian day. Morning (top left): Newly synthesized, unphosphorylated WCC enters the nucleus. It is active and binds to target DNA regions, for example in the *frq* gene locus, but active WCC is rapidly degraded. Noon (top right): By inactivating WCC through phosphorylation, FRQ stabilizes WCC in the nucleus and supports the accumulation of the transcription factor. Evening (bottom left): FRQ accumulates in the cytosol and FRQ-mediated phosphorylation dominates over dephosphorylation. Consequently, the import kinetics of WCC decrease and the stable complex is retained in the cytoplasm. Night (bottom right): FRQ is degraded and then WCC is dephosphorylated—thereby activated—and re-enters the nucleus. CK, casein kinase; FRQ, FREQUENCY; P, phosphate; PP, protein phosphatase; WCC, white collar complex.

progressive FRQ phosphorylation occurs through a 'two steps forward, one step back' mechanism. CK-1a could phosphorylate FRQ at several sites before targeting itself and thereby inactivating its kinase activity. A phosphatase could then remove some of the phosphates from both proteins, allowing the reactivation of CK-1a and ongoing phosphorylation of FRQ.

As a result of progressive phosphorylation, the stability, function and subcellular localization of FRQ can be modulated in an hourglass-like manner throughout the day. This would provide a simple yet elegant molecular timer for a 24-h period (Fig 2).

Similarly to the *Neurospora* FRQ protein, the mammalian negative clock element PER2 has almost 250 putative phosphorylation sites. Proteomic analyses have found that at least 21 sites are phosphorylated in murine PER2 and that these appear in clusters (Schlosser *et al*, 2005; Vanselow *et al*, 2006). Phospho-clusters have also been identified in the *Drosophila* PER protein (Chiu *et al*, 2008). This suggests that both PER proteins and FRQ are regulated by clustered phosphorylation, but there is a discrepancy between the number of phosphorylation sites that have been identified. Future research might attempt to identify more sites in the PER proteins of flies and mammals. Otherwise, although FRQ seems to be regulated by an hourglass mechanism, the regulation of PER might occur by sequential phosphorylation of distinct sites, or through clusters that have specific effects.

WC-1 matures over the course of a day as a consequence of the equilibrium between the activities of kinase and phosphatase, which function to delay phosphorylation. For WC-1 and WC-2, only a few phosphorylation sites have been identified so far (He *et al*, 2005; Sancar *et al*, 2009). WCC localization is therefore probably not regulated by hourglass-like hyperphosphorylation, but rather by sequential phosphorylation at specific sites, similar to the regulation of the

 ©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

activity and subcellular localization of the *Drosophila* CLK protein (Hung *et al*, 2009).

Both types of regulation by phosphorylation—switches and hourglasses—seem to be able to have a role in the control of circadian clocks.

New ways to control nuclear import in circadian clocks?

Recent studies have shown that the *Neurospora* clock proteins FRQ and WCC undergo rapid cycles of nucleo-cytoplasmic shuttling, which are modulated by phosphorylation (Diernfellner *et al*, 2009; Schafmeier *et al*, 2008). The phosphorylation-dependent import, export, activation or inactivation of transcription factors commonly regulates gene expression (Cyert, 2001). The activity or subcellular localization of a transcription factor is often modified in a switch-like manner in response to environmental or internal stimuli.

One exception to this switch-like modulation is the calcineurin-responsive transcription factor Crz1 in budding yeast, which mediates the expression of genes in response to calcium stress. An extracellular increase in calcium concentration has been found to lead to the dephosphorylation and nuclear localization of Crz1 (Stathopoulos-Gerontides *et al*, 1999). However, a recent study has shown that Crz1 is localized to the nucleus in short bursts when calcium is present and that as the calcium concentration increases, the frequency of these bursts also increases. As a result, the nuclear accumulation of Crz1 and the eventual transcriptional activation of Crz1 target genes is faster (Cai *et al*, 2008). The nuclear accumulation of WCC might be regulated in a similar manner; an increased frequency of active WCC import in the subjective morning is then hypophosphorylated, driving the expression of clock-controlled genes.

Cai *et al* (2008) also reported in the Crz1 study that Msn2—another transcription factor that exhibits oscillations of nuclear translocation in response to stress or nutritional changes (Jacquet *et al*, 2003)—shows similar import bursts and nuclear accumulation in response to calcium.

NF-κB shows a similar pattern; its activity is generally linked to oscillations of nuclear entry frequency. The frequency of nuclear NF-κB translocation is decreased in response to increased amounts of the inhibitor IκB (Nelson *et al*, 2004). These observations from distinct pathways in different organisms suggest that import frequency modulation of shuttling transcription factors is a common, yet unconventional mechanism of regulating gene expression in response to stimuli.

What is the advantage of rapid shuttling cycles that can be modulated, rather than a time-of-day-specific 'import on demand' mechanism? In *Drosophila*, newly synthesized PER is rapidly degraded in the subjective morning until it can bind to its interaction partner TIM in the subjective evening. This regulatory mechanism ensures a delay that contributes to the timing of the approximately 24-h period, but it seems energetically wasteful as PER continues to be synthesized and degraded throughout the day until the 'import' signal is activated. By comparison, the seemingly futile cycles of translocation in *Neurospora* might be energy saving; the *Neurospora* negative element—FRQ—is not permanently degraded in the subjective morning and so its biosynthesis rate can be lower than that of PER. Furthermore, modulation of the shuttling frequency might make the circadian oscillation more robust, as perturbations can be buffered.

In other clock-model organisms there is evidence for nucleo-cytoplasmic shuttling—that is, of PER, TIM and CLK in *Drosophila*, and of PER2 and BMAL1 in mammals (Fig 1; Ashmore *et al*, 2003; Hung *et al*, 2009; Kwon *et al*, 2006; Vielhaber *et al*, 2001; Yagita *et al*, 2002). BMAL1 shuttles when it is transcriptionally active and is stored in the nucleus when it is inactivated by CRY1 or CRY2 (Kwon *et al*, 2006). This is similar to the regulation of WCC in *Neurospora*, which shuttles rapidly until FRQ levels increase, slowing down import kinetics and leading to the cytoplasmic accumulation of WCC.

Sidebar A | In need of answers

(i) Are there post-translational modifications other than phosphorylation that regulate the subcellular distribution of clock proteins?
(ii) Is rapid shuttling a general feature of the regulation of subcellular localization in circadian clocks?
(iii) Why might rapid shuttling cycles be advantageous, compared to strictly directed import of clock components?
(iv) Which kinases control the shuttling of FRQ and WCC in *Neurospora*?
(v) Could the nuclear abundance of rapid-shuttling clock proteins be regulated by import frequency modulation?
(vi) Is the hourglass-like progressive phosphorylation of FRQ a unique phenomenon among circadian clock proteins?

Conclusions

The proteins that constitute the circadian feedback loops in organisms from fungi to humans are modified post-translationally by phosphorylation. In addition to its well-characterized functions in regulating the turnover of clock proteins, phosphorylation also determines their subcellular localization. There seem to be more differences than similarities in the regulation of nuclear translocation between fungi, mice and flies, but investigations have also shown several common aspects. Both the import and export of clock proteins to and from the nucleus are required to sustain the circadian oscillation, and these processes contribute to molecular timekeeping. Furthermore, the transcription factors of the mammalian and *Neurospora* circadian system undergo rapid cycles of shuttling that are slowed down or abolished when transcriptional activity is low. The negative elements PER and FRQ both undergo progressive, clustered phosphorylation at several sites, which seems to be important for maturation; although, at least in the case of FRQ in *Neurospora*, hyperphosphorylation results in cytoplasmic accumulation of the protein. Further analysis of the subcellular shuttling of clock proteins is an important avenue for research, in order to fully understand circadian clock regulation (Sidebar A).

ACKNOWLEDGEMENTS

We thank Michael Brunner for helpful comments on the manuscript. Work was supported by the Deutsche Forschungsgemeinschaft grant BR 1375-3-1 to T.S. and a PhD fellowship of the Excellence Cluster CellNetworks to Ö.T, who is also a member of the Hartmut Hoffmann-Berling International Graduate School of Molecular and Cellular Biology (HBIGS).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Akashi M, Tsuchiya Y, Yoshino T, Nishida E (2002) Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. *Mol Cell Biol* **22:** 1693–1703

Akten B, Jauch E, Genova GK, Kim EY, Edery I, Raabe T, Jackson FR (2003) A role for CK2 in the *Drosophila* circadian oscillator. *Nat Neurosci* **6:** 251–257

Ashmore LJ, Sathyanarayanan S, Silvestre DW, Emerson MM, Schotland P, Sehgal A (2003) Novel insights into the regulation of the timeless protein. *J Neurosci* **23:** 7810–7819

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Bae K, Edery I (2006) Regulating a circadian clock's period, phase and amplitude by phosphorylation: insights from *Drosophila*. *J Biochem* **140:** 609–617
Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC (2009) Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol Cell* **34:** 354–363
Ballario P, Talora C, Galli D, Linden H, Macino G (1998) Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. *Mol Microbiol* **29:** 719–729
Bao S, Rihel J, Bjes E, Fan JY, Price JL (2001) The *Drosophila* double-timeS mutation delays the nuclear accumulation of period protein and affects the feedback regulation of period mRNA. *J Neurosci* **21:** 7117–7126
Blau J (2008) PERspective on PER phosphorylation. *Genes Dev* **22:** 1737–1740
Brunner M, Kaldi K (2008) Interlocked feedback loops of the circadian clock of *Neurospora crassa*. *Mol Microbiol* **68:** 255–262
Brunner M, Schafmeier T (2006) Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and *Neurospora*. *Genes Dev* **20:** 1061–1074
Cai L, Dalal CK, Elowitz MB (2008) Frequency-modulated nuclear localization bursts coordinate gene regulation. *Nature* **455:** 485–490
Castro-Longoria E, Ferry M, Bartnicki-Garcia S, Hasty J, Brody S (2010) Circadian rhythms in *Neurospora crassa*: dynamics of the clock component frequency visualized using a fluorescent reporter. *Fungal Genet Biol* **47:** 332–341
Cha J, Chang SS, Huang G, Cheng P, Liu Y (2008) Control of WHITE COLLAR localization by phosphorylation is a critical step in the circadian negative feedback process. *EMBO J* **27:** 3246–3255
Cheng P, He Q, Wang L, Liu Y (2005) Regulation of the Neurospora circadian clock by an RNA helicase. *Genes Dev* **19:** 234–241
Cheng P, Yang Y, Gardner KH, Liu Y (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of *Neurospora*. *Mol Cell Biol* **22:** 517–524
Cheng P, Yang Y, Liu Y (2001) Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. *Proc Natl Acad Sci USA* **98:** 7408–7413
Chiu JC, Vanselow JT, Kramer A, Edery I (2008) The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes Dev* **22:** 1758–1772
Cyert MS (2001) Regulation of nuclear localization during signaling. *J Biol Chem* **276:** 20805–20808
Cyran SA, Yiannoulos G, Buchsbaum AM, Saez L, Young MW, Blau J (2005) The double-time protein kinase regulates the subcellular localization of the *Drosophila* clock protein period. *J Neurosci* **25:** 5430–5437
Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* **72:** 517–549
Diernfellner AC, Querfurth C, Salazar C, Hofer T, Brunner M (2009) Phosphorylation modulates rapid nucleocytoplasmic shuttling and cytoplasmic accumulation of *Neurospora* clock protein FRQ on a circadian time scale. *Genes Dev* **23:** 2192–2200
Dunlap JC, Loros JJ (2006) How fungi keep time: circadian system in *Neurospora* and other fungi. *Curr Opin Microbiol* **9:** 579–587
Edery I, Zwiebel LJ, Dembinska ME, Rosbash M (1994) Temporal phosphorylation of the *Drosophila* period protein. *Proc Natl Acad Sci USA* **91:** 2260–2264
Etchegaray JP *et al* (2009) Casein kinase 1 delta regulates the pace of the mammalian circadian clock. *Mol Cell Biol* **29:** 3853–3866
Field MD, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH (2000) Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* **25:** 437–447
Franchi L, Fulci V, Macino G (2005) Protein kinase C modulates light responses in *Neurospora* by regulating the blue light photoreceptor WC-1. *Mol Microbiol* **56:** 334–345
Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol* **8:** 139–148
Garceau NY, Liu Y, Loros JJ, Dunlap JC (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. *Cell* **89:** 469–476
Gorl M, Merrow M, Huttner B, Johnson J, Roenneberg T, Brunner M (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. *EMBO J* **20:** 7074–7084
Guo J, Cheng P, Yuan H, Liu Y (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. *Cell* **138:** 1236–1246
Hardin PE (2005) The circadian timekeeping system of *Drosophila*. *Curr Biol* **15:** R714–722
He Q, Cha J, He Q, Lee HC, Yang Y, Liu Y (2006) CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the *Neurospora* circadian negative feedback loop. *Genes Dev* **20:** 2552–2565
He Q, Shu H, Cheng P, Chen S, Wang L, Liu Y (2005) Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. *J Biol Chem* **280:** 17526–17532
Hong CI, Ruoff P, Loros JJ, Dunlap JC (2008) Closing the circadian negative feedback loop: FRQ-dependent clearance of WC-1 from the nucleus. *Genes Dev* **22:** 3196–3204
Huang G, Chen S, Li S, Cha J, Long C, Li L, He Q, Liu Y (2007) Protein kinase A and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. *Genes Dev* **21:** 3283–3295
Hung HC, Maurer C, Zorn D, Chang WL, Weber F (2009) Sequential and compartment-specific phosphorylation controls the life cycle of the circadian CLOCK protein. *J Biol Chem* **284:** 23734–23742
Jacquet M, Renault G, Lallet S, De Mey J, Goldbeter A (2003) Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*. *J Cell Biol* **161:** 497–505
Jolma IW, Falkeid G, Bamerni M, Ruoff P (2006) Lithium leads to an increased FRQ protein stability and to a partial loss of temperature compensation in the *Neurospora* circadian clock. *J Biol Rhythms* **21:** 327–334
Jolma IW, Laerum OD, Lillo C, Ruoff P (2010) Circadian oscillators in eukaryotes. *Wiley Interdiscip Rev Syst Biol Med* **2:** 533–549
Kim EY, Edery I (2006) Balance between DBT/CKIepsilon kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. *Proc Natl Acad Sci USA* **103:** 6178–6183
Kim EY, Ko HW, Yu W, Hardin PE, Edery I (2007) A DOUBLETIME kinase binding domain on the *Drosophila* PERIOD protein is essential for its hyperphosphorylation, transcriptional repression, and circadian clock function. *Mol Cell Biol* **27:** 5014–5028
Kivimae S, Saez L, Young MW (2008) Activating PER repressor through a DBT-directed phosphorylation switch. *PLoS Biol* **6:** e183
Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet* **15:** R271–R277
Ko HW, Jiang J, Edery I (2002) Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature* **420:** 673–678
Kondratov RV, Chernov MV, Kondratova AA, Gorbacheva VY, Gudkov AV, Antoch MP (2003) BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. *Genes Dev* **17:** 1921–1932
Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, Lee KH (2006) BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. *Mol Cell Biol* **26:** 7318–7330
Lee C, Bae K, Edery I (1998) The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER–TIM complex. *Neuron* **21:** 857–867
Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107:** 855–867
Lee H, Chen R, Lee Y, Yoo S, Lee C (2009) Essential roles of CKIdelta and CKIepsilon in the mammalian circadian clock. *Proc Natl Acad Sci USA* **106:** 21359–21364
Lee K, Loros JJ, Dunlap JC (2000) Interconnected feedback loops in the *Neurospora* circadian system. *Science* **289:** 107–110
Lin JM, Kilman VL, Keegan K, Paddock B, Emery-Le M, Rosbash M, Allada R (2002) A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature* **420:** 816–820
Liu Y, Loros J, Dunlap JC (2000) Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. *Proc Natl Acad Sci USA* **97:** 234–239
Luo C, Loros JJ, Dunlap JC (1998) Nuclear localization is required for function of the essential clock protein FRQ. *EMBO J* **17:** 1228–1235
Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. *Cell* **105:** 769–779

 ©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Maurer C, Hung HC, Weber F (2009) Cytoplasmic interaction with CYCLE promotes the post-translational processing of the circadian CLOCK protein. *FEBS Lett* **583:** 1561–1566
McDonald MJ, Rosbash M (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107:** 567–578
Mehra A, Baker CL, Loros JJ, Dunlap JC (2009a) Post-translational modifications in circadian rhythms. *Trends Biochem Sci* **34:** 483–490
Mehra A, Shi M, Baker CL, Colot HV, Loros JJ, Dunlap JC (2009b) A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. *Cell* **137:** 749–760
Meyer P, Saez L, Young MW (2006) PER–TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. *Science* **311:** 226–229
Miyazaki K, Mesaki M, Ishida N (2001) Nuclear entry mechanism of rat PER2 (rPER2): role of rPER2 in nuclear localization of CRY protein. *Mol Cell Biol* **21:** 6651–6659
Miyazaki K, Nagase T, Mesaki M, Narukawa J, Ohara O, Ishida N (2004) Phosphorylation of clock protein PER1 regulates its circadian degradation in normal human fibroblasts. *Biochem J* **380:** 95–103
Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. *Science* **308:** 414–415
Nawathean P, Rosbash M (2004) The doubletime and CKII kinases collaborate to potentiate *Drosophila* PER transcriptional repressor activity. *Mol Cell* **13:** 213–223
Nawathean P, Stoleru D, Rosbash M (2007) A small conserved domain of *Drosophila* PERIOD is important for circadian phosphorylation, nuclear localization, and transcriptional repressor activity. *Mol Cell Biol* **27:** 5002–5013
Nelson DE *et al* (2004) Oscillations in NF-κB signaling control the dynamics of gene expression. *Science* **306:** 704–708
Nitabach MN, Taghert PH (2008) Organization of the *Drosophila* circadian control circuit. *Curr Biol* **18:** R84–R93
Oishi K *et al* (2003) Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* **278:** 41519–41527
Pregueiro AM, Liu Q, Baker CL, Dunlap JC, Loros JJ (2006) The *Neurospora* checkpoint kinase 2: a regulatory link between the circadian and cell cycles. *Science* **313:** 644–649
Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94:** 83–95
Querfurth C, Diernfellner A, Heise F, Lauinger L, Neiss A, Tataroglu O, Brunner M, Schafmeier T (2007) Posttranslational regulation of *Neurospora* circadian clock by CK1a-dependent phosphorylation. *Cold Spring Harb Symp Quant Biol* **72:** 177–183
Reppert SM, Weaver DR (2001) Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* **63:** 647–676
Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418:** 935–941
Saez L, Young MW (1996) Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. *Neuron* **17:** 911–920
Sancar G, Sancar C, Brunner M, Schafmeier T (2009) Activity of the circadian transcription factor White Collar Complex is modulated by phosphorylation of SP-motifs. *FEBS Lett* **583:** 1833–1840
Schaffer R, Landgraf J, Accerbi M, Simon V, Larson M, Wisman E (2001) Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell* **13:** 113–123
Schafmeier T, Diernfellner A, Schafer A, Dintsis O, Neiss A, Brunner M (2008) Circadian activity and abundance rhythms of the *Neurospora* clock transcription factor WCC associated with rapid nucleo-cytoplasmic shuttling. *Genes Dev* **22:** 3397–3402
Schafmeier T, Haase A, Kaldi K, Scholz J, Fuchs M, Brunner M (2005) Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. *Cell* **122:** 235–246
Schafmeier T, Kaldi K, Diernfellner A, Mohr C, Brunner M (2006) Phosphorylation-dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor toward a cytoplasmic activator. *Genes Dev* **20:** 297–306
Schlosser A, Vanselow JT, Kramer A (2005) Mapping of phosphorylation sites by a multi-protease approach with specific phosphopeptide enrichment and NanoLC-MS/MS analysis. *Anal Chem* **77:** 5243–5250
Schwerdtfeger C, Linden H (2000) Localization and light-dependent phosphorylation of white collar 1 and 2, the two central components of blue light signaling in *Neurospora crassa*. *Eur J Biochem* **267:** 414–422
Shafer OT, Rosbash M, Truman JW (2002) Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster*. *J Neurosci* **22:** 5946–5954
Shearman LP *et al* (2000) Interacting molecular loops in the mammalian circadian clock. *Science* **288:** 1013–1019
Smith KM *et al* (2010) Transcription factors in light and circadian clock signaling networks revealed by genome-wide mapping of direct targets for *Neurospora* WHITE COLLAR COMPLEX. *Eukaryot Cell* 9:1549–1556
Stathopoulos-Gerontides A, Guo JJ, Cyert MS (1999) Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. *Genes Dev* **13:** 798–803
Takano A, Isojima Y, Nagai K (2004) Identification of mPer1 phosphorylation sites responsible for the nuclear entry. *J Biol Chem* **279:** 32578–32585
Talora C, Franchi L, Linden H, Ballario P, Macino G (1999) Role of a white collar-1–white collar-2 complex in blue-light signal transduction. *EMBO J* **18:** 4961–4968
Tamanini F, Yagita K, Okamura H, van der Horst GT (2005) Nucleocytoplasmic shuttling of clock proteins. *Meth Enzymol* **393:** 418–435
Tamaru T, Hirayama J, Isojima Y, Nagai K, Norioka S, Takamatsu K, Sassone-Corsi P (2009) CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. *Nat Struct Mol Biol* **16:** 446–448
Tamaru T, Isojima Y, van der Horst GT, Takei K, Nagai K, Takamatsu K (2003) Nucleocytoplasmic shuttling and phosphorylation of BMAL1 are regulated by circadian clock in cultured fibroblasts. *Genes Cells* **8:** 973–983
Tang CT, Li S, Long C, Cha J, Huang G, Li L, Chen S, Liu Y (2009) Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. *Proc Natl Acad Sci USA* **106:** 10722–10727
Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM, Ptacek LJ, Fu YH (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291:** 1040–1043
Tomita J, Nakajima M, Kondo T, Iwasaki H (2005) No transcription–translation feedback in circadian rhythm of KaiC phosphorylation. *Science* **307:** 251–254
Vanselow K, Vanselow JT, Westermark PO, Reischl S, Maier B, Korte T, Herrmann A, Herzel H, Schlosser A, Kramer A (2006) Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes Dev* **20:** 2660–2672
Vielhaber EL, Duricka D, Ullman KS, Virshup DM (2001) Nuclear export of mammalian PERIOD proteins. *J Biol Chem* **276:** 45921–45927
Weber F, Hung HC, Maurer C, Kay SA (2006) Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE-dependent transcription. *J Neurochem* **98:** 248–257
Woolum JC (1991) A re-examination of the role of the nucleus in generating the circadian rhythm in *Acetabularia*. *J Biol Rhythms* **6:** 129–136
Xu X, Hotta CT, Dodd AN, Love J, Sharrock R, Lee YW, Xie Q, Johnson CH, Webb AA (2007) Distinct light and clock modulation of cytosolic free Ca^{2+} oscillations and rhythmic CHLOROPHYLL A/B BINDING PROTEIN2 promoter activity in *Arabidopsis*. *Plant Cell* **19:** 3474–3490
Yagita K, Tamanini F, Yasuda M, Hoeijmakers JH, van der Horst GT, Okamura H (2002) Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. *EMBO J* **21:** 1301–1314
Yagita K, Yamaguchi S, Tamanini F, van Der Horst GT, Hoeijmakers JH, Yasui A, Loros JJ, Dunlap JC, Okamura H (2000) Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes Dev* **14:** 1353–1363
Yang Y, Cheng P, He Q, Wang L, Liu Y (2003) Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the *Neurospora* circadian clock. *Mol Cell Biol* **23:** 6221–6228
Yang Y, Cheng P, Liu Y (2002) Regulation of the *Neurospora* circadian clock by casein kinase II. *Genes Dev* **16:** 994–1006
Yang Y, Cheng P, Zhi G, Liu Y (2001) Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the *Neurospora* circadian clock protein FREQUENCY. *J Biol Chem* **276:** 41064–41072
Yang Y, He Q, Cheng P, Wrage P, Yarden O, Liu Y (2004) Distinct roles for PP1 and PP2A in the *Neurospora* circadian clock. *Genes Dev* **18:** 255–260
Yu W, Hardin PE (2006) Circadian oscillators of *Drosophila* and mammals. *J Cell Sci* **119:** 4793–4795
Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev* **20:** 723–733

©2010 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION