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Review Article

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A Role for Timely Nuclear Translocation of Clock Repressor Proteins in Setting Circadian Clock Speed

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By means of a circadian clock system, all the living organisms on earth including human beings can anticipate the environmental rhythmic changes such as light/dark and warm/cold periods in a daily as well as in a yearly manner. Anticipating such environmental changes provide organisms with survival benefits via manifesting behavior and physiology at an advantageous time of the day and year. Cell-autonomous circadian oscillators, governed by transcriptional feedback loop composed of positive and negative elements, are organized into a hierarchical system throughout the organisms and generate an oscillatory expression of a clock gene by itself as well as clock controlled genes (ccgs) with a 24 hr periodicity. In the feedback loop, hetero-dimeric transcription factor complex induces the expression of negative regulatory proteins, which in turn represses the activity of transcription factors to inhibit their own transcription. Thus, for robust oscillatory rhythms of the expression of clock genes as well as ccgs, the precise control of sub-cellular localization and/or timely translocation of core clock protein are crucial. Here, we discuss how sub-cellular localization and nuclear translocation are controlled in a time-specific manner focusing on the negative regulatory clock proteins.

Key words: circadian rhythms, nuclear translocation, phosphorylation, posttranslational modification, O-GlcNAcylation

INTRODUCTION

The molecular clock present in nearly every cell is composed of transcriptional/translational feedback loop, namely TTFL [1]. Although specific components of TTFL are different, the governing rules of TTFL are well conserved from fungi to vertebrate, including humans [2]. Current understanding of the underlying biochemical mechanisms in animal circadian clockworks is largely based on earlier studies using the *Drosophila* as a model system [3, 4]. In 1971, the pioneering behavioral geneticists Seymour Benzer and Ron Konopka searched for mutant flies having defects in daily rhythmic eclosion, a process of flies coming out of the pupae that happens mostly early in the morning [5]. During this screening, they identified 3 lines of mutant flies with affected eclosion rhythm in the population. One mutant was arrhythmic; another had a short (~19 hr) period; the third had a long period (~28 hr). These mutants were named per^0, per^S, and per^L, respectively. A decade later, all three mutations turned out to be present on a single gene. This gene was named period after the mutants and became the first "clock gene." Many more clock genes were identified through genetic analysis in the following years in the *Drosophila*. Also, homology search revealed

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the mammalian clock genes, except the *Clock*. *Clock* (*Circadian Locomotor Output Cycles Kaput*) was identified in 1994 by Takahashi and his colleagues through forward ENU mutagenesis screen and is homologous to the *Drosophila Clk* [6, 7].

In the *Drosophila*, two basic-helix-loop helix and PAS containing transcription factors, dCLOCK (dCLK) and CYCLE (CYC) dimerize and induce the transcription of *period* (*per*) and *timeless* (*tim*) by binding to the E box (CACGAG) sequence element of genome at midday [8]. The rise in the newly made *per* and *tim* mRNA leads to the accumulation of PER and TIM proteins in the cytoplasm as the herterodimer form during the early evening. After a ~4 hr delay in the cytoplasm, PER and TIM translocate to the nucleus, presumably in a separate manner, to repress the transcriptional activity of dCLK/CYC resulting in a down-regulation of their own mRNA levels constituting namely the "core-loop." In the so-called "stabilizing-loop," the expression of *dClk* is controlled and interlocks with the "core-loop." dCLK-CYC stimulates the expression of two bZip containing transcription factors, *vrille* (*vri*) and *PAR domain protein 1 ε* (PDP1ε). While VRI represses the expression of *dClk* at early night, PDP1ε mediated stimulation of *dClk* is followed 3~4 hrs later generating a daily rhythmic oscillation of *dClk* mRNA levels. Due to this relationship among the proteins in the feedback loops, *dClk* mRNA levels cycle in an anti-phasic fashion to mRNA levels of *per*, *tim*, *vri*, and *pdp1ε* in a day. On the other hand, overall daily levels of *cyc* mRNA manifests no daily oscillation. Another dCLK/CYC downstream clock gene, bHLH orange domain putative transcription factor *clock work orange* (*cwo*) works as an oscillator amplifier by repressing and/or activating dCLK/CYC mediated transcription [9-12].

In the mammalian system, a similar circuitry operates. CLK and CYC homolog BMAL1, posit in the center of interlocked TTFL produce *Drosophila per* gene homolog *mPer1*, *mPer2*, and *mPer3*. CLK/BMAL1 dimer also turns on the expression of *mCryptochrome1* (*mCry1*) and *mCryptochrome2* (*mCry2*) which dimerize with mPER proteins to inhibit the transcriptional activity of CLK/BMAL [13]. One twist of the mammalian TTFL is that while CRY proteins play a role in transmitting light signals to the molecular clock in *Drosophila*, the mammalian CRY proteins work as a repressor for mCLK/BMAL1. Although the mammalian TIM protein is also produced, its role in the clock system is not yet evident. In the stabilizing loop, retinoid–related orphan receptors (RORa, b, c) [14] activate and REV-ERBα inhibits the transcription of *Bmal1* rather than *Clk* as in the *Drosophila*. Clock controlled rhythms in many physiological processes and behavior are generated by a cyclical gene expression governed by this interlocked feedback loop either directly or indirectly [15, 16] (For review, see [17]).

At a systemic level, cell-autonomous oscillator is orchestrated in a hierarchical network of master and peripheral oscillators. In the *Drosophila*, about 150 neurons in the brain work as a master clock driving its circadian behavior [18]. In mammals, suprachiasmatic nucleus (SCN) is the master clock synchronizing all other peripheral oscillators in various tissues (e.g. liver, heart, lung, etc.) [19, 20]. Although peripheral oscillators generate self-sustained rhythms, without the SCN, as in SCN-lesioned animals, rhythms of peripheral tissues become out of phase to an external LD cycle and to oscillations in other tissues [21]. Thus, the main role of a master clock is considered to be synchronizing peripheral oscillators by sending time cues via hormonal and neural pathway.

Although transcriptional control via interlocked feedback loop posits as a framework for the molecular clock, diverse regulations employed after the clock genes are transcribed also play crucial roles to finely adjust the clock speed to a 24 hr period. Post-translational modification, most notably phosphorylation of the clock proteins, has been extensively studied so far [22-26]. The first example was the *Drosophila* PER, which manifested timely progressive phosphorylation and hyper-phosphorylated isoforms degraded through a ubiquitin-proteasome system at the early day leading to the de-repression of dCLK/CYC transactivation [27-30]. In turn, another round of the cycle could start the next day. Casein kinase 1ε homolog DOUBLETIME (DBT), Glycogen synthase kinase 3β (GSK 3β), casein kinase 2 (CK2), and NEMO (NMO) are identified as kinases for PER to regulate its levels, activity as a repressor, and subcellular localization [31-39]. TIM is also phosphorylated by Glycogen synthase kinase 3β and CK2 regulating its levels and nuclear entry time [40, 41]. More recently, numerous studies revealed the diverse regulation of molecular clock at the post-transcriptional level. Please refer to the excellent recent review for more information [42].

One important issue in circadian rhythm is to generate oscillation in such a long 24 hr period. Based on a simple oscillator model [43], synthetic feedback loop only generates rhythmic oscillation with a 2 hr period; thus, imposing a time delay between transcriptional activation and repression is inevitable to generate such a long rhythm period [44-51]. The observation that nuclear accumulation of PER is lagged in both *Drosophila* and mammals by approximately 4~6 hours with respect to the peak mRNA levels support this notion [47, 52]. There could be various means to impose a time delay between the activation of circadian transcription factors and repression by circadian repressor proteins. Delaying the nuclear entry time of circadian repressor proteins could be employed as a time delay in the clock system. This review will focus on how clock speed is regulated by

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controlling negative circadian regulator's nuclear entry time.

SUBCELLULAR LOCALIZATION AND NUCLEAR ENTRY REGULATION BY SIGNAL SEQUENCE MOTIF

Traffic between the nucleus and the cytoplasm is carried out through specialized apertures, nuclear pore complexes (NPCs) [53, 54]. Various carrier proteins are involved in the translocation of cargo proteins through NPCs. Cargo proteins are targeted for nuclear import by a short nuclear localization signal (NLS) sequence motif. A well-known NLS is composed of one (monopartite) or two (bipartite) basic amino acid clusters. The classic nuclear import pathway uses importin β1 (Impβ1) as a carrier, which recognizes NLS as cargo and binds through the adaptor molecule importin α (Impα). It is the trimeric cargo containing protein complex Impα/Impβ1/NLS that can enter the nucleus [55-57].

The *Drosophila* PER protein has a functional bipartite NLS sequence at the C-terminus. Albeit with a functional NLS, the full-length PER protein expressed in the *Drosophila* S2 cell resides in the cytoplasm, most likely due to the cytoplasmic localization domain (CLD) at the C-terminal end of its PAS domain [58, 59]. When TIM protein was co-expressed, PER/TIM proteins were both detected in the nucleus indicating that the heterodimer formation is crucial for nuclear entry [59]. PER and TIM consistently accumulated in the cytoplasm in tim^0 and per^0 mutant flies, respectively [60-62]. Nonetheless, real-time imaging analysis revealed that PER/TIM complex formed in the cytoplasm of S2 cells dissociates before nuclear translocation and that dPER was detected in the nucleus before TIM in flies' pacemaker lateral neurons, suggesting that presumably PER and TIM independently moved to the nucleus in a short period of time [46, 63, 64]. Subcellular localization of TIM might be regulated in a slightly different manner as to the case of PER [65]. TIM could shuttle independently between the nucleus and cytoplasm both in flies and S2 cells. The role of PER was suggested to be necessary for nuclear retention of TIM in this case. Export from the nucleus to the cytoplasm is mediated through the recognition of nuclear export signal (NES) sequence. The typical NES is characterized as a leucine-rich sequence that is recognized by CRM1/exportin1, which belongs to Impβ family [57, 66]. Through a sequence scan, several putative NES of TIM were provided, although which one might be functional *in vivo* is not yet proven [65].

Similar to the *Drosophila*, the mammalian negative regulator's subcellular localization is affected by the interaction with its partner proteins. Exogenously expressed mPER1 or 2 in COS7 and NIH3T3 cells can accumulate in the nucleus in the presence of co-expressed mCRY proteins [67] or mPER3 [68]. Although the mCRY protein is retained in the nucleus when expressed in tissue cultures, the observation that the co-expression of mPER2 lacking NLS motif induced the retention of CRY in the cytoplasm supports the idea that mPER plays an important role in the nuclear localization of CRY as well. Consistently, putative NLS sequence motifs were identified from mPER1, 2, 3 and CRY1, 2 proteins [68-73]. Nonetheless, the predominant nuclear localization of mPER2 in the liver of *mCry1/mCry2* deficient mouse might suggest that the role of CRY is not necessarily for the nuclear entry *per se*, but rather for stabilizing PER in the nucleus [73]. Taken together, the nuclear accumulation of circadian repressor proteins such as dPER and TIM in *Drosophila* and mPERs and mCRYs in mammals are inter-dependent on partner proteins. How can interaction between binding partners affect nuclear localization of clock repressor proteins? The interaction between the partner proteins (e.g. PER/TIM, mPER/CRY, mPER1/mPER3, mPER2/mPER3) might adopt the conformation that allow their NLS unmasked, leading to the recognition by their carrier proteins.

TIMELY CONTROL OF NUCLEAR ENTRY BY PHOSPHORYLATION OF CLOCK PROTEINS

The *Drosophila* PER protein has 250 putative phosphorylation sites which suggest that critical functions of dPER might be controlled by the dynamic regulation of phosphorylation. Indeed, progressive phosphorylation of dPER occurs in a timely manner, and the phosphorylation status of dPER is different depending on the time of day [27]. Numerous reports indicated that phosphorylation of dPER is involved in a tight regulation of nuclear entry time. Glycogen synthase kinase 3β homolog, *shaggy* (*sgg*), promotes nuclear entry of dPER via direct phosphorylation of dPER [32] and/or indirect effects by phosphorylating TIM [41]. CK2 also promotes the nuclear entry of dPER in the *Drosophilia* pacemaker neurons [34, 36, 74]. CK2 is a tetrameric holo-enzyme composed of a catalytic subunit α2 and a regulatory subunit β2. Both α2 subunit mutant Timekeeper (*Tik*) and β subunit mutant andante flies manifest delayed nuclear entry time of dPER. When GSK 3β or CK2 activity was down-regulated either by mutation or decreased protein expression, circadian periods lengthened with concomitant delayed nuclear entry of dPER in pacemaker cells [34, 36, 41, 74]. On the other hand, DBT hindered the nuclear accumulation of dPER in the pacemaker neurons of *Drosophilia*. This notion is supported by the observation that in dbt^P and dbt^{AR} mutant flies, of which kinase activity is severely compromised, dPER is predominantly present in the nucleus even without TIM [33,75]. However, dbt^S flies which have reduced

DBT kinase activity, exhibited delayed nuclear entry of dPER [76] which complicate the role of DBT on dPER nuclear entry. Given that another DBT allele which supposedly has decreased kinase activity-*dbt*^{L}- manifested lengthened circadian periods [77], we prefer the idea that *dbt*^{S} and *dbt*^{L} flies might exert the effects in a more qualitative and not in a quantitative manner; suggesting DBT^{S} and DBT^{L} mutant proteins induced the alteration of phosphorylation sites on dPER, ultimately leading to the different outcomes in the length of the circadian periods. Taken together, while de-novo synthesized, hypo-phosphorylated dPER by DBT at specific sites, is retained in the cytoplasm, and the interaction with TIM would somehow relieve the cytoplasmic retention via antagonizing DBT mediated phosphorylation on dPER in *Drosophila* [75, 78]. This antagonizing effect could be aforementioned GSK3, CK2 mediated phosphorylation. Nonetheless, the duration of cytoplasmic retention of dPER might also be controlled by other posttranslational modifications (see below).

In mammals, the regulation of nuclear entry via phosphorylation of mPER is inconsistent depending on types of cells and kinds of proteins studied. Casein kinase 1 delta (CKIδ) and CKIε are two paralogs of mammalian CKI, both target mPERs as substrates regulating their stability and subcellular localization [79, 80]. Although some degree of functional redundancy of CKIε and CKIδ was observed, depending on the tissues, one might act more dominant than the other, as shown in the study where the depletion of CKIδ resulted in a long circadian period in the absence of behavioral effect with the depletion of CKIε [81]. In HEK293T cells, ectopically expressed mPER1 enters the nucleus while mPER2 resides in the cytoplasm [72]. This observation provided the idea that there must be a mechanism to prevent the premature nuclear entry of mPER1. This turned out to be a CKIε mediated phosphorylation of mPER1 via masking of NLS motif on mPER1 [72]. Interestingly, the co-expression of mCRY1 in the presence of CKIε and mPER1 brings all three components in the vicinity, and this trimeric complex can enter the nucleus [82]. Thus, mCRY1 is able to negate the CKIε mediated cytoplasmic retention of mPER1. This phenomenon is very closely related to the situation where DBT dependent phosphorylation retards the nuclear entry of dPER in *Drosophila* lateral neurons. In contrast, in other cell types, e.g. COS7 cells, CKIε mediated phosphorylation of Ser-661 and Ser-663 is a prerequisite for the nuclear entry of mPER1 [83]. Similarly CKIε and CKIδ induced phosphorylation of mPER3 accelerated nuclear translocation in COS7 cells while mPER1 and mPER2 were not affected by the co-expression of these kinases [79]. The inconsistent results regarding the effects of CKI mediated phosphorylation of mPERs in nuclear translocation might be attributed to the *in vitro* cell culture system of studies.

O-GLCNACYLATION MODULATES CLOCK PROTEIN LOCALIZATION

Recent findings have indicated that the extent of *O*-GlcNAc modification on dPER was correlated with nuclear translocation of dPER in *Drosophila* [84]. Aside from phosphorylation, the hydroxyl groups of Ser/Thr residues on proteins can also be modified with *O*-GlcNAc (*O*-GlcNAcylation) [85, 86]. Two enzymes mediate reversible addition of the β-N-acetylglucosamine moiety to the hydroxyl side chains of Ser/Thr residues of protein substrates; namely, glycosyltransferase (*O*-GlcNAc transferase; OGT) and β-*N*-acetylglucosaminidase (*O*-GlcNAcase; OGA) [87, 88]. Numerous findings reveal a complex interplay between phosphorylation and *O*-GlcNAcylation [89,90]. In the case of MYC, threonine 58 in the transactivation domain (TAD) is either *O*-GlcNAcylated or phosphorylated by GSK3 in a competitive manner regulating transactivation potential of MYC [91,92]. Stability of p53 is regulated by other modes of interplay between *O*-GlcNAcylation and phosphorylation; competitive occupancy at different sites. Treatment of MCF-7 cells with streptozotocin-OGA inhibitor- increased *O*-GlcNAcylation at serine 149 on p53. This *O*-GlcNAcylation represses the phosphorylation on threonine 155 leading to the inhibition of the interaction with the UPS system, ultimately resulting in the accumulation of p53 proteins in the cells [93]. *O*-GlcNAcylation of proteins might lead to an increase in phosphorylation at other sites as seen in the example of IRS-1 [94]. In the mouse liver, *O*-GlcNAcylation of IRS-1 directly correlates with an increase in serine 307, 632/635 phosphorylation, which are sites known to attenuate insulin signaling [94].

O-GlcNAc modification of dPER was evident in *Drosophila* S2 cells and in flies. More interestingly, *O*-GlcNAcylation of the dPER protein is temporally regulated. In flies, peak level in *O*-GlcNAcylation of dPER was observed during the first half of the night before a massive phosphorylation of dPER occured [84]. Remarkably, genetic manipulation of *O*-GlcNAc levels by either down- or up-regulating OGT in clock cells speeds up or slows down the pace of circadian behavioral rhythms, respectively. In the key pacemaker neurons in flies, the timing of dPER nuclear translocation is advanced in *ogt* knockdown flies and delayed in *ogt* overexpressing flies. Because *O*-GlcNAcylation of dPER mainly occured when it was retained in the cytoplasm, authors suggested the compelling hypothesis that *O*-GlcNAcylation gates the timing of when dPER translocates from the cytoplasm to the nucleus possibly via the interplay between phosphorylation and *O*-GlcNAcylation.

mPER2 is also modified with *O*-GlcNAc. Consistent with the observation in flies, conditional knockout of OGT shortened the

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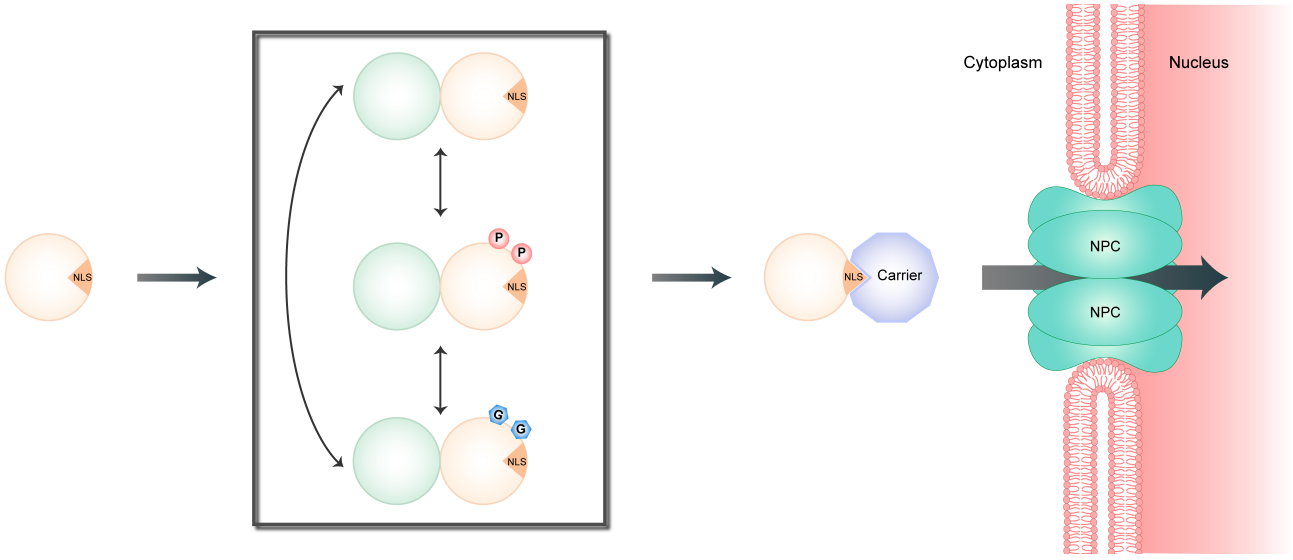

Fig. 1. A model for the timely nuclear entry of circadian repressor proteins in *Drosophila* and mammals. Newly synthesized circadian repressor protein (orange circle) is modified in the cytoplasm (gray box) over the course of time resulting in conformational changes. Conformational changes might unmask the NLS, which is recognized by carrier proteins mediating the transport through the nuclear pore complex (NPC). Mainly, phosphorylation (P) of repressor protein seems to control the timely nuclear translocation of repressor protein. Nonetheless, interaction with the other repressor protein (green circle) and crosstalk with other posttranslational modifications (e.g. *O*-GlcNAcylation, G) might regulate timely phosphorylation of repressor protein (orange circle).

circadian locomotor period [95]. Interestingly, *O*-GlcNAcylation can occur on S662 always together with *O*-GlcNAcylation on S671 [95]. *O*-GlcNAcylation on S662 decreased the phosphorylation on S662 suggesting the possible antagonism between *O*-GlcNAc and phospho- modification. It has been well known that phosphorylation on S662 is tightly linked to the familial advanced sleep phase syndrome (FASPS) in humans. People having FASPS disorders have a phenotype of early morning awakening and early sleep times together with a shortened circadian rhythm [96]. Two genes have been identified to be related with the FASPS disorder-S662G mutation on dPER or hypomorphic T44A mutation on CKIδ [96, 97]. A later study further revealed that phosphorylation of S662 was necessary for serial phosphorylation of S662-S674 cluster, which somehow leads to the increase in mPer2 transcript levels [95]. Another study suggests different roles for S662 phosphorylation, which is stabilizing mPER2 from degradation by blocking nuclear export [98]. Although the role of S662 phosphorylation on the metabolism of mPER is still controversial, the *mPER2* nuclear localization might also be regulated by controlling O-GlcNAc modification of mPER2, as similar to that of the *Drosophilia*.

CONCLUSION

To be able to sustain a 24 hr rhythm period, timely control of nuclear translocation is crucial in both *Drosophila* and mammals [99]. Strong nuclear accumulation of major circadian repressor protein-PER- is lagged several hours to the times of peak transcript levels. This delayed nuclear entry was controlled via post-translational modification of PER, namely phosphorylation that may be affected by the interaction with other partner proteins (TIM in Drosophila and mPERs or CRYs in mammals) and/or dynamic interplay with other post-translational modifications (e.g. *O*-GlcNAcylation) (Fig. 1). One important feature of the circadian clock system is that it may be able to re-synchronize to changes in time-cues when travelling through different time-zones. To be able to re-synchronize, cellular oscillators may be able to easily adapt to the extracellular time-cues. Recently, studies about PTM crosstalk in regulating the function of a protein have been accumulating (for review, [100]). Currently, more than 450 PTMs are listed in the protein data base [101]. As shown in the control of nuclear entry of PER proteins by possible crosstalks between *O*-GlcNAcylation and phosphorylation, other PTM crosstalks might play crucial roles in controlling nuclear entry of clock proteins and other functions as well. Future studies in this direction will shed light on understanding the detailed biochemical underpinnings of nuclear entry regulation of clock proteins.

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