Role for *LSM* genes in the regulation of circadian rhythms

Soledad Perez-Santángelo[a], Estefanía Mancini[a,1], Lauren J. Francey[b,1], Ruben Gustavo Schlaen[a], Ariel Chernomoretz[a], John B. Hogenesch[b], and Marcelo J. Yanovsky[a,2]

[a]Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas de Buenos Aires–Consejo Nacional de Investigaciones Científicas y Técnicas, C1405BWE, Buenos Aires, Argentina; and [b]Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

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Growing evidence suggests that core spliceosomal components differentially affect RNA processing of specific genes; however, whether changes in the levels or activities of these factors control specific signaling pathways is largely unknown. Here we show that some SM-like (*LSM*) genes, which encode core components of the spliceosomal U6 small nuclear ribonucleoprotein complex, regulate circadian rhythms in plants and mammals. We found that the circadian clock regulates the expression of *LSM5* in *Arabidopsis* plants and several *LSM* genes in mouse suprachiasmatic nucleus. Further, mutations in *LSM5* or *LSM4* in *Arabidopsis*, or down-regulation of *LSM3*, *LSM5*, or *LSM7* expression in human cells, lengthens the circadian period. Although we identified changes in the expression and alternative splicing of some core clock genes in *Arabidopsis lsm5* mutants, the precise molecular mechanism causing period lengthening remains to be identified. Genome-wide expression analysis of either a weak *lsm5* or a strong *lsm4* mutant allele in *Arabidopsis* revealed larger effects on alternative splicing than on constitutive splicing. Remarkably, large splicing defects were not observed in most of the introns evaluated using RNA-seq in the strong *lsm4* mutant allele used in this study. These findings support the idea that some *LSM* genes play both regulatory and constitutive roles in RNA processing, contributing to the fine-tuning of specific signaling pathways.

posttranscriptional | alternative splicing | circadian clock | *Arabidopsis* | mammals

Circadian rhythms are persistent 24-h oscillations in biological processes that occur under constant environmental conditions. They allow organisms to coordinate multiple physiological processes with periodic or seasonal changes that occur in the environment. At the heart of the eukaryotic circadian system lies a complex set of interconnected transcriptional and translational feedback loops, in which a group of core clock genes regulate each other to ensure that their mRNA levels oscillate with a period of ∼24 h (1). The core oscillator in *Arabidopsis thaliana* involves two MYB domain-containing transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), that repress the expression of *TIMING OF CAB2 EXPRESSION 1* (*TOC1*) at the beginning of the day. In turn, TOC1, a member of the PSEUDO-RESPONSE REGULATOR (PRR) family, represses *CCA1*/*LHY* expression at the end of the day (2). Other clock components expressed throughout the day form multiple interconnected transcriptional feedback loops (2).

Mounting evidence indicates that alternative splicing (AS), the process by which pre-mRNA molecules are differentially spliced to yield multiple mRNA isoforms from a single gene, plays a key role in the regulation of circadian networks in a variety of organisms, including *Drosophila melanogaster* (3), *Neurospora crassa* (4–6), and *Arabidopsis* (7-11). For example, the core clock genes *period* in *Drosophila* and *frequency* in *Neurospora* give rise to different mRNA isoforms through AS, which helps these organisms adjust to different temperature conditions (4, 5, 12). Four different *BMAL2* transcripts, encoding proteins with varying levels of transcriptional activity, are generated by AS in humans; however, it remains to be determined whether these transcripts have different physiological functions (13). In *Arabidopsis*, several AS events associated with core clock genes have been reported, many of which seem to play a significant role in adjusting gene expression according to temperature variations. Even though multiple clock genes have been reported to undergo AS in organisms ranging from plants to humans, few splicing factors controlling clock function have been characterized (14–16).

Splicing of pre-mRNA molecules is carried out by the spliceosome, a large ribonucleoprotein complex consisting of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6, and several accessory factors. U1, U2, U4, and U5 snRNPs are composed of a specific snRNA (U1–U5 snRNA), a common ring of seven Sm proteins (SmB/B′, D1, D2, D3, E, F, G), and 3–15 snRNP-specific proteins. By contrast, U6 snRNP is composed of U6 snRNA, a ring of seven SM-like (LSM) proteins (LSM2–8), and 3–15 snRNP-specific proteins (17). The regulation of AS is associated with changes in levels and/or activities of splicing regulators or auxiliary factors, such as SR and hnRNP proteins, which either promote or inhibit the recruitment of spliceosomal snRNPs to donor and/or acceptor splice sites (18). In addition, recent evidence indicates that AS, a cotranscriptional process, is also regulated by complex interactions between spliceosomal factors, the transcriptional machinery, and chromatin structure (19, 20). Interestingly, several recent reports revealed that mutation or inhibition of core splicing factors, such as SmB in human cells and U1C in zebrafish, have a greater effect on AS than on constitutive splicing

Significance

There is increasing evidence that previously considered core constituents of multi-subunit complexes involved in RNA processing play regulatory rather than passive roles in the control of gene expression, but specific signaling pathways in which they participate are not known. Here we show that SM-like (*LSM*) genes, which encode core components of the spliceosome, are regulated by the circadian clock and control clock function in plants and mammals, revealing convergent evolutionary mechanisms mediating posttranscriptional regulation of circadian networks across kingdoms.

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[1]E.M. and L.J.F. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: myanovsky@leloir.org.ar.

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(21–23). Thus, changes in the levels and/or activities of core-spliceosomal proteins may play a regulatory role in AS, although specific signaling pathways where this is relevant are not known.

To identify genes controlling AS of the *Arabidopsis* clock genes, we characterized the circadian rhythms of clock-regulated splicing factor mutants. We found that a hypomorphic mutation in the *Arabidopsis LSM5* gene, a member of the U6 snRNP complex, lengthens the period of the circadian rhythm of leaf movement by more than 3 h. Similar circadian defects were seen in an *Arabidopsis lsm4* null mutant. Interestingly, we found that several *LSM* genes are regulated by the circadian clock in mouse suprachiasmatic nucleus (SCN) and that down-regulation of their homologs in human cells also increases the period of circadian rhythms. Our results support the idea that clock-regulated transcription of core splicing factors is a mechanism by which transcriptional and posttranscriptional regulation is linked to control clock function in eukaryotes.

Results

Characterization of Clock-Regulated Splicing Factors in *Arabidopsis*. To identify connections between the circadian clock and AS in plants, we investigated which splicing factors were clock-regulated (24–26). We found 83 splicing-related genes that cycle at the mRNA level, and thus represent candidate regulators of clock-mediated AS (Dataset S1). We evaluated the mRNA levels of five representative genes encoding clock-regulated splicing factors using real-time quantitative PCR (RT-qPCR) analysis (Fig. 1) and validated their circadian expression as described by Edwards et al. (27) (Fig. S1). We examined the circadian rhythm of leaf movement in mutants for 13 of the 83 splicing-related genes to determine whether any of these factors influence clock function. Mutations in most of these genes did not affect clock function, suggesting that these components regulate clock output genes and not the clock itself, or that they act redundantly with other genes to control clock function (Fig. S2).

A Mutation in *Arabidopsis LSM5* Confers a Long Period Phenotype and Differentially Affects the Expression and Splicing of Core Clock Genes. Interestingly, one of the mutants, *sad1/lsm5*, had a significant change in the period of the circadian rhythm of leaf movement under free-running conditions. *LSM5*, which encodes an Sm-like protein, was originally identified in a screen for genes that control abiotic stress responses, and was named supersensitive to abscisic acid and drought *(sad1)* (28). The *sad1/lsm5* mutant has a point mutation that changes a conserved glutamic acid residue to lysine (29). We found that the period length in *sad1/lsm5* mutants was more than 3 h longer than in wild-type plants (29.09 ± 1.83 h and 24.20 ± 0.32 h, respectively, $P < 0.05$) (Fig. 2*A*). To corroborate that the observed circadian phenotype was due to the point mutation in *LSM5*, we complemented the *sad1/lsm5* mutant with wild-type *LSM5* driven by its own promoter. We analyzed leaf movements of transgenic plants and found that the complementation restored the wild-type period (Fig. 2*A*).

To further analyze the role of *LSM5* in the clock, we used RT-qPCR to examine the expression of core clock genes in the *sad1/lsm5* mutant. Two-week-old plants were grown under 16-h light/8-h dark cycles and then transferred to constant light (LL) for 3 d. Samples were collected every 4 h for 1 d. We found alterations in the expression pattern of almost all clock genes analyzed in *sad1/lsm5* compared with wild-type plants (Fig. 2 *B–D* and Fig. S3 *G–I*). Furthermore, the overall mRNA levels of the early morning genes *CCA1* and *LHY* were significantly reduced (Fig. 2*B* and Fig. S3*G*). In turn, peak expression of the midday genes *PRR9* and *PPR7* was delayed ~8 h (Fig. 2*C* and Fig. S3*H*), whereas expression of the late afternoon genes *PRR5* and *TOC1* was delayed 4 h, without significant changes in overall mRNA levels (Fig. 2*D* and Fig. S3*I*). Although the delayed expression of *PRR* genes is consistent with the increased period length of the *sad1/lsm5* mutant, it is uncertain which of these changes is primarily responsible for the circadian phenotype of *sad1/lsm5*.

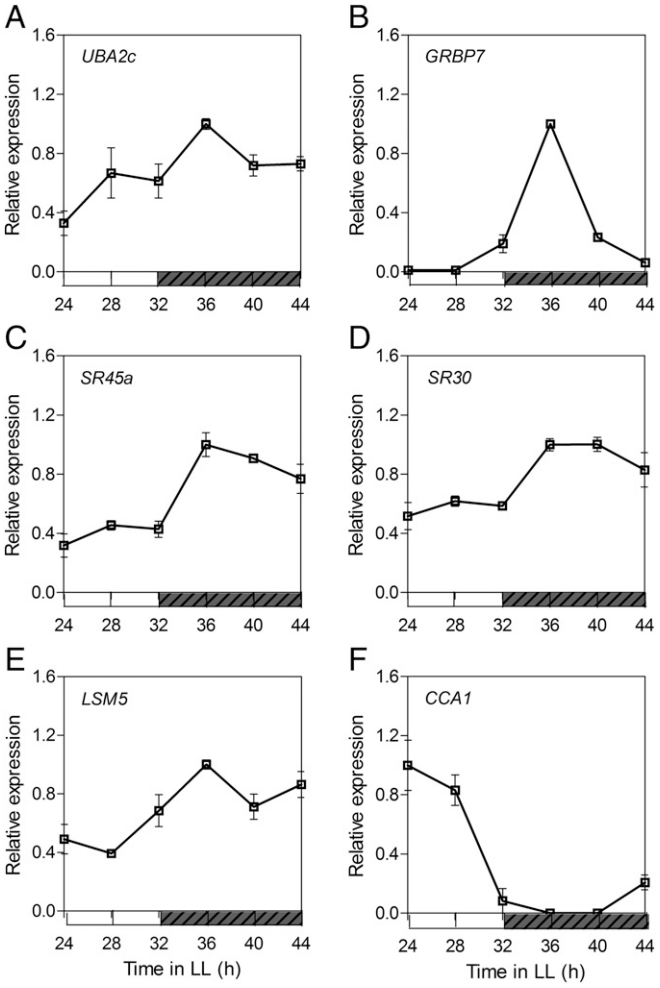

Fig. 1. Confirmation of circadian expression of selected splicing-related genes. Gene expression measured by RT-qPCR in wild-type (Col) plants entrained under short-day photoperiods (8 h light/16 h dark) before being moved to constant light (LL). (*A* and *B*) *hnRNP* genes; (*C* and *D*) *SRp* genes; (*E*) *LSM*; and (*F*) a core clock gene used as a control. Data are the average of three biological replicates expressed relative to *PP2A* and normalized to the maximum value. White boxes represent light, and gray striped boxes represent subjective night. Error bars represent SD.

We also examined mRNA levels of core clock genes in *sad1/lsm5* under light/dark conditions. A clear phase delay was found in the mutant for *CCA1* (Fig. S3*A*), *PRR7* (Fig. S3*E*), and *PRR5* (Fig. S3*F*), which is consistent with its longer circadian period phenotype. A reduction in *LHY* and *TOC1* overall mRNA levels was also observed (Fig. S3 *C* and *D*), although it is uncertain whether and how this causes the long period phenotype of the mutant.

We then evaluated whether there were changes in AS of core clock genes in *sad1/lsm5*. We found splicing changes in a subset of these genes in *sad1/lsm5*, including enhanced retention of intron 4 of *TOC1* (Fig. 2*G*), which undergoes AS in wild-type plants exposed to low temperature conditions (8). We also found a slightly enhanced retention of intron 2 of *CCA1* in *sad1/lsm5*, which is constitutively spliced in wild-type plants (Fig. 2*E*). These results were obtained with plants grown under long-day photoperiods and harvested at the time of peak expression of each gene. Similar alterations in splicing were observed in samples collected every 4 h during a 24-h cycle and then pooled for analysis (Fig. S4 *A* and *B*) or in plants grown always under continuous light and temperature (Fig. S4 *C* and *D*), indicating that the intron retention events observed were not simply the

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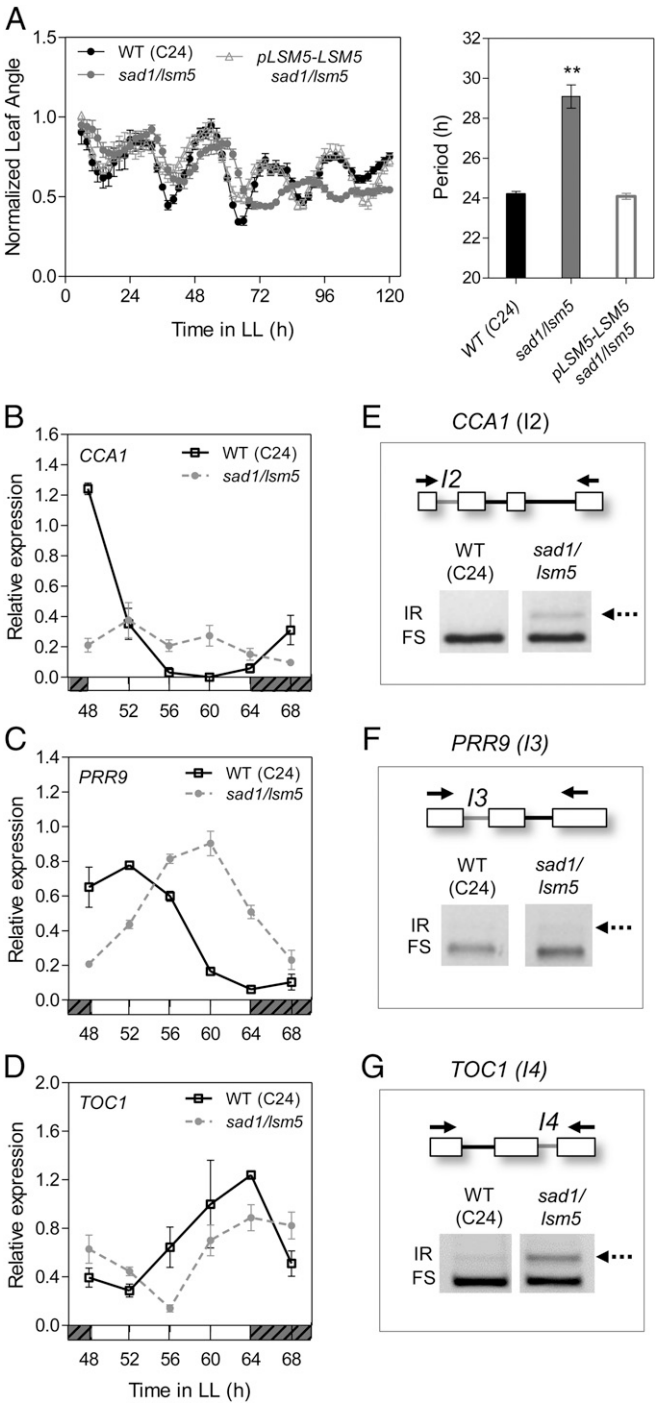

Fig. 2. A mutation in *LSM5* affects the clock. (*A*, *Left*) Circadian rhythm of leaf movement. Leaf angles were measured for the first pair of leaves in seedlings entrained under long-day conditions (16 h light/8 h darkness), and the plants were then transferred to constant light (LL). WT (C24), wild-type C24 ecotype (black circles; $n = 6$). *Sad1/lsm5* mutant (gray circles; $n = 10$). *pLSM5-LSM5* sad1/*lsm5*, *sad1/lsm5* mutant plants transformed with a wild-type version of genomic *LSM5* (gray empty triangle; $n = 9$). (*A*, *Right*) Period length of leaf movement rhythms estimated by fast Fourier transform–nonlinear least test (FFT-NLLS). Error bars represent SD. $^{**}P < 0.001$ (Student *t* test). (*B–D*) Gene expression measured by RT-qPCR in plants entrained under long days (16 h light/8 h darkness) before being moved to constant light (LL). WT (C24) (solid black line), sad1/*lsm5* mutant (dashed gray line). Data are the average of three biological replicates normalized to the maximum value. Error bars represent SD. (*E–G*) RT-PCR analysis of AS events. The analysis was conducted on samples harvested under long-day conditions at the time of peak gene expression of each gene. Exons and introns are displayed as boxes and lines, respectively, with gray lines representing the intron of interest. The solid arrows show the primer position. FS, fully spliced. The dashed arrow marks the IR event. Image represents one of three biological replicates measured.

result of changes in the phase of expression of the different transcripts caused by the circadian defect of *sad1/lsm5*.

Interestingly, no significant alterations were observed in AS events previously reported for intron 3 of *PRR9* (Fig. 2*F*), intron 5 of *LHY* (Fig. S4*E*), or intron 1 of *RVE8* (Fig. S4*F*) (30). Therefore, *LSM5* has specific, rather than global, effects on the regulation of splicing of core clock genes in plants.

Circadian Rhythm Defects in a Strong Loss of Function *lsm4* Mutant Allele in *Arabidopsis*. LSM5 is part of a multisubunit complex that forms the core of the U6 snRNP, together with six other LSM proteins. Prompted by the finding that some of the other *Arabidopsis LSM* mRNAs also oscillated (Fig. S5 *A–C*), we sought to determine whether mutations in other *LSM* genes also affected the clock. Most T-DNA insertions in *lsm* mutants cause lethality. However, we analyzed circadian rhythms in the transfer DNA (T-DNA) insertional mutant of *LSM4* (SALK_063398), which was previously characterized by Zhang et al. in 2011 (31). Homozygous *lsm4-1* mutant plants displayed severe developmental retardation compared with wild-type plants and were infertile (Fig. S6*A*). Given the small size of their leaves, it was not possible to measure rhythms in leaf movement in these homozygous mutant plants. Therefore, we transformed heterozygous plants with different bioluminescent reporters to measure oscillations in gene expression driven by the *CCA1* and *CCR2* promoters in young homozygous mutants in the next generation. We found that the circadian period of expression of these genes was lengthened by more than 2 h in *lsm4* homozygous mutants relative to wild-type plants (26.24 ± 0.70 h and 23.91 ± 0.73 h, respectively, for *CCA1::LUC* and 27.07 ± 1.47 h and 23.64 ± 0.50 h, respectively, for *CCR2::LUC*, $P < 0.05$) (Fig. 3*A* and Fig. S6*C*), and this phenotype fully cosegregated with the T-DNA insertion in *LSM4*.

A Subset of *LSM* Genes Is Regulated by the Circadian Clock and Controls the Circadian Period in Mammals. Given the importance of the U6 snRNP complex in regulating splicing in eukaryotic organisms, we evaluated the circadian regulation of mammalian *LSM* genes using CircaDB (32), which contains circadian transcriptional profiles of human and mouse cells and many mouse organs. Interestingly, we found strong circadian oscillations in the expression of *LSM3*, *LSM4*, and *LSM7* in the SCN (Fig. S5 *D–F*), the "master" oscillator in mammals that coordinates circadian rhythms throughout the body (33). To establish whether these genes influence clock function in human U2 OS cells, we used data from the BioGPS portal (34, 35). Interestingly, two independent RNAi knockdown experiments of *LSM7* generated a long period phenotype similar to that observed when knocking down the *CRY2* core clock gene (Fig. S7 *G* and *H*). A long circadian period phenotype in cells was also seen after knocking down the expression of *LSM3* or *LSM5* with one pair of siRNAs (Fig. S7 *A* and *F*). In another experiment using a second pair of siRNAs, knocking down the expression of these genes greatly dampened circadian oscillations (Fig. S7 *B* and *E*), a phenotype also observed after knocking down *LSM4* (Fig. S7 *C* and *D*). Therefore, to verify the effect of *LSM3* and *LSM5* on clock function, we repeated the analysis with a pool of four siRNAs and confirmed that down-regulation of *LSM3* and *LSM5* in human cells resulted in longer circadian period phenotypes compared with control cells (Fig. 3 *B* and *C* and Dataset S2). These results provide strong evidence that *LSM* genes also regulate circadian rhythms in mammals.

Mutations in *LSM4* and *LSM5* Result in Large Alterations in Expression and Splicing of a Subset of Genes in *Arabidopsis*. Recent studies revealed that the *sad1/lsm5 Arabidopsis* mutant has intron-

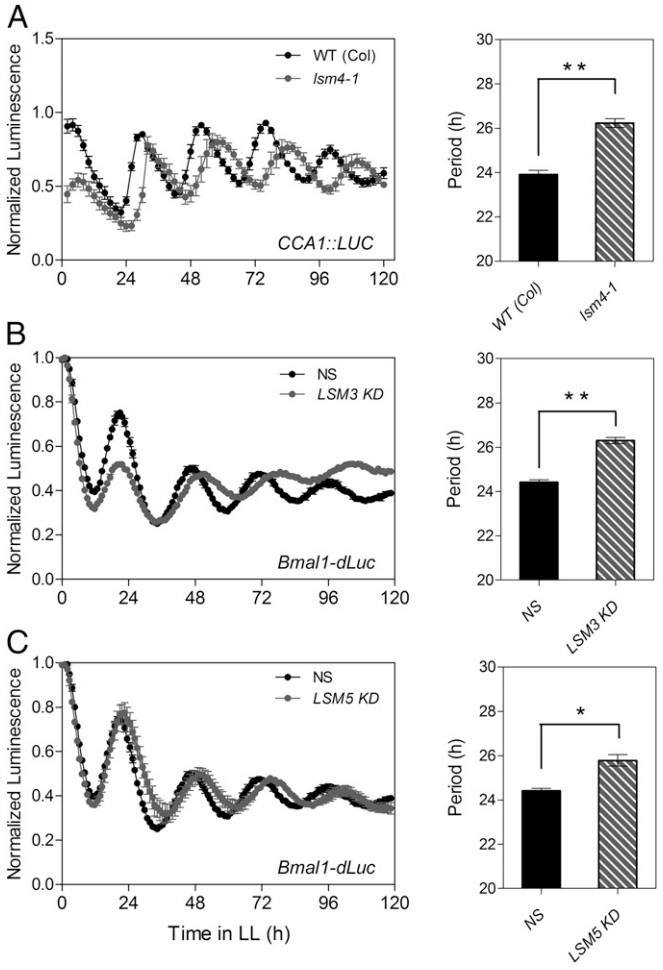

Fig. 3. Mutations in plant *LSM4 and* down-regulation of human *LSM3* and *LSM5* affect the clock. (*A*) CCA1::LUC activity measured for 5 d in constant light (LL) after entrainment under long-day conditions. WT (Col), wild-type Col ecotype (black circles, $n = 14$); *lsm4-1*, Salk_063398 mutant (gray circles, $n = 11$). Bioluminescence was recorded every 2 h over 5 d and analyzed by FFT-NLLS, and period values were plotted. (*B* and *C*) *BMAL1-dLuc* activity measured in U2OS cells after siRNA transfection and dexamethasone synchronization using a Synergy microplate reader. NS, Allstars negative siRNA control from Qiagen (black circles); *KD*, knock-down (gray circles). Bioluminescence was recorded every hour over 5 d, and period length was quantified using *Waveclock* (43) implemented in R. Data represent the average bioluminescence of three replicates. $*P < 0.05$; $**P < 0.001$ (Student *t* test).

specific splicing defects (29). One of these studies analyzed splicing alterations at the genome-wide level using high-throughput RNA sequencing (RNA-seq) and found that *LSM5* controls the splicing efficiency and splice-site recognition of many splicing events, but is particularly required for regulating the splicing efficiency of stress-responsive genes (29). Interestingly, splicing of most other introns was not affected. These discriminating splicing events either suggest a specific splicing role for some LSM proteins or reflect the weak nature of the *sad1/lsm5* mutation. To examine these possibilities, we used RNA-seq to analyze the transcriptome of both the weak *sad1/lsm5* mutant and a strong mutant with a T-DNA insertion in the coding region of *LSM4* in *Arabidopsis*, *lsm4-1* (SALK_063398). For this, we sequenced cDNA libraries prepared from three independent biological replicates of each of the two different *lsm* mutants and their corresponding wild-type accessions. We mapped the resulting reads to the *Arabidopsis* genome (TAIR10 version) and evaluated changes in the gene expression and splicing of annotated AS events associated with the expressed genes. We also performed an in depth analysis of splicing of all introns present in genes expressed above a threshold level in all genotypes.

For this analysis, plants were grown from germination until harvest under continuous light and temperature without any prior exposure to light or temperature cycles. Under this condition circadian rhythms are strongly attenuated, and differences in expression are unlikely to result simply from differences in the phase of clock-regulated transcripts caused by the long period phenotype of the mutants. We compared the transcriptomes of the two *lsm* mutants and identified 759 genes that had similar changes in expression out of a total of 21,806 expressed genes ($P = 2.1E^{-26}$) (Fig. S8*A* and Dataset S3). Among these, we searched for genes that affect the clock itself or are involved in pathways that are affected by the circadian signaling network, such as flowering time and light-regulated developmental responses. Whereas no core clock genes were found to be affected in a similar manner in the two different *lsm* mutants, we found that the expression of *FT* and *MAF5*, two key flowering time genes, was up-regulated and down-regulated, respectively, in both mutants (Fig. S8*B*). These data are consistent with the early flowering time phenotype we observed in *sad1/lsm5* (Fig. S9*A*). Similarly, we found common changes in expression of *RRC1* and *LAF3*, two genes involved in the regulation of photomorphogenic responses in *Arabidopsis*, which were up-regulated and down-regulated in both mutants, respectively (Fig. S8*B*). These observations are consistent with the hyposensitivity observed in the *sad1/lsm5* mutant for light control of hypocotyl elongation (Fig. S9*B*). An analysis of gene ontologies associated with genes commonly altered in the mutants revealed an enrichment in genes involved in the regulation of transcription and stress responses (Fig. S8*C*). Among these, we found that *DREB19*, a transcription factor involved in the activation of stress responses, was up-regulated in both mutants, and *ABA4*, a gene required for ABA biosynthesis under stress conditions, was down-regulated in both mutants (Fig. S8*B*). These findings are consistent with the alterations in abiotic stress responses present in both *sad1/lsm5* and *lsm4* mutants (28, 31).

We then evaluated alterations in annotated AS events in the *sad1/lsm5* and *lsm4* mutants relative to wild-type plants. We analyzed a total of 6,567 annotated AS events corresponding to genes expressed above a minimal threshold level in the mutants and their corresponding wild-type plants (*SI Materials and Methods*). Of these, 363 events were altered in the *lsm4* mutant and 57 in the *sad1/lsm5* mutant, with 24 events being similarly changed in both mutants (an overlap that was 7.6 times larger than expected by chance; $P < 4.4E^{-16}$) (Fig. 4*A* and Dataset S4). In wild-type plants, the most abundant AS events were those associated with the use of alternative 3′ splice sites (alt 3′; 33%), followed by intron retention (IR; 32%), alternative 5′ splice sites (alt 5′, 22%), and exon skipping (13%) (Fig. 4*C*). Among the AS events affected in the *lsm4* and *sad1/lsm5* mutants, we found an increase in the proportion of IR events relative to their frequency in wild-type plants, which is consistent with previous findings reported for the *sad1/lsm5* mutant (Fig. 4*C*) (29, 36). Representative examples of alterations in AS observed in the mutants using the Integrative Genome Browser (IGB) (37) are shown in Fig. 4*E*.

We also evaluated splicing of all introns present in genes expressed above a minimal threshold level (*SI Materials and Methods*). Among the 87,241 introns analyzed, we detected alterations in splicing of 553 and 4,354 introns in *sad1/lsm5* and *lsm4* mutants, respectively, with 212 being similarly affected in both mutants (an overlap that was 7.7 times larger than expected by chance; $P < 1.1E^{-127}$) (Fig. 4*B* and Dataset S5). Interestingly, the proportion of increased intron retention events detected in the *lsm* mutants among "constitutively spliced" introns (i.e., introns not annotated as alternatively spliced) was approximately half that observed among annotated IR events (Fig. 4*D*). This observation indicates that *LSM* genes contribute preferentially, although not exclusively, to the regulation of AS. Two examples of "constitutively spliced" introns with defective splicing in the mutants are shown using the IGB (Fig. 4*F*). An additional example

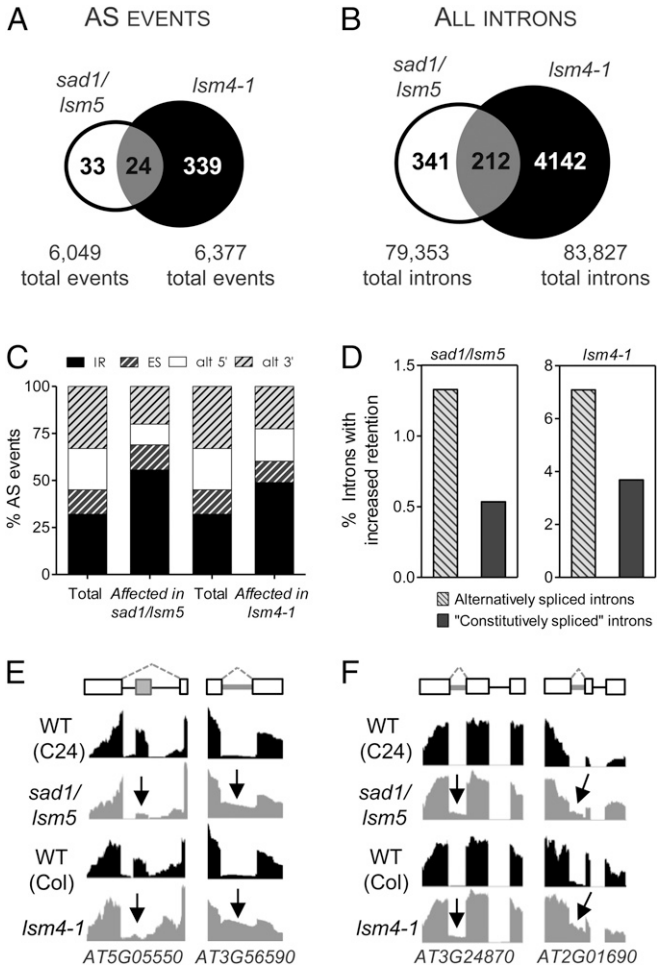

Fig. 4. RNA-seq genome-wide analysis of AS and constitutive splicing in *lsm4* and *lsm5* mutants. (*A* and *B*) Venn diagrams showing splicing alterations affecting alternative events (*A*) or all introns (*B*) in the *lsm4* and *lsm5* mutants. (*C*) Relative frequencies of different AS types for all detected AS events; alt 3′ and alt 5′, alternative acceptor and donor splice sites, respectively; ES, exon skipping. (*D*) Percentage of introns showing increased retention in *lsm5*/*sad1* or *lsm4* mutants relative to their corresponding wild-type plants among AS or "constitutively" spliced introns. (*E* and *F*) Examples of IR and ES using the IGB. Black arrows mark the event of interest.

of the selectivity of splicing defects associated with *lsm* genes can be observed for the *CCA1* core clock gene (Fig. S10*A*). The slight retention of intron 2 detected for this gene by RT-PCR (Fig. 2*E*) can also be observed in the RNA-seq data from both *lsm* mutants (Fig. S10 *A* and *B*). Furthermore, enhanced removal of intron 4 of *CCA1*, which is alternatively retained in wild-type plants of the Col accession, was observed in the *lsm4* mutant, revealing that this mutant is not simply defective in intron removal but has defects in the regulation of a subset of AS events (Fig. S10*C*). Finally, splicing of the remaining introns in *CCA1* was not affected in the mutants, revealing the selectivity of their splicing defects (Fig. S10*A*).

Discussion

Increasing evidence suggests that the core constituents of multisubunit complexes involved in transcription or RNA processing play regulatory rather than passive roles in controlling gene expression. Indeed, core components of the promoter recognition complex, generally considered to have a passive role in controlling gene expression, were recently shown to regulate cell-specific transcriptional programs during development (38). Similarly, down-regulation of SmB, a core component of U1–U5 snRNPs, in human cells, or mutations in the zebrafish *U1C* gene, which encodes a component of U1SnRNP, result in stronger alterations in AS than in constitutive splicing (21–23). A genome-wide characterization of RNA processing reported recently for *sad1*/*lsm5* also revealed alterations in a subset of splicing events (29). These findings suggest that changes in the levels and/or activities of core spliceosomal proteins might be associated with the regulation of specific signaling pathways. Indeed, *Arabidopsis lsm5* and *lsm4* mutants have reduced tolerance to abiotic stress, a phenotype that is consistent with alterations in the expression and AS of stress-related genes. However, whether abiotic stress-related signals control the expression and/or activity of LSM proteins in plants is not known. Here we report that the circadian clock controls the expression of several *LSM* genes in plants and mammals, and that mutations in some of these genes in plants, or down-regulation of their expression in human cell lines, results in defective control of circadian period length. Thus, our results provide strong evidence that some *LSM* genes regulate circadian signaling networks across kingdoms.

Eukaryotic LSM proteins are organized into two different heptameric complexes localized in the cytoplasm and nucleus. The nuclear complex consists of LSM2 to LSM8 and is required for U6 snRNA stabilization and proper pre-mRNA splicing. The cytoplasmic complex consists of LSM1 to LSM7 and is required for P-body formation, mRNA decapping, and accurate mRNA decay in the cytoplasm (36, 39). LSM4 and LSM5 are part of both complexes and, therefore, although alterations in circadian period are likely to result in part from defective splicing of specific clock genes, part of the circadian phenotype may result from the role of LSM proteins in regulating the mRNA stability of a subset of clock genes.

Although previous reports showed that down-regulation of *SmB* in humans, or mutations in *LSM5* in *Arabidopsis*, have limited effects on constitutive splicing and stronger effects on AS, the lack of strong effects on constitutive splicing might be due to the incomplete down-regulation of *SmB* mRNA levels in human cells or the weak nature of the mutation affecting the *sad1*/*lsm5* gene in *Arabidopsis*. In this study we provide strong evidence that LSM proteins have a greater effect on AS than on constitutive splicing by characterizing RNA processing in a strong *Arabidopsis lsm4* mutant allele that lacks full-length functional *LSM4* mRNA (Fig. S6*B*). Although the effect of this mutation on RNA processing was stronger than that observed for the weak *sad1*/*lsm5* mutation, splicing of only 5% of all introns evaluated by RNA-seq was significantly affected in the *lsm4* mutant, even though this is a single copy gene in *Arabidopsis*.

Although AS is known to regulate circadian networks in plants and animals, the splicing factors involved in this process are only beginning to be elucidated (14–16). In 2010 our group reported that PROTEIN ARGININE METHYL TRANSFERASE 5 (PRMT5), which transfers methyl groups to arginine residues present in histones and in some Sm and LSM spliceosomal proteins, links the circadian clock to the control of AS (11). The circadian alterations found in *prmt5* are partially due to the effect of *PRMT5* on the regulation of AS of the core clock gene *PRR9*. However, the underlying splicing regulatory mechanism remains unknown. Given that LSM4 is methylated by PRMT5, mutations in *Arabidopsis LSM* genes might alter the clock affecting splicing of *PRR9*. However, whereas the splicing of intron 3 of *PRR9* is strongly reduced in *prmt5* mutants (Fig. S10*G*), splicing of this intron is normal in *sad1*/*lsm5* (Fig. 2*F*) and *lsm4* (Fig. S10*F*), suggesting that this is not the mechanism through which *LSM* genes control the circadian period. Although we identified alterations in the AS of other clock genes, such as increased retention of intron 4 of *TOC1* (Fig. 2*G* and Fig. S10 *D* and *E*) or intron 2 of *CCA1* (Fig. 2*E* and Fig. S10*B*), these defects should shorten rather than prolong the circadian period, suggesting that they are not the primary defects responsible for the circadian phenotype of the *lsm* mutants. Therefore, we conclude that *LSM* genes affect the circadian period of *Arabidopsis* by regulating the RNA processing of one or more unidentified clock genes.

LSM4, together with LSM2, LSM3, and LSM8, participate directly in the recognition of the 3′ end of U6 snRNA, contributing

to the assembly of mature U6 snRNP complexes (40). How LSM4 regulates the RNA processing of a subset of transcripts is unknown, but it must involve additional activities of this protein. Interestingly, some Sm proteins have been shown to bind to a specific subset of transcripts (41), and function in additional RNA processing regulatory mechanisms (42). This may also be the case for some LSM proteins.

In summary, our findings indicate that some *LSM* genes play a regulatory role in RNA processing and thereby contribute to the adjustment of organisms to periodic changes in environmental conditions.

Materials and Methods

Plant Material. The mutants used, including *sad1/lsm5* (CS24935), *lsm4-1* (SALK_063398), *atsr45a* (SALK_052345), *grbp1e* (SALK_038244), *sr30* (SALK_116747), and *grbp7* (SALK_039556), were obtained from the *Arabidopsis* Biological Resource Center (ABRC).

Growth Conditions. Plants were grown in pots containing a mixture of organic substrate, vermiculite, and perlite [1:2:2 (vol/vol)] at 22 °C under long days (LD; 16-h light/8-h dark cycles; 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), short days (SD; 16-h light/8-h dark cycles; 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), or continuous light (LL; 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light), depending on the experiment.

Cell Culture and Reverse Transfection. Detailed information is presented in *SI Materials and Methods*.

Physiological Measurements. Detailed information is presented in *SI Materials and Methods*.

RT-qPCR and RNA-Seq Analysis. Detailed information is presented in *SI Materials and Methods*. Primers used for amplification of each gene are described in Dataset S6.

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