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A New Twist on Clock Protein Phosphorylation: A Conformational Change Leads to Protein Degradation

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Progressive phosphorylation of circadian clock proteins is a hallmark of time-keeping. In this issue of *Molecular Cell*, Querfurth et al. (2011) demonstrate that phosphorylation of *Neurospora* FRQ induces a conformational change, which can account for its temporally gated degradation.

Molecular circadian clocks enable organisms to adapt their physiology and behavior to daily variations in their environment. Eukaryotic clocks rely on negative feedback loops, namely the rhythmic synthesis of transcriptional repressors, which then subsequently rhythmically repress their own transcription. These transcription factors are also posttranslationally modified (for example, by phosphorylation, sumoylation, acetylation, and ubiquitination), and many reports have highlighted the importance of these events to circadian timing. Genetic studies in a number of organisms indicate that these modification events not only help set the pace of the clock to approximately 24 hr but are also necessary for the generation of circadian rhythms (Mehra et al., 2009). Yet important aspects of these events and their regulation remain enigmatic. In the current issue of *Molecular Cell*, Querfurth et al. (2011) provide substantial mechanistic detail by indicating a new role for phosphorylation: it regulates the timing of a conformational switch in the *Neurospora* circadian repressor protein FREQUENCY (FRQ), which leads to its degradation.

Phosphorylation is the best-understood and perhaps the major posttranslational mechanism that modifies clock proteins. It is also an evolutionarily conserved phenomenon; clock proteins from bacteria to human display daily rhythms of phosphorylation. Importantly, several kinases that phosphorylate clock components function broadly in eukaryotes, such as the casein kinases *Ck1* and *Ck2*. As some of these enzymes also function in plant clocks, it is highly likely that of these mechanisms were already functioning in the circadian clock of a common ancestor.

The phosphorylation of circadian transcriptional repressors is particularly important. The synthesis of these proteins is under temporal control, and they are then progressively phosphorylated. These events appear to control the temporal regulation of repressor function, as they influence protein subcellular localization, dimerization, activity, and ultimately degradation (Mehra et al., 2009). Several kinases are involved, and mutations in these enzymes lead to strong circadian phenotypes, including period lengthening, shortening, and even arrhythmicity (Mehra et al., 2009). One interpretation is that these phenotypes reflect misregulation of kinase activity, i.e., wild-type activity is temporally regulated, which contributes directly to period determination.

Understanding the roles and the functions of individual phosphorylation sites on target clock proteins is critical, and numerous papers have mapped phosphorylation sites and correlated them with biological function. These efforts are exemplified by studies of the Ptacek and Fu groups. For example, a single amino acid change in the human circadian repressor PERIOD2 (hPER2) causes advanced sleep phase syndrome (FASPS). Affected individuals have a serine to glycine mutation within the casein kinase Iε binding region of the protein, which causes hypophosphorylation by CKIε (Toh et al., 2001). In addition, proper phosphorylation of serines in the N-terminal region of *Drosophila* PERIOD (dPER) (particularly serine 47) leads to the binding of the F box protein SLIMB and dPER degradation (Chiu et al., 2008). More recently, phosphorylation of a small domain of dPER (called the Per-Short cluster) was shown to be responsible for the proper timing of dPER degradation (Chiu et al., 2011). Intriguingly, this small domain includes serine 589 (Ser589), which is phosphorylated and is also the amino acid mutated in the original per^S allele of Konopka and Benzer (Konopka and Benzer, 1971; Chiu et al., 2011), which has a short circadian period of 19 hr. These examples lead to the notion that most phosphorylation sites control specific aspects of clock protein function with precise temporal roles.

However, the recent mapping of phosphorylation sites with mass spectrometry has challenged this notion. Indeed, there are a large number of characterized phosphorylation sites in dPER (Chiu et al., 2008). A staggering 113 sites were found within *Neurospora* FRQ (Baker et al., 2009; Tang et al., 2009)! Is it possible that each subserves a specific role within the molecular clock?

In the present issue of *Molecular Cell*, Querfurth and collaborators propose a more appealing explanation for many of these sites. They demonstrate that the progressive phosphorylation of FRQ by *Ck1a* leads to a buildup of charge in one region of the protein, which results in a conformational change and then FRQ

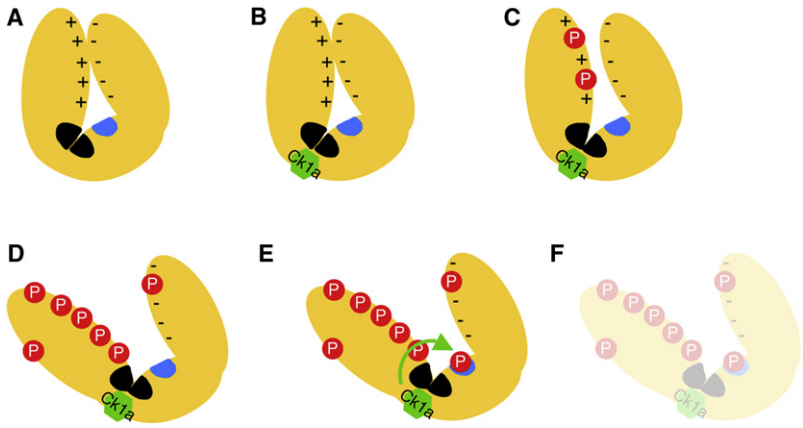

Figure 1. Phosphorylation-Induced Conformational Change of FRQ Is Responsible for Its Temporally Gated Degradation

Following its translation, FRQ (orange) folds into a close conformation in which the basic N-terminal portion of FRQ (plus signs) interacts with the acidic C-terminal portion (minus signs) (A). This close conformation also leads to the association of two domains organized in amphipathic α helices (displayed in black) (A). This association between the two α helix domains allows the binding of CK1a (shown in green) to FRQ (B). CK1a binding to FRQ is then responsible for the progressive phosphorylation (displayed in red) of the N-terminal basic portion of FRQ (C). The increasing number of phosphorylation sites on the N-terminal portion of FRQ decreases its isoelectric point and creates a charge repulsion between the N-terminal and C-terminal portions of FRQ (D). Phosphatases can counterbalance the effect of kinases. The charge repulsion induced by progressive phosphorylation of the N-terminal portion of FRQ creates a conformational change of the protein and results in an open conformation. This open conformation allows CK1a to access a previously hidden PEST sequence (blue) (E) and leads to FRQ degradation (F).

degradation. Importantly, the change does not require a precise role for each phosphorylation event but is an aggregate property (Figure 1).

The authors first show that many portions of FRQ are predicted to be unstructured but that two domains are organized into amphipathic α helices; they associate to create a CK1a-interaction domain, which then promotes the progressive phosphorylation of FRQ on many phosphorylation sites. Removal or mutation of the two domains abolishes the recruitment of CK1a and results in an unphosphorylated form of FRQ, which does not undergo degradation (Querfurth et al., 2011). Interestingly, PER also has a CK1 (*doubletime*) binding site (Kim et al., 2007; Nawathean et al., 2007), which is important for normal PER phosphorylation.

By inspecting the isoelectric point of different FRQ regions, the authors note that charge distribution is heterogenous, with a basic N-terminal portion, and acidic middle and C-terminal portions. Using different biochemical and molecular tools, the authors show that the N-terminal portion associates with the C-terminal portion when unphosphorylated. Progressive phosphorylation of the many sites in the N-terminal portion decreases its isoelectric point and creates charge repulsion between the two regions, which ultimately leads to a conformational change of the protein. This was visualized by partial protease digestion, which shows that hypophosphorylated FRQ is less prone to proteolysis, i.e., more compact, whereas hyperphosphorylation enhances proteolysis. The inferred conformational change allows CK1a to access a previously hidden PEST sequence and leads to FRQ degradation (Figure 1). Remarkably, the described series of events—progressive phosphorylation, the structural change, and then degradation—extends to PER (Chiu et al., 2011).

One curiosity is the phenotype of a failure to phosphorylate several serines within the Per-Short domain of dPER. This region contains Ser589, which is the site of the per^S mutation, as mentioned above. It is normally phosphorylated (Chiu et al., 2011), and all missense mutations of this amino acid (except for threonine—pleasingly) shorten circadian period (Rutila et al., 1992; Chiu et al., 2011). Why does the failure to phosphorylate this serine and a few others in its vicinity lead to such dramatic, short period phenotypes? Do these specific phosphorylation events serve a precise timing role, which then delays the hyperphosphorylation program (Chiu et al., 2011)? An alternative possibility, inspired by the findings of Brunner and colleagues, is that mutations in this region just disrupt structure, a not unprecedented role for missense mutations. What is unusual is that short period *per* mutants are relative rare. Perhaps most missense mutations that disrupt structure fail to provide sufficient function for rhythmicity, i.e., they are arrhythmic or long period. This would be because their poor function gives rise to a failed clock or one that marches through the circadian program only weakly—and slowly. In this view, the unusual thing about the PERS mutant is its temporal effect: the PERS structure is predicted to be normal or nearly so throughout much of its daily program and then is disrupted earlier than WT PER to accelerate the structural transition that causes PER degradation. For PER and PERS as well as FRQ, it would be interesting to track structural transitions as a function of circadian time; for example, are they gradual or discrete, and when during the in vivo cycle do they occur?

The study by Querfurth et al. (2011) challenges the notion that each phosphorylation site has a precise role, controlling a specific aspect of protein function. In this new view, it is the number of phosphorylated residues, rather than the particular sites, that determines the transition kinetics between the closed and open conformations. This perspective could account for the poor conservation of overall PER sequence between species, i.e., what is important for much of the protein is not a precise sequence but local charge density and its change with time. In this context, it may be relevant that the perS region is not conserved in mammals.

Still remaining, however, is a classic problem present in many different biological contexts. How does a continuous process result in a discrete event, i.e., how does a gradual increase in charge density result in a conformational change at a specific time? Are there as-yet-undiscovered feedback loops that contribute to this timing event? Understanding this problem will almost certainly require biochemical studies, where the slow, gradual phosphorylation events as well as the

presumptive discrete structural transition can be reconstructed in vitro. Eukaryotic circadian clocks await this kind of biochemical breakthrough.

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HDAC3 at the Fulcrum of an Epithelial-Mesenchymal Balance

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In this issue of *Molecular Cell*, Wu et al. (2011) reveal an essential role for a chromatin modifier, histone deacetylase 3 (HDAC3), in hypoxia-induced epithelial-mesenchymal transition (EMT); HIF-activated HDAC3 integrates with WDR5 to impose chromatin modifications that culminate in EMT.

The epithelial-mesenchymal transition (EMT) has received significant attention in the last several years. Following the initial discovery that epithelial-like cells transiently acquire mesenchymal properties and undergo EMT during embryonic development, numerous studies uncovered similar events in multiple pathological conditions, including tissue pathogenesis, wound healing, and cancer (Kalluri and Neilson, 2003; Thiery et al., 2009). These studies have led to an improved understanding of how these conditions arise and progress. Of these pathological conditions, EMT events are perhaps best described in cancer metastasis, where there has been significant progress toward the identification of molecular alterations that are sufficient and/or necessary for EMT. Although molecular markers of EMT may be observed in tumor specimens, causal evidence for their role in EMT in vivo is largely lacking. During embryonic development, EMT events are transient in nature, but whether this is also true in cancer is not clear. While some tumor cells may exhibit a permanent EMT phenotype, for example claudin-low breast cancer (Hennessy et al., 2009), other cancer cells likely undergo a transient EMT, as suggested by the epithelial nature of macroscopic metastases. Therefore, some EMT events are likely sustained by transient molecular changes induced by extracellular cues from the tumor microenvironment, hypoxia for example, and not by permanent genetic alterations. Consequently, it is likely that epigenetic adjustments and chromatin modifications play a key role in EMT and cancer metastasis. To address these questions, Wu et al. (2011) pursued the identification and characterization of epigenetic regulators, particularly chromatin modifiers, which play essential roles in hypoxia-induced EMT. Their findings are published in this issue of *Molecular Cell*.

EMT has been linked to regulation by oxygen demand or hypoxia in both developmental and pathological processes (Haase, 2009; Yang et al., 2008). Wu et al. (2011) initiated in silico and molecular studies to uncover hypoxia-regulated genes that encode histone modifiers and reveal essential mechanisms of epigenetic alterations during hypoxia-induced EMT. They found that HIF-1α-mediated activation of class I histone deacetylase HDAC3 expression in response to hypoxia occurs in both epithelial and mesenchymal cells and is essential for EMT and metastasis (Figure 1). In epithelial cells, hypoxia induces HDAC3 expression and its enzymatic activity toward H3K4ac for removal of acetyl groups and repression of epithelial-associated gene chromatin structure. Transcription factors Snail and Twist are likewise induced by hypoxia, independently of HDAC3, and bind chromatin with HDAC3 to repress epithelial genes such as E-cadherin, leading to loss of