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Genomics and Systems Approaches in the Mammalian Circadian Clock

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Summary

The circadian clock is an endogenous oscillator that regulates daily rhythms in behavior and physiology. In recent years, systems biology and genomics approaches re-shaped our view of the clock. Our understanding of outputs that regulate behavior and physiology has been enhanced through gene expression profiling and proteomic analyses. Systems approaches uncovered underlying principles of transcriptional regulation and robustness of the oscillator through perturbation analysis and synthetic methods. Finally, new clock components and modifiers were identified through cell-based screening efforts and proteomics.

Introduction

Circadian clocks drive daily rhythms in physiology and behavior such as the sleep/wake cycle, cardiovascular function, hormonal rhythms, and metabolism [1,2]. In mammals, these clocks are found throughout the body, with a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus that synchronizes peripheral oscillators throughout the body [3, 4]. Inputs such as light, temperature, hormones, and feeding can entrain these oscillators, which, in turn, drive daily output rhythms in a cell type or organ specific fashion[5]. Interestingly, the discovery that these rhythms persist in cells, including immortalized cell lines, provides an exciting model system to study clock function at multiple levels including synchronization/entrainment, core clock mechanisms, and cell autonomous clock outputs [6–9]. Moreover, clocks in cultured cells have been useful in real-time imaging of circadian gene expression using reporter genes such as luciferase [10–12]. These autonomous cellular clock models are ideal for perturbation experiments using cell biology techniques such as RNA interference (RNAi).

The discovery of clockwork components enabled biochemical, cellular, and *in vivo* studies that describe the detailed mechanism of rhythm generation in mammals (and other species such as *Neurospora crassa*, *Drosophila melanogaster*, and *Arabidopsis thaliana*). The mammalian clock is made of two interlocking negative feedback loops (summarized in Figure 1) that regulate precisely timed circadian gene expression. The transcription factors Bmal1/Bmal2 and Clock/Npas2 heterodimerize and bind E-box regulatory elements in the promoters of the circadian repressors Period (Per1, Per2, Per3) and Cryptochrome (Cry1, Cry2), thereby

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activating their expression. Once translated, PER and CRY repressor proteins form large cytoplasmic complexes and later translocate to the nucleus to repress BMAL/CLOCK - mediated transcription (reviewed in [13]). To relieve this repression, CRYs are targeted for proteasomal degradation through association with FBXL3 E3 ubiquitin ligase complexes [14–16], which allows a new cycle to begin. A second, stabilizing transcriptional loop regulates Bmal1 (and other clock gene) expression through factors that bind the ROR element (RRE) such as Rev-erb-alpha, Rev-erb-beta, Rora, Rorb, and Rorc [17–21]. These two feedback loops, combined with regulated events including protein stability, protein complex formation, nuclear translocation, and post translational modification of clock gene products, create a robust 24 hour timekeeper that drives oscillations in output gene expression. These molecular rhythms culminate in observed rhythms in physiology and behavior (see more details in [22,23]).

Historically, identification of mammalian clock components benefitted from forward and reverse genetics in flies and mice. Typically, fly clock genes were identified, mouse homologues were characterized, then mouse knockouts were generated and confirmed in behavioral, cellular, and molecular assays (summarized in [23]). Circadian gene discovery was not confined to Drosophila, however, as forward genetics in mice identified Clock and Fbxl3 through mapping of N-ethyl-N-nitrosurea (ENU)- induced mutations in mice with abnormal locomotor behavior [15,16,24–26]. More recently, genomics based approaches have been used to identify modulators of circadian rhythms and have significantly broadened our understanding of mammalian clock output at the transcriptional and post-transcriptional levels [21,27,28].

In this review, we detail recent studies using functional genomics and systems biology approaches to understand mammalian clock function. We start with output regulation, where molecular clock components regulate gene expression networks that convey physiological and behavioral rhythms, and then extend our review to examine how functional genomics approaches are uncovering the circuitry that dictates this clock output. We then detail how systems approaches are beginning to describe complex properties of the clock such as its robustness. Finally, we discuss large-scale functional genomics screens to find novel candidate clock components and modifiers. Collectively, these studies are revolutionizing our understanding of circadian clocks by generating and analyzing data at genome scale and addressing circadian biology at the network level.

Output Regulation: how molecular rhythms generate physiological and behavioral changes

One of the key questions in clock biology is how molecular rhythms translate into observed circadian regulation of behavior and physiology. Early on, circadian clock researchers appreciated the technological advances that enable large-scale gene expression profiling. One of these technologies, DNA array expression profiling, had, and continues to have, a remarkable impact on our understanding of circadian output. Early studies, excellently reviewed elsewhere [29], identified hundreds of transcripts that are under circadian regulation and found distinct subsets of transcripts oscillating in different tissues. As the cost of microarrays has decreased, increasing sampling frequency to intervals of every 1 or 2 hours has allowed for increased statistical power to identify more high-confidence rhythmic transcripts and fewer false positives [30,31]. Moreover, recent studies have not only identified oscillating transcripts with high confidence, but have also enabled detection of genes with shorter time frequencies of expression. For example, high density time sampling in liver identified genes with second and third order harmonics (i.e. 12- and 8-hour oscillators, respectively) [31]. Oscillations with a 12-hour frequency were shown to be driven by systemic cues, as they were lost or reduced in amplitude in *ex vivo* liver cultures and under restricted feeding conditions [31]. Additionally, this sampling paradigm was used to accurately identify

feeding- and fasting- induced transcripts in the liver, as well as a handful of circadian clock driven transcripts that still oscillate under prolonged fasting conditions [32].

Recent microarray studies also addressed the role of specific clock genes in peripheral oscillator function. For example, oscillations were lost in 90% of transcripts when the circadian clock was selectively disrupted in mouse hepatocytes, suggesting that the remaining 10% of rhythms were driven by systemic cues originating outside the liver but impinging on its function [33]. Transcriptional profiling from *Clock* mutant mice identified an abundance of CLOCK-regulated transcripts involved in cell cycle and cell proliferation [34]. Finally, the role of Rev-erb-alpha in regulation of cholesterol and bile acid synthesis through SREBP (sterol regulatory element-binding protein) was discovered by profiling mice with over-expressed or knocked-out *Rev-erb-alpha* [35].

Identifying hundreds of oscillating transcripts prompted studies aimed at understanding how rhythmic gene expression is regulated. The role of *cis*-regulatory elements such as the E/E'-box (targets of BMAL1-CLOCK/NPAS2), D-box (DBP/E4BP4-binding elements) and RRE (REV-ERBalpha/ROR-regulatory elements) in generating oscillations in sixteen cycling transcripts were characterized by Ueda *et al.*, as described below [36]. Other meta analysis studies of DNA array data identified known regulatory motifs (E-box, RRE, D-box, and CRE) as well as some tissue-specific regulatory motifs in liver, heart, and skeletal muscle [37,38]. In addition, novel E-box like motifs consisting of tandem E-box like elements, termed the E1 and E2 motifs or the EE element, were identified by both modeling of CLOCK/BMAL1-regulated homologs in Drosophila and comparative analysis of mammalian promoter regions [39,40]. Interestingly, the EE element found in a subset of oscillating transcripts requires both of the E-box like elements (E1 and E2) for rhythms in cell culture models [39].

Recently, proteomic approaches were applied to identify proteins with rhythmic abundance or release. Although mass spectrometry approaches don't yet provide as much coverage as transcriptional analyses, diurnal or circadian variation in protein levels have been reported in retina, pineal, blood, liver, and SCN [41–45]. In addition, rhythmic release of neuropeptides was recently identified in rat SCN, and application of one neuropeptide, little SAAS, caused phase delays of SCN rhythms [46]. Furthermore, the availability of microarray and proteomic datasets allowed comparisons between protein and transcript regulation. These comparisons revealed the following: only about one third to one half of rhythmically expressed proteins from SCN or liver also have oscillations in their mRNA [41,44]. Therefore, post transcriptional regulation must play a significant role in regulating circadian rhythms.

Synthetic and systems approaches to the study of clocks

In mammals, systems biology approaches have been used to better understand transcriptional control and oscillator function. Ueda *et al.* identified and characterized the regulatory elements driving circadian oscillations in sixteen clock components and clock-controlled genes (CCGs) and also uncovered a critical role of E/E'-box regulation in circadian gene expression [36]. Principles uncovered from studying the regulatory elements in the context of the cellular oscillator were then applied to design artificial transcriptional circuits; by driving the expression of artificial activators and repressors at different times of day, both daytime and night-time specific gene expression could be generated [47]. Building on the identification and experimental validation of functional clock controlled elements, synthetic E-box, D-box, and RRE circadian elements were constructed to better understand amplitude of circadian transcription oscillations [48]. Synthetic D-box and RRE elements with robust, high amplitude oscillations were bound by transcriptional activators and repressors with high affinity. Mathematical modeling of this data suggested this was due to balanced binding affinities between activators and repressors. However, E-box elements showed an unexpected

difference: high affinity binding of transcriptional activators resulted in low amplitude rhythms. This may point to a role for DNA binding or the lack of it in repression of the CLOCK/BMAL1 complex. Therefore, a fundamental distinction exists between E-boxes and other circadian regulatory elements; the authors hypothesize this is due to the evolutionary and structural divergence between DNA binding domains of E-box bound activators and repressors [48].

Another popular systems biology approach is perturbation analysis. Our group used this approach to study a "systems" property of the clock -- robustness [49]. Using dose-dependent RNAi to deplete clock genes, we studied the levels of depletion required to disrupt clock function in cellular assays. These results showed that the clock is remarkably robust to genetic perturbation. Furthermore, by studying the biochemical changes that accompany clock gene perturbation, we uncovered two governing properties (summarized in Figure 2). The first property, proportionality, showed that the clock genetic network responded in proportion to the initial perturbation, sometimes in a nearly linear fashion. For example, after Bmal1 knockdown, one of its target genes, Rev-erb-alpha, had relative mRNA levels that were nearly identical to that of Bmal1. While the constants may have changed, the principle of proportionality was consistent across all perturbations and probably reflects the law of mass action. A second property, paralog compensation, was also evident, especially after knockdown of clock repressors. Earlier studies of many clock factors suggested significant redundancy in the clock. However, in the cellular assays, after knockdown of Rev-erb-beta, Per1, and Cry1, levels of their paralogs, Rev-erb-alpha, Per2 and Per3, and Cry2, respectively, went up dramatically. Active compensation, rather than simple redundancy, is therefore a property of the clock gene network. These results show that the clock gene network uses its wiring and properties of proportionality and compensation to maintain robustness to genetic perturbation.

Identification of clock components and modifiers

In the last 2 years, chemical and functional genomics approaches identified 100’s of new modulators of oscillator function. These screening efforts benefitted from the use of autonomous cellular models of clock function and kinetic luminescence imaging, which enable the efficient study of circadian properties such as synchronization, period length, and amplitude. To identify entraining factors, Nakahata et al. screened libraries of peptides and bioactive lipids for factors that synchronize circadian oscillations [50]. These efforts identified both known and novel entraining factors, including a natural ligand of the peroxisome proliferator-activated receptor -γ (PPAR-γ) [50]. This observation is consistent with recent evidence on the relationship between the clock and metabolism in mammals. Yagita et al. screened a kinase inhibitor library using real-time bioluminescence monitoring and identified several compounds that lengthen period length of oscillation [51]. Screening a larger library of ~1200 pharmacologically active compounds identified about a dozen drugs that effect period length [52,53]. Target validation with some of these chemicals demonstrated the role of several kinases, including CKIδ/CKIε and GSK-3β, in regulating period length [52,53]. Expanding these studies to larger, more diverse chemical libraries will help inform on other targets that contribute to oscillator function [54]. In the future, these small molecule screens may enable the discovery of therapeutics that modulate circadian properties such as amplitude, period length, and entrainment, clock properties that are disrupted in patients with circadian sleep disorders. Moreover, the tantalizing links between the clock and metabolism suggest these molecules may be useful in treating metabolic disorders.

Genomic cell-based screening approaches using RNA interference (RNAi) has also identified novel regulators of clock function. For example, Maier et al. performed an RNAi based genetic screen using short hairpin RNA expression constructs (shRNAs) targeting human kinases, phosphatases, and F-box proteins [27]. This screen identified casein kinase 2 (CK2) as a novel

component of the mammalian circadian clock [27]. They went on to find that CK2 binds to and phosphorylates PER2, which regulates the nuclear accumulation of PER2 [27]. Extending the RNAi screening to the whole human genome using synthetic siRNAs led to the identification of hundreds of modulators of oscillator function [28]. While decreased expression of most of these genes resulted in long period length, a few dozen resulted in a short period phenotype or increased amplitude of oscillator function. Analysis of protein interaction networks of the circadian modulators identified in this screen revealed only a few that directly interact with established clock factors. Many more, however, interact with proteins that also interact with clock factors, suggesting that large complexes rather than single interactions may influence clock function. Furthermore, many of the hits segregate into canonical biological pathways also regulated by the clock. For example, individual knockdown of multiple components in the insulin signaling, hedgehog signaling, cell cycle, and folate metabolism pathways influence cellular clock function. The insulin pathway was particularly striking in that nine components of this pathway are involved in regulating clock function and previous studies demonstrated that nearly half of the steps in the insulin signaling pathway are regulated by the clock [28,31,55]. These results demonstrate that the circadian clock is functionally intertwined with other pathways, rather than an independent entity.

Several groups have begun to apply protein-interaction screens to define clock regulators. For example, Brown and colleagues identified PER1 associated factors that modulate its activity [56]. Using RNAi, they went on to demonstrate that depletion or loss of function of NONO attenuated or disrupted circadian rhythms in mammalian cells and in flies [56]. Weitz and colleagues have also used these approaches. For example, they characterized Bmal1 interacting proteins and found Receptor for Activated C Kinase 1 (RACK1), which stimulated Bmal1 phosphorylation by PKCalpha in vitro [57]. In addition, two E3 ligases that modulate stability of REV-ERBalpha were identified by Yin et al. in a similar manner [58]. These studies provide good examples of combinations of genomic approaches being used to identify clock modulating factors and/or clock components (summarized in Figure 1). Their extension to other facets of clock function will undoubtedly lead to more discoveries and mechanistic insight into the clock.

Conclusions

Historically, the dominant paradigm of one-gene-at-a-time research has driven most biology including that of clocks. However, with the widespread adoption of various genome wide technologies such as gene expression profiling, RNAi screening, proteomics, informatics, and their combinations, the use of global approaches has catalyzed discovery and enabled new insights. These insights include a much broader understanding of clock output at the transcriptional and post-transcriptional levels, as well as mechanistic insight into how these programs are initiated and maintained. Small molecule and genomic cell-based screening are providing new insights as well. This includes the identification of modifiers of circadian rhythms including small molecules and genes that impact synchronization, amplitude, and period length (summarized in Figure 1). Some of these genes are *bona fide* components of the clock. Others are modifiers that impinge on clock function, but are not required for it. These studies are also leading to higher order knowledge, such as determining properties enabled by the genetic architecture of the clock such as proportionality and paralog compensation. In addition, while output studies show that the clock regulates many biological pathways, functional genomics studies show some of these pathways have a reciprocal relationship and regulate the clock. Together, these observations show that the clock is functionally intertwined with many biological pathways. Most if not all of these observations would be impossible or glacially slow with one gene at a time research -- leaving us to conclude that genomics approaches have much to teach us about how organisms including man adapt and anticipate the environment.

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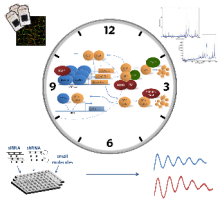

Figure 1. Simplified model of the mammalian circadian clock

The transcriptional feedback model of molecular oscillations is drawn with components that have been confirmed using in vivo methods. Clock modifiers and/or components identified through genomic methods and have a biochemically defined role in the clock are highlighted in red. Genomics approaches that have identified novel circadian outputs and modifiers of clock function are depicted and include mass spectrometry, microarrays, and cell based screening.

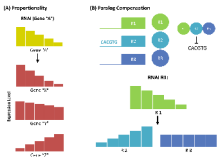

Figure 2. Perturbation analysis revealed two principles of clock function, proportionality and paralog compensation

(A) Proportionality. Knockdown of a transcriptional activator, Gene “A”, results in linear and proportional changes in genes “X”, “Y”, and “Z”. While “X” and “Y” go down in response to knock down of gene “A”, consistent with a role of gene “A” as an activator of those genes, gene “Z” goes up, indicating a repressive relationship between gene “A” and gene “Z”. (B) Paralog Compensation. Given a family of paralogous transcriptional repressors, R1, R2, and R3, and a regulatory architecture where R2 harbors the response element bound by this family, the following responses occur following knockdown of R1: R2 goes up, R3 is unchanged.