Jumonji domain protein JMJD5 functions in both the plant and human circadian systems

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Circadian clocks are near-ubiquitous molecular oscillators that coordinate biochemical, physiological, and behavioral processes with environmental cues, such as dawn and dusk. Circadian timing mechanisms are thought to have arisen multiple times throughout the evolution of eukaryotes but share a similar overall structure consisting of interlocking transcriptional and posttranslational feedback loops. Recent work in both plants and animals has also linked modification of histones to circadian clock function. Now, using data from published microarray experiments, we have identified a histone demethylase, jumonji domain containing 5 (JMJD5), as a previously undescribed participant in both the human and Arabidopsis circadian systems. Arabidopsis JMD5 is coregulated with evening-phased clock components and positively affects expression of clock genes expressed at dawn. We found that both Arabidopsis jmjd5 mutant seedlings and mammalian cell cultures deficient for the human ortholog of this gene have similar fast-running circadian oscillations compared with WT. Remarkably, both the Arabidopsis and human JMJD5 orthologs retain sufficient commonality to rescue the circadian phenotype of the reciprocal system. Thus, JMJD5 plays an interchangeable role in the timing mechanisms of plants and animals despite their highly divergent evolutionary paths.

KDM8 | JMJD3 | TOC1 | coexpression

Circadian rhythms are endogenous oscillations that attune the behavior and physiology of organisms to regular changes in their environment, such as dusk and dawn. It is estimated that 1/10th of mammalian genes and one-third of Arabidopsis genes are regulated in a circadian fashion (1, 2). Not surprisingly, the circadian clock modulates a broad range of processes, ranging from the regulation of growth and flowering time in plants to the control of body temperature and the sleep-wake cycle in mammals (3, 4). The coordination of these molecular and physiological processes with the external environment has been shown to provide an adaptive advantage in organisms as diverse as cyanobacteria, higher plants, and insects (5–7).

Although it is thought that circadian clocks have arisen multiple times during eukaryotic evolution (8), molecular oscillators in a diverse range of lineages share a broad overall structure consisting of interlocking transcriptional and posttranslational feedback loops (3, 4). In plants, the morning-phased Myb-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYOCOTYL (LHY) repress the expression of TIMING OF CAB1 EXPRESSION 1 (TOC1) by binding to a conserved motif within its promoter known as the evening element (9–11). TOC1, in turn, promotes expression of CCA1 and LHY via an indirect mechanism, forming a negative feedback loop (12). This central CCA1/LHY/TOC1 loop interlocks with additional morning- and evening-phased circuits to provide more robustness and flexibility to the circadian mechanism (reviewed in 3, 13).

Because genes that act together are often coregulated (14), we have characterized a gene that is coexpressed with TOC1 on both developmental and daily time scales. This gene, JMJD5, contains a jumonji-C (jmc) domain that is often found in proteins with histone demethylase activity (15); indeed, its human ortholog (also known as KDM8) has recently been shown to have this function (16). Although originally described as a permanent modification, histone methylation marks are now acknowledged to be dynamic alterations that can fine-tune transcription in a broad range of eukaryotic phyla (15). Components of histone methyltransferase complexes have been associated with mammalian circadian clock function (17, 18), and changes in histone methylation have been correlated with modifications in circadian gene expression in plants (19).

Here, we report that jmjd5 mutant plants have a short-period circadian phenotype and demonstrate that JMJD5 works in concert with TOC1 to promote CCA1 and LHY expression. Remarkably, we also found that mammalian cells deficient for the human JMJD5 ortholog have a short-period phenotype very similar to that of jmjd5 plants and that both JMJD5 orthologs retain sufficient commonality to rescue the circadian phenotype of the reciprocal system. These data suggest that JMJD5 may play a similar role in both the Arabidopsis and human circadian systems despite the wide evolutionary distance separating plants and mammals.

**Results**

**TOC1 and JMJD5 Are Coexpressed.** Despite recent advances (12), the mode of action of the central plant clock gene TOC1 is currently enigmatic. Coexpressed genes frequently share biological function (14), prompting us to identify genes that are developmentally coregulated with TOC1. We therefore obtained microarray expression data generated from 79 different Arabidopsis samples (representing a wide variety of developmental stages and diverse organs) (20) and calculated the Pearson correlation coefficients between TOC1 and each gene on the ATH1 array. The gene most highly correlated with TOC1 (r = 0.77) was EARLY FLOWERING 3 (ELF3; Table S1), a gene involved both in light input to the clock and in central clock function (21–23), thereby validating our approach. Because genes involved in the circadian clock are themselves frequently expressed with a circadian oscillation (3, 13), we examined the daily patterns of expression of our candidate genes using previously published microarray data (2). Of the 10 genes most highly correlated with TOC1, only ELF3 and a previously uncharacterized gene, JMJD5 (Ar3920810), were expressed with a circadian rhythm (Fig. 1B). The expression patterns of TOC1 and JMJD5 were the third most highly correlated across all genes on the microarray (r = 0.75; Fig. 1B and Table S1). Empirical sampling of randomly per-


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expression was regulated

Expression data were obtained from the work of Covington and Harmer (51). Data are the mean of three technical replicates; SE is shown. Presented results are representative of three independent experiments.

muted data shows that the chance for this level of correlation occurring between two randomly selected genes is only 1.3% (2).

We next investigated whether JMJ5 expression was regulated by known clock genes. In toc1-2 mutants, JMJ5 expression levels gravitated toward a median level relative to the peak of expression in WT plants (Fig. 1C). In contrast, JMJ5 mRNA was not detectable by quantitative RT-PCR (qRT-PCR) in seedlings over-expressing CCA1 (Fig. S1D). This repression was similar to but more pronounced than the reduction of TOC1 expression previously reported in these CCA1-OX plants (9) (Fig. S1E). Like TOC1, the JMJ5 promoter contains an evening element sequence immediately upstream of the transcriptional start site (TSS) in addition to two within the 5′ UTR (Fig. S1F), suggesting that CCA1 might bind to this region and directly repress JMJ5 expression. These data demonstrate that not only are JMJ5 and TOC1 co-regulated across developmental and circadian time but they are both repressed by the morning-phased clock protein CCA1.

jmdj5, Like toc1, Is Conditionally Arrhythmic. To determine whether JMJ5 is simply an output of the clock or if it is involved in the clock mechanism, we introduced the CCR2::LUC luciferase reporter construct into two lines with T-DNA insertions within the At3g20810 locus (jmdj5-1 and jmdj5-2; Fig. S1A). qRT-PCR using oligos spanning the first exon–exon junction revealed no detectable full-length mRNA in either mutant but a low level of a truncated form of the message in jmdj5-1 (Fig. 1C and Fig. S1B). Because the conserved jmjC domain is located at the C terminus of the protein (Fig. S1A), both alleles are likely loss-of-function mutations. Consistent with this possibility, circadian rhythms in luciferase activity in both mutants were ≈0.5–1.0 h shorter than in WT (Fig. 2A and B; P < 0.05, Student’s t test). This phenotype was highly reproducible and was observed when plants were grown in a variety of light conditions (constant red light, constant blue light, constant white light, monochromatic red light). The jmdj5-2 allele displayed reduced luciferase activity despite expressing normal levels of JMJ5 mRNA (Fig. 2B). These results are consistent with the notion that JMJ5 is involved in the circadian clock mechanism.

Fig. 1. JMJ5 is coexpressed with TOC1 over developmental and diurnal timescales. (A) Daily expression patterns of the 10 genes most highly developmentally correlated with TOC1. TOC1, JMJ5, and ELF3 are shown in black, with the remaining genes shown in gray. Accession numbers for each of these genes are listed in Table S1. Expression data were obtained from the work of Covington and Harmer (51). (B) Scatter plot of JMJ5 compared with TOC1 mRNA expression levels across a range of developmental time points. Each symbol represents the mean of each different biological sample; error bars represent SEM. Data were obtained from the work of Schmid et al. (20). (C) qRT-PCR analysis of JMJ5 expression under constant red light. WT Columbia (Col), jmjd5-1, jmjd5-2, and toc1-2 seedlings were compared using oligos spanning intron 1 (Fig. S1A and Table S4). Plants were entrained to 12/12-h light/dark cycles for 6 d before being moved to constant conditions with red light. The jmdj5-2 alleles displayed reduced luciferase activity despite expressing normal levels of CCR2 mRNA (Fig. 2C). Similar phenomena have previously been described in other T-DNA lines (49). Error bars indicate SEM and are displayed every 20 h for clarity (n ≥ 20). (B) Scatter plot of data shown in A comparing circadian period with relative amplitude error, a measure of rhythmic robustness, with a perfect cosine wave having a value of 0 as calculated by Fourier fast transform-nonlinear least squares (50). Data points are presented for WT Col, jmjd5-1, and jmjd5-2. Plants were treated as described in A. (C) Bioluminescence of WT, jmjd5-1, and toc1-2 seedlings maintained in higher intensity red light. Plants were entrained to 12/12-h light/dark cycles for 6 d before being moved to constant conditions with 120 µmol m⁻² s⁻¹ red light. Data are representative of two independent experiments. Error bars are plotted every 20 h and indicate SEM (n ≥ 11).
or constant red plus blue light; Table S2). Neither insertion allele displayed an altered rhythm under constant darkness, indicating that JMJD5 function is light-dependent (Table S2). Similar light-dependent circadian phenotypes have been reported for other clock mutations (24, 25).

Loss-of-function toc1-2 mutants have a strong short-period phenotype in most conditions but become arrhythmic in constant red light (26). We therefore examined clock regulation of luciferase activity in jmjd5 mutants maintained in different fluence rates of constant red light. When held under low levels of red light (<30 μmol·m⁻²·s⁻¹), jmjd5 mutants had a short period but preserved robust rhythms (Fig. 2 A and B). Intriguingly, at higher fluence rates of monochromatic red light (120 μmol·m⁻²·s⁻¹), jmjd5 seedlings became arrhythmic or had greatly dampened oscillations compared with WT plants, which had robust rhythms under these conditions (Fig. 2C and Fig. S2A). The jmjd5 mutants continued to cycle on days 4 and 5 of free-run under a high fluence rate of white light (Fig. S2B), suggesting that this phenotype was specific to red light. Thus, the jmjd5 and toc1 mutant phenotypes are similar: Both have short free-running periods in constant blue or white light but become arrhythmic in constant red light.

**JMD5 Is a Positive Regulator of CCA1 and LHY Expression.** Although it is known that TOC1 promotes CCA1 and LHY expression, the underlying mechanism is not yet fully understood (9, 12). TOC1 and JMJD5 are coexpressed and have comparable mutant phenotypes; thus, we speculated that JMJD5, like TOC1, might play a role in the regulation of CCA1 and LHY expression. To investigate this possibility, we used qRT-PCR to examine the expression of core circadian genes in toc1-2 and jmjd5 mutants. Because both the toc1 and jmjd5 phenotypes were most severe in monochromatic red light (Fig. 2C and Fig. S2A), we chose to examine clock gene expression in plants grown in high-intensity constant red light, collecting samples over a full 2 d. Consistent with the luciferase data, qRT-PCR analysis revealed that expression of the evening-phased genes GIGANTEA (GI) and TOC1 was disrupted, with a decrease in rhythmic amplitude (Fig. 3A and B).

We next examined expression of CCA1 and LHY, factors known to repress GI and TOC1 expression (9, 27). In jmjd5 mutants, peak levels of CCA1 and LHY mRNA were reduced relative to WT (Fig. 3 C and D and Fig. S3), similar to the reduced expression seen in toc1-2 plants (Fig. 3 C and D). These data indicate that like TOC1, JMJD5 promotes expression of the morning-phased clock genes CCA1 and LHY.

**JMJD5 and TOC1 Interact Genetically.** Given that TOC1 and JMJD5 are coexpressed and have related mutant phenotypes, we speculated they might act at a similar step in the clock mechanism and would show genetic interactions. We therefore generated plants mutant for both genes and assessed circadian function in multiple light conditions. When monitored in constant red plus blue light, jmjd5-1 toc1-2 double mutants had a free-running period ~1.5 h shorter than toc1-2 single mutants; in comparison, jmjd5-1 single mutants had a period only 0.5 h shorter than WT controls (Fig. 4A). Therefore the combination of jmjd5-1 and toc1-2 mutations had a synergistic effect on period length. When maintained under constant red light of a moderate fluence rate (30 μmol·m⁻²·s⁻¹), jmjd5-1 toc1-2 mutants also showed a synergistic effect on rhythmicity. Under this condition, jmjd5-1 single mutants were robustly rhythmic, whereas ~70% of toc1-2 mutants had a detectable rhythm in luciferase activity (Fig. 4C). In the double mutants, only 30% of plants had a significant circadian rhythm. Thus, TOC1 and JMJD5 showed synergistic genetic interactions for multiple clock phenotypes. In contrast, only additive effects on period and rhythmicity were observed in jmjd5-1 lhy-100 double mutants, with the double mutants having a free-running period 0.5 h shorter than the lhy-100 single mutants (Fig. 4 B and D).

**Human Cells Deficient for JMJD5 also Have a Short-Period Phenotype.** Although the core circadian transcription factors are not shared across higher taxa, some proteins (such as casein kinase and cryptochrome) and protein modifications (such as histone acetylation and poly-ADP ribosylation) do play a role in the circadian clocks of diverse eukaryotes (11, 28, 29 and reviewed in 30, 31). JMJD5 orthologs can be found in many eukaryotic genomes (Fig. S4 and Table S3). For example, the human JMJD5 protein (also known as KDM8) (16) is highly related to the Arabidopsis protein, with 39% identity and 56% similarity at the amino acid level and similar predicted secondary and tertiary protein structures (Fig. S4 B and C). Amino acid similarity rises to 68% within the conserved jmjC domain. Given the similarities between the human and Arabidopsis JMJD5 genes, we investigated whether the human ortholog might also be involved in clock function. We designed an siRNA construct against the human JMJD5 gene and transfected this into U2OS cells expressing luciferase under the control of the BMAL1 promoter (U2OS-B6) (32). Transformation with the siRNA construct specifically knocked down endogenous levels of JMJD5 to 20% of WT (Fig. S5 A and B). Transformed cells had a significantly shorter period than U2OS-B6 cells transfected with a scrambled siRNA.

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**Fig. 3.** qRT-PCR analysis of circadian clock-regulated genes. WT Columbia (Col), jmjd5-1, and toc1-2 mutants were compared by qRT-PCR. Levels of TOC1 (A), GI (B), CCA1 (C), and LHY (D) mRNA were assessed. Plants were entrained to 12/12-h light/dark cycles for 6 d before being moved to constant conditions with 120 μmol·m⁻²·s⁻¹ red light. mRNA levels for each gene were normalized to PP2a. Data are the mean of three technical replicates; SE is shown. Results are representative of three independent replicates.
control (Fig. 5A and B; *P = 0.022, Student’s t test) but continued to show robust rhythmicity (Fig. 5A). This period shortening was strikingly similar to the circadian phenotype observed for Arabidopsis jmjd5 mutants in most light conditions (Table S2).

**JMJD5 Homologs Act in Both Plant and Human Circadian Systems.** Because loss of either ortholog caused similar effects on clock function in plants and human cells, we speculated that the proteins might share a conserved cellular function. To test this possibility, we created a cell line (U2OS + AtJMJD5) that stably expressed the Arabidopsis gene in a U2OS-B6 background and monitored its circadian periodicity following knockdown of the endogenous human gene (Fig. 5B). Remarkably, U2OS + AtJMJD5 cells transfected with a human JMJD5 siRNA had a significantly longer period than U2OS-B6 cells (Fig. 5B; *P = 0.0026, Student’s t test), indicating that the plant protein is able to function within the circadian system. This rescue is highly significant but not complete, because a modest decrease in period length was observed in U2OS + AtJMJD5 cells when transfected with JMJD5 siRNA compared with a scrambled siRNA control (Fig. 5B).

We also investigated whether the human ortholog was functional in planta. To test this hypothesis we introduced either the Arabidopsis or the human JMJD5 gene, expressed under the control of the plant JMD5 promoter (Fig. S4D), into the Arabidopsis jmjd5-1 mutant background and tested for rescue of the period defect. The circadian periods of T1 seedlings transformed with either the human or plant gene were then compared with those of WT and jmjd5-1 plants (Fig. 5C and Fig. S4E). Intriguingly, seedlings transformed with either JMJD5 ortholog displayed a significantly lengthened circadian period compared with the untransformed parental control (*P = 0.0013 and 0.0210 for the Arabidopsis and human genes, respectively). Use of T1 plants permitted characterization of at least 20 independent insertion events for each transgenic population but necessitated the use of hygromycin selection. Such treatment is unlikely to alter circadian period, however, because hygromycin-resistant homozygous T3 transgenics demonstrated no difference in period length in the presence or absence of hygromycin (Fig. S4F). In combination, these cross-kingdom rescue experiments demonstrate that JMJD5 functions in both the Arabidopsis and human circadian clocks via a conserved molecular mechanism.

**Discussion**

**Characterization and Placement of JMJD5 Within the Arabidopsis Circadian System.** We have successfully used a data-mining strategy to identify JMJD5, an evening-phased putative histone demethylase that acts within the Arabidopsis central clock. Similar to TOC1, JMJD5 expression is strongly repressed by CCA1 (9) (Fig. S1D and E). Indeed, it appears that the effect of CCA1 overexpression on JMJD5 expression levels may be more pronounced than that on TOC1; unlike TOC1, JMJD5 mRNA was not detectable by qRT-PCR in seedlings overexpressing CCA1 (9) (Fig. S1D and E). Although JMJD5 expression was altered in a toc1-2 mutant background (Fig. 1C), this might be an indirect consequence of reduced CCA1 and LHY mRNA levels in this genetic background (9). Similarly, mutation of the TOC1 homologs PSEUDORESPONSE REGULATOR 5, 7, and 9 (PRR5, PRR7, and PRR9) profoundly increased expression of CCA1 and LHY and decreased expression of JMJD5, suggesting the PRRs may indirectly regulate JMJD5 expression (33). TOC1 expression was also significantly reduced in the prr5, prr7, and prr9 mutants but to a lesser extent than that of JMJD5 (33).

We found that JMJD5 and TOC1 showed a synergic genetic interaction, whereas the interactions between JMJD5 and LHY were additive (Fig. 4). Formally, synergism may arise either when two genes share overlapping functions within the same process or when they act in convergent signaling pathways. The reduced CCA1 and LHY expression seen in both jmjd5 and toc1 single mutants (Fig. 3 C and D and Fig. S3) suggests that they may act together to promote expression of these morning-phased clock genes. Similar synergic effects on regulation of gene expression

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**Fig. 4.** JMJD5 and TOC1 interact genetically. (A) Period estimates of WT Columbia (Col), jmjd5-1, toc1-2, and jmjd5-1 toc1-2 genotypes. Plants were entrained to 12/12-h light/dark cycles for 6 d before being held in continuous 35 μmol·m⁻²·s⁻¹ red and blue light. Each genotype had a significantly different period length (*P < 0.001, Tukey range test). Error bars represent SE (n ≥ 11). Presented data are representative of three independent experiments. (B) Period estimates of WT Col, jmjd5-1, lhy-100, and jmjd5-1 lhy-100 genotypes. Seedlings were treated and analyzed as described in A. Error bars represent SE (n ≥ 30). Presented data are representative of three independent experiments. (C) Rhythmicity of jmjd5-1 toc1-2 seedlings held under 30 μmol·m⁻²·s⁻¹ red light. Percent rhythmicity was defined as the fraction of seedlings that returned a period estimate with a relative amplitude error < 1 as determined by Fourier fast transform-nonlinear least squares (50). Seedlings and entrainment conditions were as described in A. Populations of toc1-2 and jmjd5-1 toc1-2 double-mutant seedlings had significantly worse rhythms than WT or toc1-2 populations, respectively (*P < 0.05, Student’s t test). Error bars indicate SE from three independent experiments. (D) Rhythmicity of jmjd5-1 lhy-100 seedlings held under 30 μmol·m⁻²·s⁻¹ red light. Seedlings and entrainment conditions were as described in C. Error bars indicate SE from three independent experiments.

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**Table S2.**
ELF4, which are all evening-phased genes that have yet to be ascribed specific positions within the circadian circuitry (21, 23, 38, 39). Like JMJD5, loss of ELF3, ELF4, or LUX causes decreased expression of CCA1 and LHY without strongly affecting TOC1 expression levels (38, 40–42). It is possible that these coexpressed genes act in concert in some manner to promote morning-phased clock gene expression.

**JMJD5 Functions in Both the Human and Plant Circadian Systems.** Orthologs of JMJD5 are found across eukaryotes (Fig. S4A and Table S3). Phylogenetic analyses suggest that these homologs originated before the separation of the animal and plant phyla, ≈1.5 billion years ago (43). Despite this ancient divergence, the human JMJD5 gene can rescue the circadian defect of Arabidopsis jmjd5 mutants. Given the apparent independent origins of the plant and human circadian clocks (8, 44), it would appear that JMJD5 has been recruited independently into each of these molecular oscillators.

The human JMJD5 protein has recently been shown to demethylate histone H3 dimethylated at lysine 36 (H3K36me2), both in vitro and in vivo (16). Although the enzymatic activity of the Arabidopsis ortholog is not yet determined, our data suggest that human and plant proteins can fulfill similar functions within the plant and human circadian systems, respectively (Fig. 5 B and C and Fig. S4E). All the cofactor-binding residues thought necessary for histone demethylase activity (15) are found in the Arabidopsis JMJD5 protein (Fig. S4B), suggesting that it may also have this biochemical function. Despite this conservation, we cannot exclude the possibility that the human JMJD5 acts simply as a scaffold to facilitate the function of endogenous plant protein complexes rather than supplying needed enzymatic activity.

Although H3K36me2 modifications are predominantly found toward the 3' end of actively transcribed eukaryotic genes (45–47), enrichment of this modification is also found near the TSS and within exons, where it acts as a repressive mark to suppress erroneous transcription (45, 47, 48). Both CCA1 and LHY loci carry extensive H3K36me2 marks within their coding regions (48), suggesting that JMJD5 may directly demethylate the CCA1 and LHY loci and thereby promote their transcription. Alternately, JMJD5 could promote gene expression less directly by modifying the combination of histone modifications present at the affected loci. Histone modifications are increasingly acknowledged to act in concert to direct transcriptional activity (15, 16, 47).

In summary, we have identified a previously undescribed component of both the Arabidopsis and human circadian systems that may link the clock with dynamic histone modification and thereby provide additional regulation of gene expression. Our coexpression and genetic analyses indicate that Arabidopsis JMJD5 acts in concert with TOC1 to positively regulate expression of CCA1 and LHY. Although we do not presume that the inclusion of JMJD5 in the molecular oscillators of both the Arabidopsis and human circadian systems is a retained ancestral function of this protein, it is intriguing that it functions in the clocks of these highly divergent species. At the very least, this suggests that histone methylation is an important regulatory mechanism in two very different eukaryotes. Future work will further elucidate the relationship between JMJD5 and the core clock components of both animals and plants.

### Materials and Methods

**Plant Materials and Growth Conditions.** Details on the construction of the binary vectors and the mutants used in this study are included in SI Materials and Methods.

**Mammalian Cell Culture.** U2OS cell lines used in this study are described in SI Materials and Methods.

**Luciferase Assays.** Luciferase assays were performed as described (32, 49, 50). Additional information is provided in SI Materials and Methods.
RNA isolation, cDNA preparation, and qRT-PCR techniques are described in SI Materials and Methods.

Gene Expression Correlation and Phylogenetic Analyses. Details on the statistical and phylogenetic tools used in this study are included in SI Materials and Methods.


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Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. jmjds-1 (SAIL-811_H12) and jmjds-2 (GK-454C10) were obtained from the SAIL and Gabi-Kat T-DNA insertion collections, respectively (1, 2). Homozygous T-DNA insertion lines were identified using oligo sets 1 and 2 as shown in Table S4. jmjds-1 toc1-2 and jmjds-1 by-100 plants were generated by crossing and then genotypically for the requisite mutations as previously described (3). CCA1-OX lines have been described previously (4).

Binary vectors for the generation of phenotypic rescue plants were generated as follows. A genomic fragment containing JMJDS and a 1.5-kb upstream region was PCR-amplified using oligo set 3 (Table S4) and introduced into pENTR/D-TOPO (Invitrogen) via the TOPO cloning method to create pENTR At JMJDS. This construct was then used in conjunction with pGBWB4 (5) to create pGBWB4 At JMJDS by attL x attR (LR) recombination as described in the manufacturer’s protocol (Invitrogen). To generate pENTR Hs JMJDS, the coding sequence of At JMJDS, the coding sequence of JMJDS using MslI and AscI (New England Biolabs). Hs JMJDS cDNA was PCR-amplified from pGWB4. The pENTR Hs JMJDS vector was then sequenced and used in conjunction with pGWB4 (5) to create pGWB4 At JMJDS by LR recombination.

All clones were moved into Agrobacterium strain GV3101 and transformed into WT U2OS cells on six-well plates before LR recombination as described by the manufacturer (Invitrogen). To generate pENTR At JMJDS, the coding sequence of Hs JMJDS was PCR-amplified from pENTR Hs JMJDS using MslI and AscI (New England Biolabs) before being ligated into the compatible ends within the cut pENTR vector. The pENTR Hs JMJDS vector was then sequenced and used in conjunction with pGBWB4 (5) to create pGBWB4 At JMJDS by LR recombination. Hygromycin-resistant control plasmids (Col + pMDC32) were generated by introducing At4g00050 cDNA into pMDC32 (6) by LR recombination.

Mammalian Cell Culture. U2OS-B6 cells have previously been described (8). At JMJDS CDNA was inserted into pCDNA3.2 JMJDS using oligo set 4 (Table S4) and digested with EcoRV and Ascl (New England Biolabs) before being ligated into the compatible ends within the cut pENTR vector. The pENTR Hs JMJDS vector was then sequenced and used in conjunction with pGBWB4 (5) to create pGBWB4 At JMJDS by LR recombination. Hygromycin-resistant control plasmids (Col + pMDC32) were generated by introducing At4g00050 cDNA into pMDC32 (6) by LR recombination.

For seedlings, plants were grown, RNA was isolated, and qRT-PCR was performed as previously described (3). Samples were run in triplicate, and starting quantity was estimated from critical thresholds using the standard curve of amplification. Data for each sample were normalized to the respective PP2a expression level. For U2OS-B6 analysis, four wells of a 384-well plate were transfected per siRNA; 36–48 h posttransfection, RNA extraction was performed with an RNAeasy kit (Qiagen). cDNA levels were measured via SYBR Green-based qRT-PCR (Roche) and normalized against GADPH expression. Primer sets used are described in Table S4.

PCR Techniques. For seedlings, plants were grown, RNA was isolated, and qRT-PCR was performed as previously described (3). Samples were run in triplicate, and starting quantity was estimated from critical thresholds using the standard curve of amplification. Data for each sample were normalized to the respective PP2a expression level. For U2OS-B6 analysis, four wells of a 384-well plate were transfected per siRNA; 36–48 h posttransfection, RNA extraction was performed with an RNAeasy kit (Qiagen). cDNA levels were measured via SYBR Green-based qRT-PCR (Roche) and normalized against GADPH expression. Primer sets used are described in Table S4.

Gene Expression Correlation and Phylogenetic Analyses. Genes with expression similar to that of TOCI1 were identified using AtGenExpress (10). Genes orthologous to Arabidopsis JMJDS were identified using BLAST (11, 12). Protein sequences were aligned using MUSCLE (13) and manually curated to remove clearly erroneous regions of alignment. A maximum likelihood phylogenetic tree was constructed using CIPRES PORTAL (14), utilizing RAXML (15) and an “LG + G + F” model of protein evolution as prescribed by ProtTest (16). Data were manipulated in JALVIEW (17).

**Fig. S1.** Characterization of Arabidopsis jmjd alleles. (A) Semiquantitative PCR analysis of JMJD5 mRNA expression in WT Columbia (Col) and jmjd5 mutant plants using primers spanning the full-length cDNA (oligo set 6; Table S4). Actin mRNA was amplified as a loading control. The positions of the T-DNA insertions within the JMJD5 locus (At3g20810) are indicated below. Gray squares indicate exons, whereas black triangles demonstrate the approximate position of the T-DNA insertion for each allele. A cartoon of the translated protein is also shown, with the conserved mjc domain indicated in blue. Numbered arrows indicate oligo sets used for qRT-PCR (Table S4). (B) qRT-PCR analysis of JMJD5 message levels in jmjd5-1 mutants. JMJD5 message was assayed in WT Col and jmjd5-1 mutants using oligos that amplified cDNA on either side of the T-DNA insertion point as in A (oligo set 6; Table S4). Plants were entrained to 12/12-h light/dark cycles for 10 d before tissue was harvested at dusk. Data are the mean of three technical replicates; SE is shown. Presented results are representative of two independent experiments. (C) qRT-PCR analysis of CCR2 expression in WT Col, jmjd5-1, and jmjd5-2 seedlings. Plants were treated and analyzed as described in Fig. 1C. JMJD5 (D) and TOC1 (E) expression analysis in WT Col and seedlings overexpressing CCA1 (CCCA1-OX). JMJD5 and TOC1 mRNA levels were normalized to PP2a. Plants were treated as described in Fig. 1C. Data are the mean of three technical replicates; SE is shown. Presented results are representative of two independent experiments. (F) Promoter analysis of JMJD5. The region directly upstream of the JMJD5 start codon contains three evening elements (AAAA-TATCT). The 5’ UTR and TSS are indicated in italics and with capital letters, respectively.
Fig. S2. *jmjd5-1* mutants have reduced rhythmicity under monochromatic red light. (A) Rhythmic amplitude of luciferase activity in *jmjd5-1* and *toc1-2* seedlings held under 120 μmol·m⁻²·s⁻¹ red light. Mean amplitude of seedlings that returned any rhythm in the circadian range (15 < τ < 30 h; rhythmicity determined by Fourier fast transform-nonlinear least squares) (9) was normalized against the WT Columbia (Col) sample mean. Seedlings and entrainment conditions were as described in Fig. 2C. Error bars indicate SE (n ≥ 8). Presented data are representative of two independent experiments. (B) Bioluminescence of WT Col and *jmjd5-1 CCR2::LUC* seedlings under high-intensity white light. Plants were entrained to 12/12-h light/dark cycles for 6 d before being moved to constant conditions with 130 μmol·m⁻²·s⁻¹ white light. Seedlings were imaged for 2 d after 3 d in free-run. Error bars are plotted every 6 h and indicate SE (n ≥ 7). Presented data are representative of two independent experiments.

Fig. S3. qRT-PCR analysis of circadian clock-regulated genes in *jmjd5-2* plants. WT Columbia (Col), *jmjd5-1*, and *jmjd5-2* were compared by qRT-PCR. Levels of CCA1 (A) and LHY (B) mRNA were assessed. Plants were entrained to 12/12-h light/dark cycles for 6 d before being moved to constant conditions with 120 μmol·m⁻²·s⁻¹ red light. mRNA levels for each gene were normalized to *PP2a*. Data are the mean of three technical replicates; SE is shown. Presented results are representative of two independent experiments.
Fig. S4. Comparison of plant and human JMJD5 orthologs. (A) Maximum likelihood phylogenetic tree showing “jmjC-only” proteins in model species; note that JMJD5 orthologs form a distinct clade compared with other proteins containing an orphan jmjC domain. *Arabidopsis* JMJD5 was utilized as a template to identify candidate orthologs using BLAST (1, 2). Additional jmjC-only proteins were selected according to previous analyses (3, 4). Sequences were aligned using MUSCLE (5), and a maximum likelihood phylogenetic tree was then constructed using CIPRES PORTAL (6). Species abbreviations are as follows: *At*, *Arabidopsis thaliana*; *Dm*, *Drosophila melanogaster*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Os*, *Oryza sativa*; *Pt*, *Populus trichocarpa*; *Xt*, *Xenopus tropicalis*. Gene locus designations are given on each tip. Percentage bootstrap values of 1,000 replications are shown on an internal node. (B) Conservation of primary and secondary structure. (C) 3D structure of Hs JMJD5. (D) Gene expression analysis. (E) Relative amplitude error. (F) Period (Hrs).
Fig. S5. Effect of $JMJD5$ siRNA treatment of human cells. (A) qRT-PCR analysis of $JMJD5$ message levels in U2OS-B6 cells transfected with either $JMJD5$ siRNA or a scrambled siRNA control (Table S4). Data are the mean of three technical replicates; SE is shown. Results are representative of three independent experiments. (B) $PER2$ message levels were unaffected by $JMJD5$ siRNA knockdown. Samples were processed and analyzed as described in A. Data are the mean of three technical replicates; SE is shown. Results are representative of three independent experiments.
Table S1. Genes correlated with **TOC1** expression over developmental time

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<th>AGI</th>
<th>Annotation</th>
<th>Pearson correlation coefficient</th>
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<td>AT5G61380</td>
<td>TOC1</td>
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<tr>
<td>AT2G25930</td>
<td>ELF3</td>
<td>0.768912673</td>
</tr>
<tr>
<td>AT4G35230</td>
<td>Putative protein kinase</td>
<td>0.765872777</td>
</tr>
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<td>AT3G20810</td>
<td>JMJD5</td>
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<td>Putative protein kinase</td>
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<tr>
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<td>Short-chain dehydrogenase/reductase (SDR) family protein</td>
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Microarray expression data generated from 79 different Arabidopsis samples [representing a wide variety of developmental stages and diverse organs (n = 20)] were examined to identify genes coexpressed with TOC1. Pearson correlation coefficients between TOC1 and each gene are presented.

Table S2. **jmjd5** clock phenotypes in a variety of light conditions

<table>
<thead>
<tr>
<th></th>
<th>cR (60 μE)</th>
<th>cB (20 μE)</th>
<th>cR + B (60 μE)</th>
<th>cD</th>
</tr>
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<tbody>
<tr>
<td>CCR2::LUC, Col</td>
<td>24.85 ± 0.07</td>
<td>24.12 ± 0.08</td>
<td>24.66 ± 0.10</td>
<td>25.75 ± 0.14</td>
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<tr>
<td>CCR2::LUC, jmjd5-1</td>
<td>23.89* ± 0.12</td>
<td>23.57* ± 0.15</td>
<td>24.10* ± 0.09</td>
<td>26.05 ± 0.29</td>
</tr>
<tr>
<td>CCR2::LUC, jmjd5-2</td>
<td>24.36* ± 0.09</td>
<td>23.09* ± 0.25</td>
<td>23.60* ± 0.08</td>
<td>25.19 ± 0.19</td>
</tr>
</tbody>
</table>

Circadian period estimates were obtained from plants entrained in 12/12-h light/dark cycles for 6 d and then moved to constant darkness (cD), constant red (cR) light, constant blue (cB) light, or the combination of constant red and blue (cR + B) light. Intensities of different light conditions are presented in μE (μmol m⁻² s⁻¹). SEM is shown. *Significant difference between the WT period and the indicated mutant (P < 0.01, Student’s t test).

Table S3. Percent identity between JMJD5 proteins in different eukaryotes

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<tr>
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<th>Annotation</th>
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<th>3</th>
<th>4</th>
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<th>7</th>
<th>8</th>
<th>9</th>
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JMJD5 orthologs were identified and aligned using BLAST and MUSCLE (11–13). Percent amino acid identity is presented. National Center for Biotechnology Information sequence references and alternate annotations are provided where applicable.
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<tr>
<th>Oligo set</th>
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<td>T-DNA insertion check (WT)</td>
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<td>4</td>
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