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Circadian regulation of physiology by disordered protein-protein interactions

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Abstract
Cellular circadian clocks, the molecular timers that coordinate physiology to the day/night cycle across the domains of life, are widely regulated by disordereddisordered protein interactions. Here, we review the disordered-disordered protein interactions in the circadian clock of Neurospora crassa (N. crassa), a filamentous fungus which is a model organism for clocks in higher eukaryotes. We focus on what is known about the interactions between the intrinsically disordered core negative arm protein FREQUENCEY (FRQ), the other proteins comprising the transcription-translation feedback loop, and the proteins that control output. We compare and contrast this model with other models of eukaryotic clocks, illustrating that protein disorder is a conserved and essential mechanism in the maintenance of circadian clock across species.

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Physiological fitness and the day/night cycle

Throughout the history of life on earth, organisms have been exposed to the steady oscillation of the day/night cycle as the earth rotates on its axis. This 24-h, diurnal rotation creates a consistent fluctuation in light and dark, which organisms had to evolve to anticipate. To account for this, most organisms living in the photic zone evolved a molecular timekeeping mechanism termed the circadian clock. This circadian clock is a timer that widely tunes cellular and organismal physiology to a 24-h pattern, creating circadian rhythms that are exhibited in almost all aspects of life, including gene expression, metabolism, reproduction, and motility [1,2].

As the importance of the circadian clock in organismal fitness became clear, so did the realization that the disruption of this molecular timer, both in the short term and in the long term, could lead to negative physiological effects for organisms [3–5]. Longevity and fitness are decreased in model organisms that are not aligned to their respective circadian cycle [3,6]. In the case of humans, chronic disruptions to the molecular timer over a lifespan greatly increase rates of disease, including conditions such as heart disease, cancer, diabetes, and Alzheimer's Disease, all of which are among the top ten diseases affecting humans as we age [3,7].

Molecular mechanisms underlying the timing of circadian rhythms

While the term circadian clock can be used to refer to a 24-h timer on several spatial scales, at its core, the clock is timed by a molecular mechanism that is architecturally conserved in fungi and animals, with a more loosely conserved architecture in plants and prokaryotes [1,5,8–10]. This clock comprises a transcription-translation feedback loop (TTFL), which, over the course of a 24-h cycle, progresses from transcriptional activation to post-translational modification to transcriptional repression to complete its cycle [1,8,9,11]. It is this basic mechanism that, in 2017, was given the Nobel Prize in Physiology or Medicine [12].

Due to its broad conservation, the principles of the molecular circadian clock have been elucidated in many organisms. While the Nobel Prize was given for work performed in *Drosophila melanogaster*, cyanobacteria, fungi, mice, and humans have all significantly contributed to the model of the molecular clock [1,12–15]. Though there are many differences in these clocks, several underlying principles are consistent among clocks across species, one of which is the importance of temporally regulated protein-protein interactions [16,17]. A key regulator in these temporal protein-protein interactions are regions of intrinsic protein disorder, regions whose amino acid sequence encodes for a heterogenous ensemble of conformations rather than a single structure, which allows for the flexibility to time the formation of the macromolecular complexes of the TTFL and beyond over the 24-h day [16,18–20]. Evidence suggests that time-of-day dependent phosphorylation, driven by the interaction with kinases, may alter the charge of positive electrostatic patches in clock proteins, changing the availability of binding regions along the protein surface and thus controlling the timing of the formation of macromolecular complexes [16,19,21]. It is on the contribution of intrinsic disorder to the molecular timing of the circadian clock that this chapter will focus. Though a great deal of insight has been gleaned in the past decade in this subfield of circadian rhythms across model systems [18,22–25], due to the brevity of this review, we will focus on the discoveries and contributions made by the repressive arm of a particular clock model organism, the fungi *Neurospora crassa* (*N. crassa*), and parallel these findings with what has been shown in other clock model systems.

Neurospora crassa as a model system for the circadian TTFL

The filamentous fungus *N. crassa* has been a quintessential molecular and genetic model for many areas of research over the past 150 years [26,27]. Relevant to this review, an overt circadian rhythm was discovered in the growth of *N. crassa* in the late 1950s, which was followed shortly by the identification of the genes involved in the TTFL, leading to the use of *N. crassa* as a model for the molecular mechanisms of the clock in eukaryotes [28–30]. The current model of the TTFL in *N. crassa* is that the cycle begins when the activating complex of the clock, the White Collar Complex (WCC) consisting of White Collar-1 (WC-1) and White Collar-2 (WC-2) drives expression of the gene *frequency*, or *frq* [30–32]. The *frq* message is then translated, giving rise to the FREQUENCY (FRQ) protein, which is the central component of the repressing complex of the TTFL [30]. FRQ binds to its partner protein, FREQUENCY-interacting RNA helicase (FRH), which prevents the nonspecific degradation of FRQ, allowing FRQ to enact its role in the TTFL [25,33]. This FRQ/FRH complex (FFC) then interacts with the kinase Casein Kinase 1a (CK1a), leading to the extensive phosphorylation of FRQ over the circadian day, with over 100 validated phosphosites that are precisely timed by the circadian clock [16,34–36]. During its life cycle, the FFC is transported to the nucleus, where it binds to the WCC, repressing the transcriptional activity of the WCC via phosphorylation of the WCC and inhibiting the production of FRQ [37]. Once FRQ becomes hyperphosphorylated, the FFC can no longer repress WCC activity and is ubiquitinated and degraded, allowing the WCC to reactivate the *frq* promoter and begin the cycle anew (Figure 1) [10,38]. The ability of the TTFL to regulate cellular physiology stems from both the capacity of the WCC to rhythmically activate promoters other than FRQ as well as the temporal formation of repressive arm-centered macromolecular complexes that impart signaling information throughout the cell [16,19,39]. Thirty years of comparative study have shown that the architecture of the clock in *N. crassa* is much the same as the clock in many eukaryotes, even if the sequence conservation of the proteins involved in the clock is limited [10,18,40].

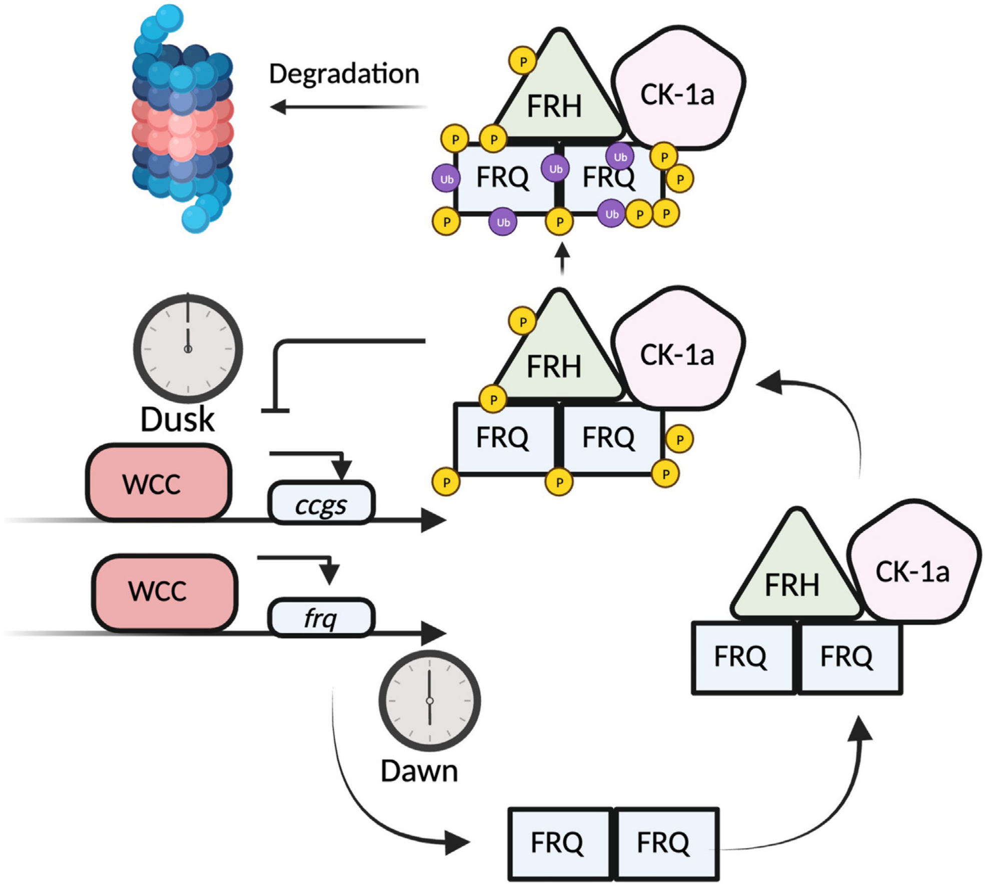
Intrinsically disordered protein regions regulate interactions within the TTFL

Though there is little sequence conservation in clock proteins from different phyla, there are some shared molecular characteristics. One of these characteristics is that the proteins that are involved in the TTFL, whether in the activating or repressing arms, are either classified as intrinsically disordered proteins (IDPs) or they contain intrinsically disordered regions (IDRs) that account for a significant portion of their sequence (Figure 2) [18,41]. The proteins of the *N. crassa* TTFL are no exception to this rule. In fact, while IDRs were previously found to control clock functions in higher eukaryotic clocks, the first IDP found to be essential for clock function was FRQ in *N. crassa* [25,42,43].

The role of protein disorder in the clock in *N. crassa* was initially discovered in the study of the interaction between FRQ and FRH [25]. While at the time little was known about the function of FRQ, FRH was predicted to be an essential helicase in *N. crassa* as it is homologous to other known DExD-box ATP-ase helicases [33]. In concordance with this, FRH was shown to have a well-ordered three-dimensional structure homologous to other DExD-box helicases and possess RNA-unwinding activity [44,45]. However, in an analysis of the interaction between FRH and FRQ, it was found that rather than the well-ordered and conserved helicase regions of FRH, it was the disordered N-terminal arm of FRH that enabled the interaction [25]. Specifically, it was the intrinsically disordered residues in the N-terminal arm of FRH, a region only conserved in fungi that maintained a circadian clock, that, when mutated, inhibited the interaction between FRQ and FRH [25]. A knockout of this region of FRH not only eliminated FRQ/FRH interaction but decreased FRQ stability and ablated the oscillation of the TTFL [25]. In concordance with this, when the core helicase regions (not in the N-terminal arm) of FRH were mutated, binding between FRQ and FRH still occurred, and the clock still oscillated with a circadian period, suggesting that the helicase activity of FRH and its well-ordered regions are not essential for the proper timing of the TTFL [25].

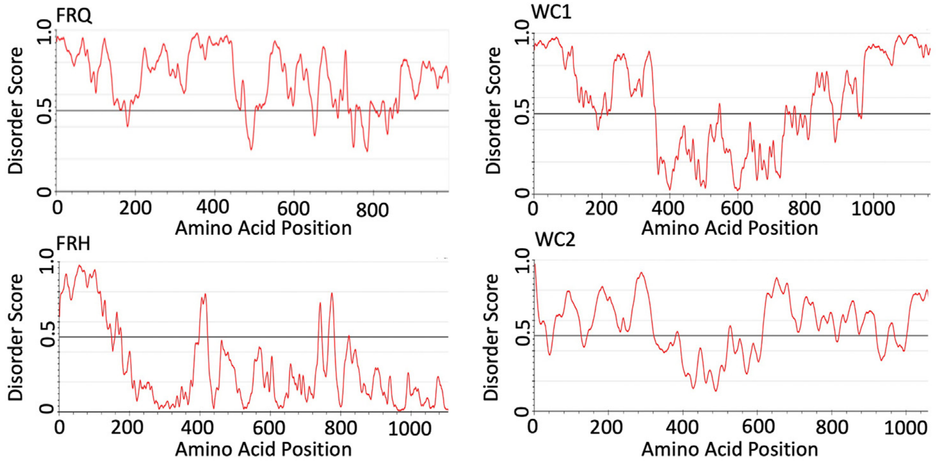
Notably, IDRs dictate interactions that occur within the TTFL that regulate the timing of the circadian clock beyond the regulation of the FFC in *N. crassa*. In a recently published example, the interaction between FRQ and the activating arm protein WC-1 has been shown to occur via an IDR-IDR interaction [21,46].

Figure 1



The transcription-translation feedback loop in the *N. crassa* circadian clock. The WCC drives the transcription and translation of FRQ. FRQ, FRH, and CK-1a form the FFC. The FFC inhibits the WCC, halting transcription of clock-controlled genes, including *frq*. CK-1a phosphorylates the FFC, leading to the separation of the FFC and the WCC, the ubiquitination of FRQ, the reactivation of the WCC, and ultimately the degradation of FRQ via the proteasome. WCC, White Collar Complex; FRQ, FREQUENCY; FRH, FREQUENCY-Interacting RNA helicase; FFC, FRQ/FRH complex.

Figure 2



IUPred3(long) disorder prediction of *N. crassa* circadian clock proteins. A low score indicates structured residues, while a high score indicates disordered residues. Predictions illustrate that FRQ and WC2 are disordered, while FRH and WC1 contain large regions of disorder [41]. FRQ, FREQUENCY; FRH, FREQUENCY-Interacting RNA helicase; WC1, White Collar1.

Like the work described below, which first demonstrated the importance of electrostatics in the clock by examining the regulation of clock output, the interaction between WC-1 and FRQ can be disrupted by exposure to high salt, illustrating that electrostatics are important to this IDR-IDR interaction, which is key to the TTFL in the *N. crassa* circadian clock [19,21]. Given the above evidence, it is likely that many of the clock protein interactions within the TTFL are enabled by intrinsic protein disorder. Similar to what is seen in *N. crassa*, the TTFL in mice is regulated by disordered protein interactions. For example, the interaction within and between the activating arm proteins basic helix-loop-helix ARNT like 1 (BMAL1) and cryptochrome (CRY) and the repressive arm proteins PERIOD proteins (PERs) in the mammalian clock are interactions between IDRs [18,24,47].

Circadian regulation of cellular physiology is enabled by regions of intrinsic protein disorder

Though the FRQ/FRH interaction domain (FFD) on FRH comprises an IDR, as do many of the interactions within the *N. crassa* TTFL, the FFD on FRQ has long been understood to reside in one of the few regions of FRQ that is predicted to have a fixed three-dimensional structure [22,25]. This FRQ FFD is recognized as the principal point of interaction and the only region of interaction between FRQ and FRH that is sequence-specific [19,48]. Further, ablation of this region eliminates the function of the TTFL [48].

However, recent work has shown that beyond the binding of FRQ to FRH via this ordered FFD, regions of disorder in FRQ may also participate in the interaction with FRH [19]. New data demonstrates that positively charged islands distributed along intrinsically disordered regions of the FRQ sequence nonspecifically bind to FRH via the primarily negatively charged solvent-accessible surface of FRH [19]. This model of nonspecific interaction via electrostatics aligns with the current model of a "fuzzy-like" biophysical complex [19]. Contrary to the function of the ordered FFD, ablation of these interactions does not eliminate the function of the TTFL. Rather, mutation of these regions ablates the overt oscillations of the *N. crassa* clock, demonstrating that these electrostatically driven interactions are important not for the oscillation of the TTFL but for the timing that the TTFL imparts onto cellular physiology [19].

The mechanism by which the fuzzy repressive arm complex of the TTFL can regulate physiology is unknown. However, clues to this mechanism stem from evidence that large macromolecular complexes form around the repressive arm of the *N. crassa* clock, including proteins not involved in the timing of the TTFL itself [16]. Research into these macromolecular complexes has shown that as many as 500 proteins bind to the repressive arm over the circadian day and that the interaction of these proteins with the repressive arm oscillates with a circadian period and correlates with the phosphorylation of FRQ [16,34]. Concordantly, when the binding regions of the non-TTFL-regulating proteins were predicted computationally along the sequence of FRQ, these proteins were primarily predicted to bind in regions of FRQ that are both intrinsically disordered and phosphorylated over the circadian day [16]. When aligned with the charge blocks on FRQ, a further correlation was noted, suggesting that FRQ interactions tended to occur in disordered and phosphorylated regions that were not electrostatically attracted to FRH [19].

Protein disorder also appears to be a key characteristic of the time at which proteins participate in repressive arm-centered macromolecular complexes [16]. Within FRQ macromolecular complexes, FRQ tends to interact more with ordered proteins than IDPs [16]. However, while ordered proteins tend to interact with FRQ when they are at their peak levels, in the case of IDPs that do interact with FRQ, the IDP/IDP interactions tend to occur at the nadir levels, suggesting that FRQ interaction may stabilize the IDPs [16].

Given this data, one can envision a model where phosphorylation of a positively charged region, harboring a target binding site of a FRQ interactor, modulates the electrostatics of that region to regulate its nonspecific binding to FRH [16,19]. In this way, phosphorylation of disordered regions could control access of the target site for the FRQ interactor temporarily. This interaction could either help to stabilize the interactor or bring together a kinase and its target in a time-of-day-specific manner. These data are supported by evidence that FRQ changes its conformation over the circadian day in concordance with both phosphorylation and protein-protein interactions [16]. The disordered nature of these regions would provide the necessary flexibility to allow FRQ to rapidly change its conformation [16]. The proposed model provides the first biophysically mechanistic explanation of how the repressive arm of the circadian clock can regulate output post-transcriptionally [16,19]. This is relevant as circadian post-transcriptional regulation has been widely described in the clock field, but little is known about the source of this regulation [39,49].

When the protein analogs of FRQ, the PERIOD proteins in flies (dPER), mice (mPER), and humans (hPER), were analyzed, positively charged islands in regions of disorder were found to be conserved [16]. In addition, when the interactomes of dPER and mPER were analyzed over circadian time, they too were found to form large macromolecular complexes that oscillate over circadian time and have differential interactions dependent on the predicted disorder of the interactor, suggesting that the function of intrinsic protein disorder in protein-protein interactions that regulate output is conserved in clocks across species [16].

Protein-protein interactions in the clock regulate circadian liquid-liquid phase separation

One of the hot topics in IDP/IDR protein-protein interactions is their ability to regulate the process of liquid-liquid phase separation (LLPS), the formation of liquid-like particles that are physiologically distinct from their surroundings [50]. These liquid-like droplets broadly assist in cellular organization, metabolism, regulation, and signaling [51,52]. IDP/IDRs within the clock are no different, as this phenomenon has been noted in circadian clock proteins across different species [22,53]. Recent evidence shows that in *N. crassa*, FRQ undergoes LLPS, which is heavily dependent on the phosphorylation state of FRQ [22]. While in liquid droplet form, FRQ phosphorylation is inhibited as CK1a is not able to access FRQ [22]. Further, FRQ can recruit FRH and CK1a, via disordered domains, to these liquid droplets, halting their enzymatic functions [22]. LLPS was also noted in the clock regulatory protein period-2 (PRD2), which is an RNA-binding protein that localizes *frq* mRNA in liquid droplets [53]. In fact, based on their amino acid composition, many of the proteins in the circadian clock in *N. crassa* may be able to exhibit LLPS [54].

Similarly, eukaryotic clocks beyond *N. crassa* exhibit LLPS in their circadian clock. The nuclear receptor and negative arm protein REV-ERBα in mice can form liquid droplets [23]. This physiological event is driven through the N-terminal IDR of REV-ERBα, which recruits nuclear receptor corepressor 1 (NCOR1) to regulate gene expression [23]. In *Arabidopsis*, EARLY FLOWERING 3 (ELF3), a repressive protein in the clock, contains a predicted prion domain (PrD) with a polyQ repeat, which is known to assemble into liquid droplets [55,56]. With increasing temperature, ELF3 forms liquid droplets dependent on this disordered PrD region [56]. Finally, in *Drosophila* clocks, the PERIOD and CLOCK proteins both form condensates during the repressive phase of the clock, and the breaking of these foci disrupts circadian rhythms [57]. Overall, LLPS is yet another mechanism by which IDPs/IDRs regulate protein-protein interactions to control clock functions across species.

Conclusion/summary

IDPs/IDRs play varied and important roles in the regulation of the circadian clock. Each of these roles is evident in the circadian model organism *N. crassa*, whose repressive arm protein FRQ is highly intrinsically disordered [18]. This characteristic allows for proper maintenance of the circadian clock via the regulation of interactions with FRH, CK1, the WCC, and other binding partners that control the regulation of physiology [16,19,22,53]. These functions are mirrored in IDPs/IDRs from circadian clocks across many eukaryotic species, including humans, mice, fungi, and plants, illustrating that characteristics granted by intrinsic disorder are essential for the circadian clock to anticipate external stimuli [18].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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