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Genomic analysis reveals novel connections between alternative splicing and circadian regulatory networks

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Abstract

Circadian clocks, the molecular devices present in almost all eukaryotic and some prokaryotic organisms, phase biological activities to the most appropriate time of day. These devices are synchronized by the daily cycles of light and temperature, and control hundreds of processes, ranging from gene expression to behavior as well as reproductive development. For a long time, these clocks were considered to operate primarily through regulatory feedback loops that act at the transcriptional level. Recent studies, however, conclusively show that circadian rhythms can persist in the absence of transcription, and it is evident that robust and precise circadian oscillations require multiple regulatory mechanisms operating at the co-/post-transcriptional, translational, post-translational and metabolic levels. Furthermore, these different regulatory loops exhibit strong interactions, which contribute to the synchronization of biological rhythms with environmental changes throughout the day and year. Here, we describe recent advances that highlight the role of alternative splicing (AS) in the operation of circadian networks, focusing on molecular and genomic studies conducted in *Arabidopsis thaliana*. These studies have also enhanced our understanding of the mechanisms that control AS and of the physiological impact of AS.

Keywords: *alternative splicing; circadian rhythms; Arabidopsis thaliana; gene expression networks*

INTRODUCTION

Alternative splicing (AS), the process by which a single gene can give rise to different mRNA isoforms, is emerging as a key regulator of numerous biological processes in eukaryotic organisms [1]. AS regulates various biological processes by controlling transcript levels and/or protein function [2]. For instance, AS can generate mRNA isoforms that contain premature termination codons (PTCs), which can lead to mRNA degradation through the nonsense-mediated decay (NMD) pathway [3, 4]. In addition, AS of the 5′- or 3′-UTR can affect mRNA stability and/or translatability through the inclusion or exclusion of miRNA-binding sites [5]. Furthermore, AS enhances proteome diversity and may serve as an explanation for why increasing

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levels of organismal complexity are not associated with corresponding increases in gene number [6]. Finally, AS can alter protein function or activity by modifying protein structure, leading to changes in properties such as post-translational modification, sub-cellular localization and protein stability [7].

The use of high-throughput sequencing technologies to characterize the transcriptomes of different organisms, tissues or cell types, under normal conditions or in response to multiple stimuli, has revealed that AS is the rule rather than the exception [8]. Approximately 95% of multiexonic genes in humans undergo AS [9, 10]. A previous analysis in the model plant *Arabidopsis thaliana* suggested the existence of alternatively spliced variants for approximately 40% of the genes [11]; however, more recent data suggest that at least 60% of the genes are alternatively spliced [12]. This number is likely to increase once experiments characterizing the transcriptome of samples obtained under various environmental conditions and from a variety of genetic backgrounds or from different tissues and cell types are analyzed.

Although the number of AS events identified in eukaryotic organisms has steadily increased over the past few years, little is known about their biological significance. An improved understanding of the regulatory functions of AS events requires a deep and thorough analysis of their contribution to the regulation of specific biological processes. Interestingly, many reports have revealed tight connections between AS and the network controlling biological timing across kingdoms [13–15]. This regulatory cross-talk affects the levels and functions of core clock genes and the output pathways that link the central oscillator to multiple biological processes under its control and regulates the levels and/or activities of *trans*-acting factors that interact with *cis*-acting elements to regulate AS.

SIGNALING NETWORKS DRIVING CIRCADIAN OSCILLATIONS

The circadian system is a complex signaling network that allows organisms to anticipate periodic changes in the environment. At the heart of the system lies an endogenous molecular device that oscillates with a period of approximately 24 h. This oscillator is coupled to output signaling pathways that control multiple biological processes, and to input signaling pathways that synchronize the clock in response to daily changes in light and temperature, allowing it to keep track of time throughout the seasons [16]. The genetic and molecular dissection of circadian networks conducted in *Drosophila*, *Neurospora*, *Arabidopsis* and mice over the last three decades has identified many of the components that are required for the proper function of the core oscillatory mechanism. Although the core clock components appear to differ greatly among fungi, plants and animals [17], a common theme across the kingdoms is that the robust and precise rhythms depend on negative transcriptional feedback loops involving a key set of transcription factors and a few additional interacting proteins [16]. In plants, the central oscillatory mechanism involves two single MYB domain proteins, CIRCADIAN CLOCK ASSOCIATED (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), whose levels peak in the morning, and which negatively regulate the expression of the evening-phased gene, *TIMING OF CAB EXPRESSION 1* (*TOC1*), also known as *PSEUDO RESPONSE REGULATOR 1* (*PRR1*) (Figure 1A) [18]. In turn, *TOC1* homologs *PRR9*, *PRR7* and *PRR5* act as negative transcriptional regulators that progressively repress *CCA1* and *LHY* expression from noon to dusk [19, 20]. Indeed, TOC1, originally considered an indirect activator of *CCA1* and *LHY* expression during the night, has recently been shown to act as a direct transcriptional repressor of these MYB transcription factors at dusk (Figure 1A) [21–23]. Several groups have reported a role for *LHY-/CCA1-like* (*LCL*) genes, also known as *REVEILLES* (*RVEs*), in the plant circadian network. The products of some of these genes regulate *TOC1* expression, while others appear to function mainly as output factors that control plant growth and development [24–26]. Furthermore, in addition to the interplay between these MYB and PRR transcriptional regulators, a recent study revealed a prominent role for a multi-protein complex, called the evening complex (EC), that involves EARLY FLOWERING 3 (ELF3), ELF4 and LUX ARRHYTHMO (LUX), in the repression of morning clock genes, such as *PRR9*, during the night [27–30].

Similar transcription networks have been shown to control circadian oscillations in fungi and animals; however, the genes involved are different [31]. In *Drosophila*, the central oscillator depends on a negative transcriptional feedback loop that involves the bHLH transcription factors CLOCK and CYCLE, known as CLOCK and BMAL in mice, which are antagonized by PERIOD (PER) and TIMELESS (TIM) in *Drosophila* or PER and CRYPTOCHROME

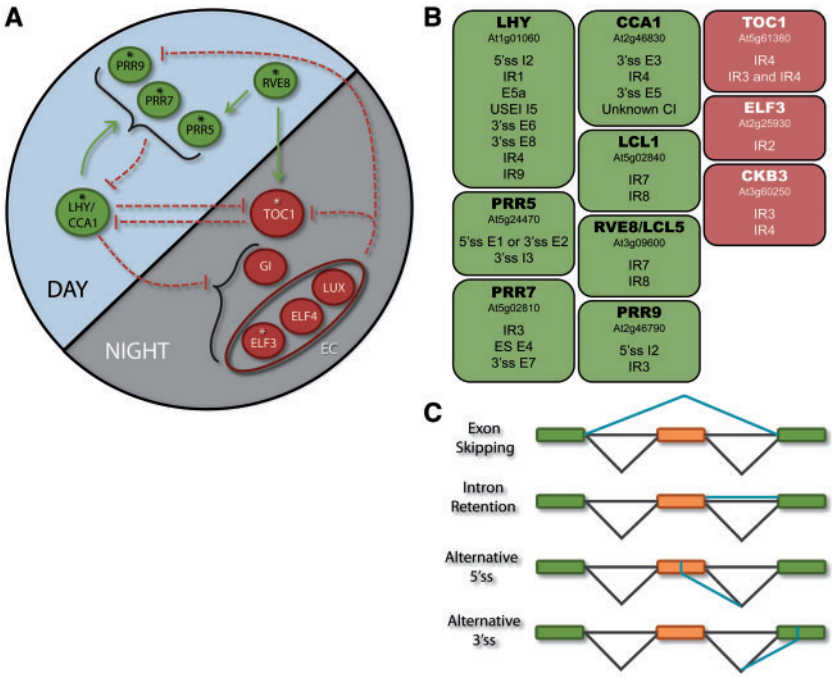

Figure 1: *Arabidopsis thaliana* core clock genes undergo extensive AS, implicating AS as a mechanism involved in the operation of the circadian clock. (**A**) Regulatory feedback loops acting at the transcriptional level in the *Arabidopsis* central oscillator are shown. Solid lines indicate positive action and dashed lines indicate negative action. Clock genes for which extensive AS has been reported are marked with an asterisk. (**B**) AS events in the core clock and clock-associated genes of *Arabidopsis* identified mostly by high-throughput and high-resolution methods. These events involve the four basic types of AS depicted in (**C**), with IR being the main one. Many of these AS events are regulated by environmental changes, suggesting that they have a physiological role. EC, evening complex; 5′-ss, alternative 5′-splice-site selection; 3′-ss, alternative 3′-splice-site selection; IR, intron retention; E5a, alternative exon E5a inclusion; USEI, upstream sub-intron and alternative exon inclusion; CI, cryptic intron.

(CRY) in mice [31, 32]. In *Neurospora*, a transcriptional complex involving the WHITE COLLAR COPMLEX (WCC) proteins promotes the expression of *frequency* (*frq*), which then antagonizes WCC activity and thereby closes the loop [33].

Despite the prominent role that transcriptional regulation appears to play in the operation of circadian networks across kingdoms, recent studies have shown that these networks can operate, albeit with altered properties, in the absence of transcription [34–38]. Indeed, numerous studies have shown that proteins that affect post-transcriptional, post-translational and metabolic pathways also have a key role in the normal operation of clock function [39–43]. Several excellent reviews have recently described the prominent role that post-transcriptional regulatory mechanisms play in the control of circadian networks [14, 15, 44, 45]. Here, we focus on recent findings that highlight the role of AS in this process.

AS OF CORE CLOCK GENES

Soon after the clock gene *period* (*per*) was identified in flies, it was reported that it could give rise to different mRNA isoforms through AS [46]. A detailed analysis revealed the existence of two mRNA isoforms of *per* in *Drosophila*, which encode identical proteins, but differ in the presence or absence of a short intron in the 3′-UTR region [47]. AS of the core clock gene *frq* was also reported several years ago in *Neurospora* [48, 49]. This gene can give rise to two different protein isoforms that differ in length by 99 amino acids [48, 49]. Plants are no exception; recent studies revealed that two core clock genes in plants, *CCA1* and *PRR9*, also undergo AS [11, 50]. Interestingly, AS of *CCA1* is conserved across many plant species [11]. These results indicate that AS of the core clock genes is a widespread phenomenon and may play a prominent role in the operation of circadian networks in fungi, plants and animals.

HIGH-THROUGHPUT METHODS EXPAND THE LIST OF AS EVENTS AMONG PLANT CLOCK GENES

Two recent reports have significantly expanded the number of clock genes known to be alternatively spliced in *Arabidopsis* (Figure 1). James *et al*. [51] used a high-resolution RT–PCR panel to characterize AS events associated with 10 core clock genes in samples harvested at different times of day and under different environmental conditions. Using this approach, the authors identified a total of 63 AS events, of which 26 showed significant abundance, suggesting that they are indeed physiologically relevant and not merely the result of incomplete splicing. In agreement with this notion, at least 13 of these AS events, which were associated with seven core clock genes, were affected by exposure to low temperatures [51]. Approximately 45% of these AS events involve intron retention (IR), which is the most common AS event in plants; however, alternative donor/acceptor sites, as well as exon skipping events, were also identified (Figure 1). Several of these events lead to the introduction of PTCs, which in some cases generate a reduction in the transcript levels through the NMD pathway [51]. A similar picture emerged from another recent study in which massively parallel RNA sequencing (RNA-seq) was used to characterize possible AS events associated with core clock and clock-associated genes in *Arabidopsis* [52]. This approach allowed the authors to identify many AS events associated with core clock or clock-associated genes, and these findings were validated by RT–PCR, qRT–PCR or Sanger sequencing. Among the identified AS events were some previously found to be associated with *CCA1* and *PRR9*, as well as novel events associated with *LHY*, *LCL1/RVE4*, *CIR1/RVE2* and *LCL5/RVE8*, including an alternative Poison Cassette Exon in intron 1 of *RVE2/CIR1* that most likely affects mRNA levels of this gene through NMD [52]. Strikingly, most of the alternatively spliced mRNAs identified in this study (14 out of 17) were associated with IR events. The high prevalence of IR, however, might be associated with the particular type of bioinformatics analysis used in this study, as some relatively abundant AS events involving alternative donor splice sites characterized in other studies were not identified. This suggests that RNA-seq is a powerful technology, but that further bioinformatics developments are needed to fully utilize it. All of these findings clearly indicate that AS is a much more widespread and quantitatively important phenomenon among clock genes than previously appreciated.

BIOLOGICAL CONSEQUENCES OF AS OF CLOCK GENES

Despite the recent appreciation of the vast extent of AS in plants and animals, an important challenge is to decipher the biological impact of these events. To achieve this goal, detailed studies characterizing the effects of the different isoforms under various environmental conditions will need to be undertaken, and the associated biological processes will need to be thoroughly investigated. Circadian networks are an ideal system in which to address these issues, given that we have a significant understanding of this system and its components.

Per was the first clock gene for which a thorough understanding of the biological significance of its regulation by AS was achieved. AS of *per* in *Drosophila* is regulated by temperature and photoperiod [53]. Although AS of *per* does not lead to changes in protein structure, this event is coupled with the regulation of *per* mRNA stability and/or translatability, and, indeed, complementation of *per* null mutants with the two different isoforms leads to differences in period length [47]. More importantly, splicing of intron 8 in the 3′-UTR of *per* accelerates PER accumulation, shifting locomotor activity toward the daytime in the short and cold days of winter, and thereby allowing the appropriate adjustment of locomotor behavior to seasonal progression [54–57].

AS of *frq* has also been extensively studied in *Neurospora*. The two different FRQ protein isoforms, LFRQ and SFRQ, result from differential usage of alternative initiation codons [48, 49]. Indeed, the AUG_L codon, which is associated with the LFRQ isoform, is present within an intron that is alternatively spliced. Retention of this intron leads to the exclusive use of the AUG_L initiation codon, while its removal by splicing leads to the usage of the alternative initiation codon called AUG_S [33, 48, 49]. This AS event is in turn regulated by temperature, which affects the ratio between these isoforms. Specific ratios of these isoforms are required for the proper operation of the clock at low and high temperatures, as well as for temperature compensation (i.e. the ability of circadian rhythms to keep a constant period of approximately 24 h under a wide range

of temperature conditions) [58]. Thus, AS of *frq* sustains robust circadian rhythms across a range of temperatures in *Neurospora*.

In plants, the role of AS of clock genes is only starting to be understood. Using a forward genetics approach, we showed that PROTEIN ARGININ METHYL TRANSFERASE 5 (PRMT5), which methylates histones and spliceosomal proteins, is required for normal operation of the plant circadian clock [50]. Although the methylated protein through which PRMT5 controls clock function remains to be identified, the circadian defect of *Arabidopsis prmt5* mutants is strongly associated with an alteration in AS of the core clock gene *PRR9*. This gene can produce four different isoforms, only one of which leads to the production of the full-length protein, and levels of this particular isoform are strongly reduced in the *prmt5* mutant. Indeed, genetic analysis indicates that the phenotype of *Arabidopsis prmt5* mutants requires the activity of *PRR9* and its close homolog *PRR7* [50]. The three remaining mRNA isoforms, whose levels are increased in *prmt5* mutants, do not have biological activity *per se*; however, the presence of PTCs coupled with NMD most likely contributes to the regulation of the precise timing of changes in *PRR9* mRNA levels. In agreement with this possibility, the most abundant alternatively spliced isoform in wild-type plants is out of phase with the canonically spliced mRNA under some environmental conditions [50]. While more studies that characterize in detail the contribution of daily changes in AS of *PRR9* to the proper regulation of the plant circadian clock are needed, additional evidence linking the regulation of AS of *PRR9* to the control of the circadian network in *Arabidopsis* has just been reported [59]. Indeed, mutations in the gene-encoding SNW/Ski-interacting protein (SKIP), a homolog of the yeast spliceosomal protein prp45, lengthens the period of circadian rhythms in *Arabidopsis* in a temperature-dependent manner, and this phenotype is associated with defective AS of *PRR9* and other genes [59].

Interestingly, Seo *et al.* [60] have recently shown that AS of *CCA1* plays a key role in coordinating the operation of the circadian clock in response to cold treatments in *Arabidopsis*. As previously reported, *CCA1* undergoes AS to produce an alternatively spliced isoform that retains intron 4, *CCA1*β, in addition to the canonically fully spliced isoform, *CCA1*α. Levels of *CCA1*β under normal growth conditions range from 35% to 65% of *CCA1* transcript levels, suggesting that the AS of this gene is physiologically relevant. Indeed, *cca1-2* mutant plants transformed with a *CCA1* pro:*CCA1*α minigene construct, which lacks functional *CCA1*β but possesses similar levels of *CCA1*α as wild-type plants, show a long circadian period phenotype [60]. Furthermore, low temperature affects the AS of *CCA1*, reducing levels of *CCA1*β, and this correlates with an enhanced freezing tolerance response and arrythmicity under cold conditions [60]. Taken together, these results suggest that AS of *CCA1* plays a major role in the integration of temperature signals and circadian circuits in plants. According to Seo *et al.*, *CCA1*β mRNA produces a protein that lacks the N-terminal DNA-binding MYB domain, but retains the C-terminal dimerization domain. This protein isoform interferes with the formation of CCA1α–CCA1α and LHY–LHY homodimers, as well as with that of CCA1α–LHY heterodimers, and its overexpression shortens the period of circadian rhythms [60]. Thus, AS of *CCA1* could affect clock function through generation of a truncated and dominant negative isoform of CCA1. Whether this is indeed the case, however, awaits demonstration of the existence of this truncated protein *in vivo*.

CIRCADIAN REGULATION OF CLOCK OUTPUTS: FROM SINGLE GENES TO GENOME-WIDE STUDIES

One of the strongest outputs of the *Arabidopsis* circadian network is the gene encoding the RNA-binding protein glycine rich protein 7 (GRP7), an heterogeneuos nuclear ribonucleoprotein (hnRNP)-like protein that controls its own transcript levels through AS coupled to NMD [61, 62]. Expression of this gene is strongly regulated at the transcriptional level by the circadian clock, with peak expression at the end of the day [61, 62], and this contributes to the control of abiotic stress tolerance and flowering time [63, 64]. Interestingly, *At*GPR7 binds to its own transcript as well as to that of its homolog AtGRP8, thereby enhancing the production of alternatively spliced isoforms that contain PTCs and causing a reduction in mRNA levels through the NMD pathway [65, 66]. Thus, *At*GRP7 constitutes a feedback loop that controls its transcript levels through AS regulation of its own gene product. In addition, we have recently shown that AS of the *RUBISCO ACTIVASE* (*RCA*) gene is also regulated

by light and the circadian clock in *Arabidopsis* [50]. Interestingly, AS of RCA generates two different mRNA isoforms [67], and the shorter encodes a protein whose activity is light independent, and the other encodes a protein whose activity is regulated by light intensity. The observation that AS of the mRNA isoform that encodes the light-regulated protein increases during daytime [50] supports the notion that AS regulation of this gene is biologically relevant.

In addition to these individual examples, genomic approaches are widely expanding our appreciation of the phenomenon of AS. A few years ago, Hazen *et al*. [68] analyzed the circadian transcriptome of *Arabidopsis* plants using tiling arrays and identified 499 introns whose levels oscillated with a 24-h periodicity, 213 of which were present in genes whose expression was also regulated by the clock. In many cases, the exons cycled in phase with the retained introns, but in others the exons and retained introns were out of phase, suggesting these were indeed IR events and not simply incompletely processed pre-mRNAs. Although most of these IR events are likely to contain PTCs, a recent study has shown that IR events containing PTCs are unlikely to be degraded through the NMD pathway in plants [3]. It is possible that some of these intron-containing transcripts are translated, giving rise to proteins with novel properties, or that they eventually act in a dominant negative manner, as suggested above for CCA1. Strikingly, the authors also found cycling introns associated with transcripts that showed no evidence of circadian expression of coding regions, revealing that the circadian clock can control transcriptional and post-transcriptional regulatory layers independently [68].Genome-wide studies of AS have also been conducted recently in flies and mice [69, 70]. In the former organism, RNA-seq analysis was used to characterize the brain transcriptome of wild-type and *per* null mutant flies in both light/dark conditions and in continuous darkness. In addition to identifying several hundred genes whose expression oscillated under the control of the circadian clock in *Drosophila*, the authors also identified many ncRNAs and several AS events that were regulated by either PER levels or by time of day [69]. Most of the clock-regulated AS events were associated with alternative transcription start sites. Curiously, lack of PER activity had a much stronger effect on AS patterns than did time of day. One possible explanation for this is that transcripts that had a stronger dependence on PER than on time of day have a long half life, which would mask any circadian oscillation. Alternatively, even though the authors studied the transcriptome of brains and not that of whole animals or fly heads, the mRNAs evaluated still correspond to a heterogenous population of cells. Therefore, circadian oscillations in AS may be obscured by differences in phase and/or amplitude across cell types. In support of this possibility, strong differences in amplitude of expression of clock genes in flies have been observed between transcriptomes of whole heads and specific subsets of pacemaker neurons [71].

Circadian oscillations in AS have also been identified in mice through the characterization of the liver transcriptome using Affymetrix exon arrays [70]. This analysis suggests that tissue-specific circadian regulation of AS is an important phenomenon, at least in mammals. The characterization of the liver transcriptome is interesting, because it constitutes a paradigm for the analysis of circadian regulation of metabolic processes and peripheral oscillators by the master clock in the suprachiasmatic nucleus, the region of the brain that controls circadian cycles. To identify clock-regulated AS events, the authors looked for deviations in the expression of individual exons and whole transcripts across a circadian cycle. Using this approach, they found 55 exons from 47 genes that oscillated in a circadian manner [70]. Many of these events were cassette exons whose associated signals peaked at different times of day. In most cases, genes that exhibited circadian regulation of AS were controlled by the clock at the whole transcript level, although in some cases differences in phase were observed between individual exons and whole transcripts. Interestingly, similarly to the finding of Hazen *et al*. [68] in *Arabidopsis*, the authors detected some genes with evidence of circadian regulation of AS that did not oscillate at the whole transcript level [70]. Gene ontology analysis revealed enrichment not only in the AS of metabolic genes but also of clock genes and of genes associated with the PPAR signal transduction pathway. Many of the AS events identified were validated in independent samples, and circadian regulation of a subset of these was also investigated in other tissues, such as lung and kidney [70]. In some cases, the expression and AS of a particular gene oscillated similarly in the liver and in other tissues, while in other cases circadian oscillations were conserved, but the phases differed. For other genes, circadian oscillations in either AS

or whole transcript levels were tissue specific. Together, these findings reveal that circadian regulation of mRNA levels and RNA processing events rely on complex networks that can act independently on individual genes.

AS REGULATION: TRADITIONAL AND CONTEMPORARY VIEWS

An important challenge now is to decipher the mechanisms underlying the circadian regulation of AS. In order to address this issue, we first review briefly what is known about AS regulation. Splicing is catalyzed by a large and dynamic RNA–protein complex, which involves five small nuclear ribonucleoproteins (snRNPs) composed of different U-rich small nuclear RNAs, seven common Sm proteins, and a variable set of additional proteins specific for each snRNP [72]. In addition, these core spliceosomal components interact with a set of auxiliary splicing factors, such as serine/arginine-rich (SR) proteins and hnRNPs, which contribute to the recognition and/or selection of different *cis*-acting sequences that define the boundaries of introns and exons [72]. The most important *cis*-acting sequences are the donor or 5′-splice site (5′-ss), the acceptor or 3′-splice site (3′-ss), the branch point (A), the polypyrimidine (py) tract, and additional exonic and intronic enhancers and silencers of splicing (ESE, ISE, ESS and ISS) [73]. The traditional view of AS regulation is that splice-site selection depends on the combinatorial effect of SR proteins that promote and hnRNPs that inhibit the ability of U1 and U2 snRNPs to recognize nearby donor and acceptor splice sites, respectively [74]. Thus, regulation of AS by endogenous or environmental cues was thought to rely on changes in the level and/or activity of specific sets of auxiliary splicing factors. However, there is increasing evidence that AS regulatory mechanisms that do not involve auxiliary splicing regulators help generate the great variety of AS events seen in eukaryotic organisms [75]. These mechanisms include epigenetic modifications that affect AS by modulating the rate of transcriptional elongation, altering the time during which weak splice sites can be recognized by U1 and U2 snRNPs. Alternatively, histone modification can modulate AS by affecting the recruitment of splicing factors through interactions that involve RNAP II and adaptor proteins [76, 77]. In addition, many reports have recently challenged our understanding of AS regulation by showing that down-regulation or mutations in specific core spliceosomal components, such as U1C in zebrafish [78] or SmB in human cells [79], or modulators of spliceosome assembly, such as survival motor neuron (SMN) in mice [80] and protein arginine methyltransferase 5 in plants and flies (PRMT5) [50], which are central components of the SMN and methylosome complexes, have only modest effects on constitutive splicing but affect particular subsets of AS events. Changes in the activity and/or concentration of core splicing factors can strongly influence AS regulation, most likely by altering the kinetics of one or more steps in spliceosome assembly. It is uncertain, however, how many AS events are regulated by general splicing factors versus auxiliary splicing factors, and why some splice sites are more susceptible than others to changes in the levels or activities of these splicing factors. We also lack a good understanding on which particular spliceosomal components are involved in this type of regulation in response to endogenous or external signals, and which particular steps in spliceosome assembly and catalysis are subject to this regulation.

REGULATING THE REGULATORS

A possible mechanism for the circadian regulation of AS involves the transcriptional regulation of splicing factors. Indeed, we have shown that PRMT5, a protein that affects splicing of both core clock and clock-output genes, is regulated by the circadian oscillator at the mRNA level in *Arabidopsis* [50]. Furthermore, AS of RCA, an AS event regulated by PRMT5, shows diurnal and circadian oscillations in wild-type plants, and these oscillations are severely disrupted in *prmt5* mutants [50]. A similar observation was made for the SR gene *RSP31,* another target of *prmt5* at the AS level in plants.

A more thorough analysis of potential mechanisms that mediate the circadian regulation of AS can be achieved by identifying the splicing factors or regulators whose expression is regulated by the circadian clock at the genome-wide level. This analysis has actually been conducted in the liver transcriptome [70] and revealed that 62 out of 227 splicing factors were rhythmic at the mRNA level. These transcripts, which correspond to several helicases, components of the U2 snRNP and numerous hnRNPs, including Cirbp, a Cold inducible RNA-binding protein that is a close homolog of AtGRP7, displayed a variety of phases, suggesting that oscillations in mRNA levels of splicing factors could

contribute to the regulation of AS throughout the day-night cycle [70]. Thus, clock- and temperature-mediated regulation of this particular family of hnRNPs is conserved in plants and animals, and presumably contributes to the circadian regulation of AS. We have conducted a similar analysis, comparing the list of genes regulated by the circadian oscillator at the mRNA level with the list of splicing factors and regulators in plants (S. Perez-Santángelo *et al.*, unpublished data), and some of the results are presented in Figure 2. This comparison revealed that the circadian clock regulates the mRNA levels of 83 genes from a list of 426 splicing-related genes. This overlap of 83 genes does not represent an enrichment of splicing factors among clock-regulated gene categories, but constitutes a list of candidate genes that may mediate the circadian regulation of AS in this species. Interestingly, the list includes not only SR and hnRNPs, but also core spliceosomal proteins, regulators of spliceosome assembly,

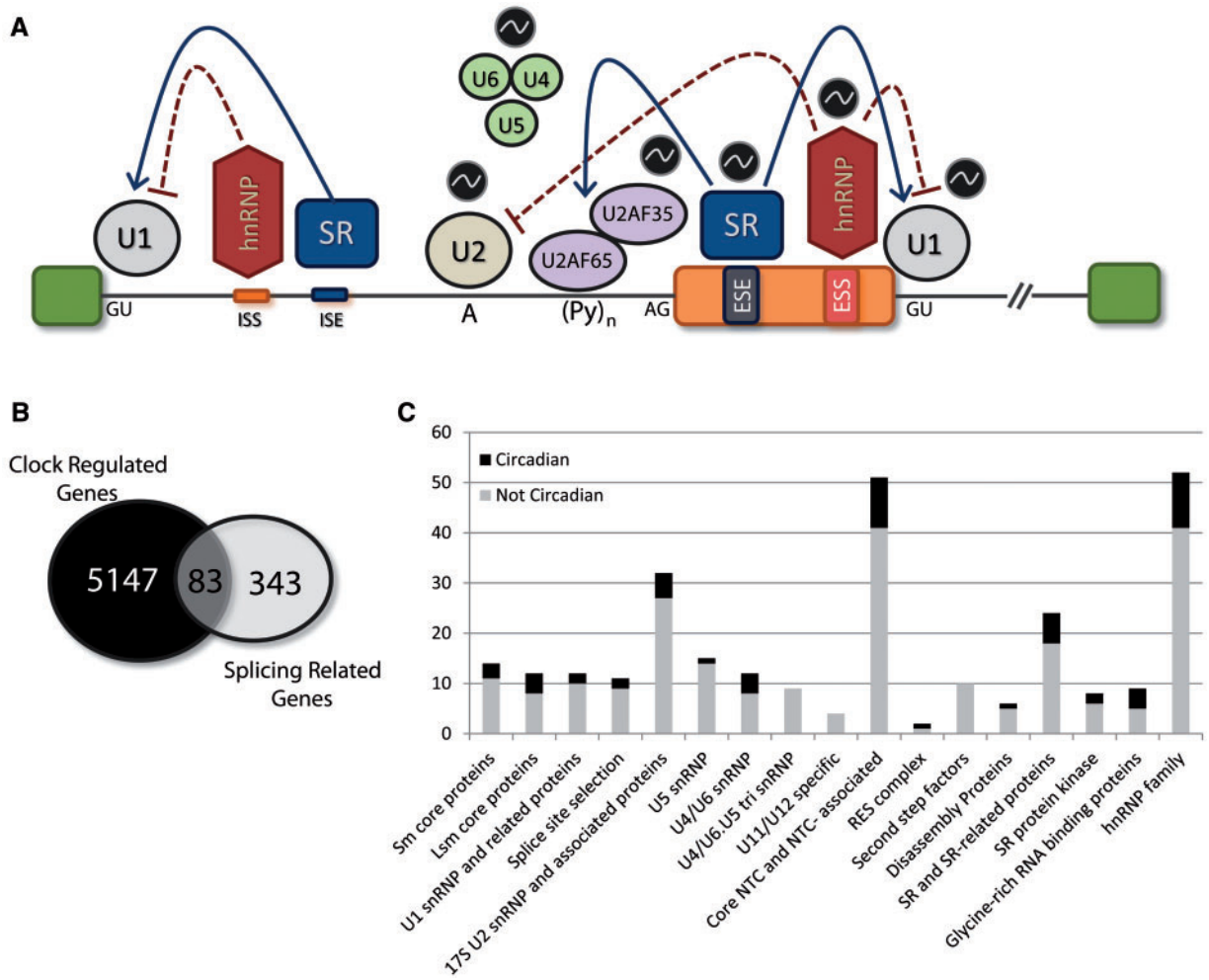

Figure 2: Many *Arabidopsis thaliana* splicing factors and regulators are under clock regulation, showing a circadian expression pattern. (**A**) Splicing is catalyzed by the spliceosome, a macromolecular complex that consists of five small nuclear ribonucleoproteins (snRNPs). Spliceosomes bind to core elements necessary for pre-mRNA splicing, including the 5′- and 3′-splice sites, a branch point sequence, and a polypyrimidine-rich tract. Auxiliary splicing factors, such as SR proteins and hnRNPs, bind to auxiliary sequences (ESE, ISE, ESS, ISS) that vary in number and location, enhancing (solid lines) or inhibiting (dashed lines) basal splicing activity, respectively. Core spliceosomal proteins and auxiliary splicing factors regulated by the circadian clock are highlighted. (**B**) Overlap between circadian-regulated genes and splicing factors or regulators listed in datasets, shown as numbers within Venn diagram circles. (**C**) Representation of members within each group of spliceosomal components, splicing factors and regulators that are regulated or not by the circadian clock. ESE, exonic splicing enhancer; ISE, intronic splicing enhancer; ESS, exonic splicing silencer; ISS, intronic splicing silencer; UI, 2, 4, 5 and 6, the five snRNPs.

components of specific snRNPs as well as some kinases that regulate SR protein function (Figures 2 and 3). Preliminary data from our laboratory indicate that mutations in a few of these components affect clock function, but that mutations in the majority do not, suggesting that most of these components mediate the circadian regulation of AS of clock output genes, and only a few feedback to regulate the clock itself. This analysis should be extended to evaluate the effect of light and temperature stimuli on the levels of splicing factors to obtain a better understanding of the mechanisms that link AS to the circadian network. Interestingly, a recent forward genetics screen revealed an important role for an SR-like gene in the regulation of the light signaling pathway in *Arabidopsis* [81]. Thus, light, such as temperature, appears to play an important role in mediating the daily and seasonal regulation of physiological processes through its effects on AS.

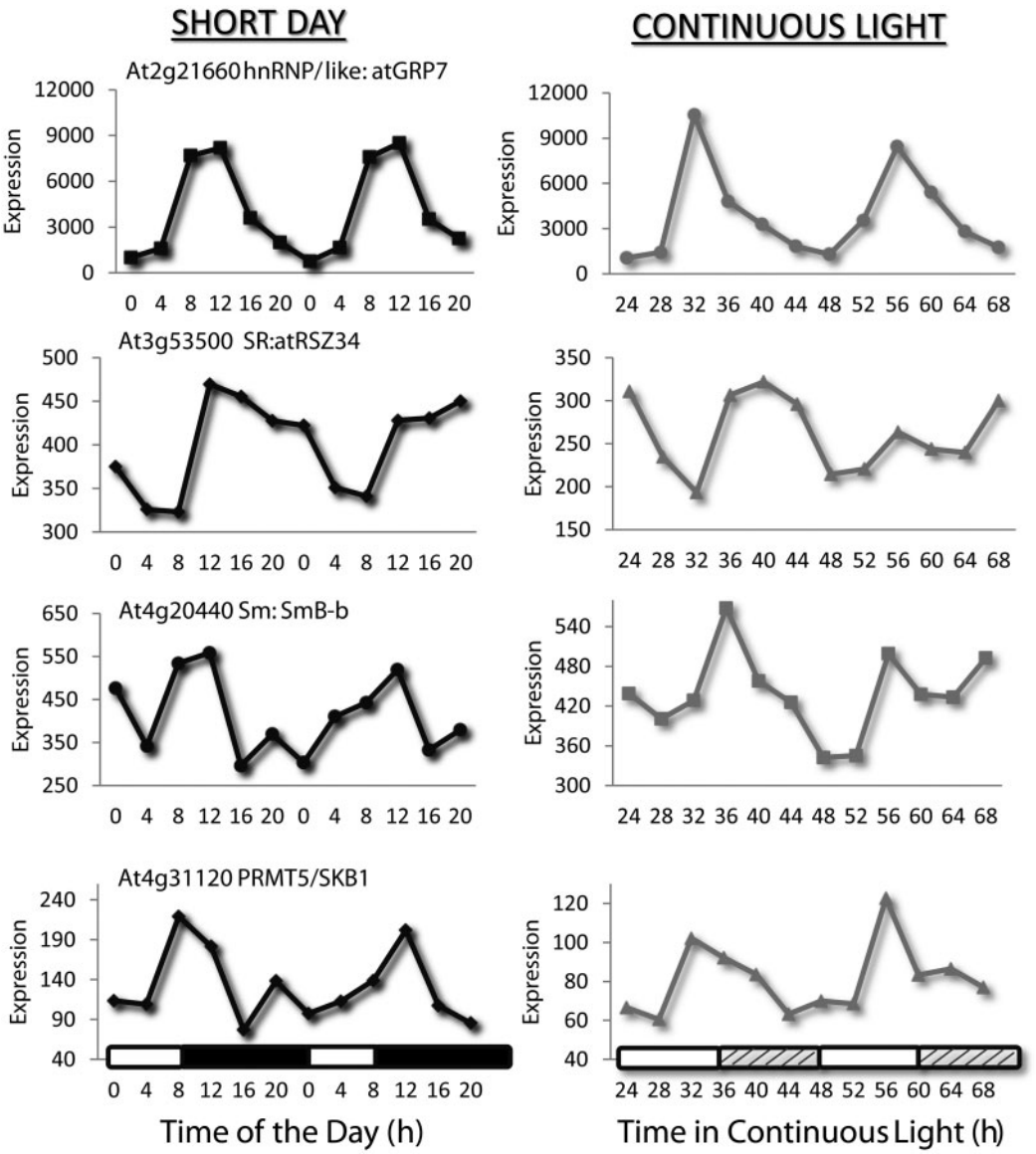

Figure 3: Examples of diurnal expression patterns of splicing factors or regulators, showing circadian regulation in *Arabidopsis thaliana*. These include auxiliary factors (atGRP7 and atRSZ34), a core component (SmB-b) and a protein linked to splicing (atPRMT5). Gene expression patterns under different environmental conditions (short day and continuous light) were obtained using the DIURNAL portal and microarray database (http://diurnal.cgrb.oregonstate.edu/).

CONCLUSIONS AND FUTURE PERSPECTIVES

Genetic and genomic studies have revealed extensive cross-talk between circadian and AS regulatory networks. Given the highly dynamic and predictable nature of circadian oscillations, the large interplay between these regulatory systems offers an incredible opportunity to decipher novel regulatory mechanisms that control the adjustment of biological processes to daily and seasonal changes, as well as to better understand the general rules controlling AS. Indeed, we have already identified many splicing-associated genes whose expression is clock-regulated, and forward and reverse genetics approaches are allowing us to identify splicing factors that affect clock function or clock output pathways in plants and animals. Future work, including RNA-seq experiments that characterize the daily changes in AS under different environmental conditions and genetic backgrounds, will allow us to identify AS regulatory modules. These modules, in combination with a detailed knowledge of the daily oscillations in the abundance of these splicing factors, as well as of the *cis*-elements that they recognize in their target pre-mRNAs, could be used to develop networks of co-regulated AS events and splicing factors, which would serve as useful tools to predict causal relationships between splicing factors and AS events [82].

Circadian clocks, which are regulated by AS, play a critical role in synchronizing reproductive events in plants, and thus have a strong impact on crop productivity [83–86]. Furthermore, mis-regulation of AS is increasingly being associated with the onset of various diseases [87]. Thus, we believe that a deeper understanding of the link between circadian clocks and AS will yield novel and important agricultural and biomedical applications.

Key Points

- Circadian clocks are based on multiple interacting feedback loops operating at the transcriptional, post-transcriptional, translational, post-translational and metabolic levels.
- There is increasing evidence that AS plays an important role in the regulation of circadian networks across eukaryotic organisms, impacting both the core clock mechanism as well as the pathways connecting the central oscillator to the hundreds of biological processes under its control.
- Genetic and genomic approaches are starting to identify the molecular components and the mechanisms linking circadian and AS regulatory networks.
- The precise and periodic nature of circadian rhythms offers a great opportunity to unravel key rules governing the process of AS in a dynamical biological context.

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