Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription

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Circadian clocks are ubiquitous molecular time-keeping mechanisms that coordinate physiology and metabolism and provide an adaptive advantage to higher plants. The central oscillator of the plant clock is composed of interlocked feedback loops that involve multiple repressive factors acting throughout the circadian cycle. PSEUDO RESPONSE REGULATORS (PRRs) comprise a five-member family that is essential to the function of the central oscillator. PRR5, PRR7, and PRR9 can bind the promoters of the core clock genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) to restrict their expression to near dawn, but the mechanism has been unclear. Here we report that members of the plant Groucho/Tup1 corepressor family, TOPLESS/TOPLESS-RELATED (TPL/TPR), interact with these three PRR proteins at the *CCA1* and *LHY* promoters to repress transcription and alter circadian period. This activity is diminished in the presence of the inhibitor trichostatin A, indicating the requirement of histone deacetylase for full TPL activity. Additionally, a complex of PRR9, TPL, and histone deacetylase 6, can form in vivo, implicating this tripartite association as a central repressor of circadian gene expression. Our findings show that the TPL/TPR corepressor family are components of the central circadian oscillator mechanism and reinforces the role of this family as central to multiple signaling pathways in higher plants.

Arabidopsis | chromatin | EAR domain | TOC1 | transcriptional repressor

The circadian clock system consists of multiple interlocked feedback loops that generally contain activating and repressive arms within the loops to sustain robust 24-h oscillations (1–3). Many of the best-characterized elements in the plant circadian system are transcriptional repressors that act during the subjective morning to allow evening expression of their targets, or are expressed during the subjective evening to keep expression of morning genes down at night (4, 5). One well-studied loop of reciprocal repression involves the inhibition of early-day expression of the evening gene *TIMING OF CAB EXPRESSION 1 (TOC1*; *PRR1*) by the morning-expressed myb transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (6). TOC1 is the founding member of five closely related PSEUDO RESPONSE REGULATORS (PRRs: PRR9, PRR7, PRR5, PRR3) and binds DNA through a conserved CCT domain at the carboxy terminus, repressing evening expression of both *CCA1* and *LHY* (7, 8). The mechanism of CCA1/LHY-mediated repression of *TOC1* requires the corepressor DE-ETIOLATED1 (DET1) to interact with CCA1 and LHY at the *TOC1*promoter, likely in the context of a larger COP10-DET1-DDB1(CDD) complex (9). *TOC1* is also regulated by the related myb-transcription factor, REV8, which binds the *TOC1* promoter and likely acts as a positive activator (10, 11). In contrast, the partners and mechanism of late-evening TOC1-mediated repression of *CCA1*/*LHY* are unknown. However, a second evening-phased repressor complex, EARLY FLOWERING 3 (ELF3)-EARLY FLOWERING4 (ELF4)-LUX ARRHYTHMO (LUX) has been identified as acting to restrict *PRR9* expression to the morning (12–14).

In addition to the role of TOC1, establishment and regulation of *CCA1* and *LHY* circadian expression relies on repression by three additional PRRs, PRR9, PRR7, and PRR5 (15). Each of these PRRs is expressed at discrete times of the circadian cycle. PRR9 accumulation begins early in the day, with maximum levels found between zeitgeber time (ZT) 2–6. PRR7 peaks next between ZT6 and ZT13 and PRR5 follows near ZT13 (15, 16). These protein expression patterns closely mirror their temporal occupancy of *CCA1* and *LHY* promoter regions (15). Mutants lacking two of the three PRR proteins often display altered patterns of *CCA1* and *LHY* expression, with increased expression of both genes coinciding with circadian times at which the missing PRRs would normally be expressed (15). These results, together with the recent demonstration of DNA binding by these PRRs (8, 17), provide compelling evidence that PRR9, PRR7, and PRR5 act in temporal sequence to keep *CCA1* and *LHY* transcription strongly repressed over most of the midmorning to early evening. However, the mechanism of how these proteins inhibit expression remains unknown.

Here we identify members of the plant Groucho/TUP1 corepressor family, TOPLESS/TOPLESS RELATED PROTEINs (TPL/TPRs), which specifically interact with three of the five members of the PRR family (PRR5, PRR7, and PRR9) and reside together at the promoters of *CCA1* and *LHY* to repress transcription and alter the circadian period. We show that diminished levels or activity of the TPL family causes increased levels of *CCA1* and *LHY* expression, and a concomitant lengthening of circadian period. We also link a complex of PRR9 and TPL to histone deacetylase 6 (HDA6), demonstrating an in vivo interaction that implicates this unique tripartite association as a central repressor of circadian gene expression.

Results

Defining the TOPLESS–PRR Interaction Domains. The PRR5, PRR7, and PRR9 proteins contain a conserved EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif (LxLxL) that is required for their repressive activity (15) (Fig. S1). This motif is shared among a wide range of plant transcription factors (18, 19) that use members of the Groucho (Gro)/TUP1 family of corepressors to facilitate inhibition of transcription (20–22). We tested whether a representative member of the five member TOPLESS family of corepressors (21) could interact with any of the five PRRs (TOC1/PRR1, PRR3, PRR5, PRR7, and PRR9). Using transient coexpression of TOPLESS-HA

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(TPL-HA) and GFP-PRRn proteins in *Nicotiana benthamiana* leaves we observed strong coimmunopreciptation of TPL-HA with GFP-PRR5, GFP-PRR7, and GFP-PRR9, but not with GFP-PRR3 or GFP-TOC1, which lack the EAR motif (Fig.1 *A* and *C*, and Fig. S2*B*). The other four closely related members of the TOPLESS family (TPR1, TPR2, TPR3, and TPR4) also interact with these three PRR proteins (Fig. S2 *C*–*F*). When the EAR domain of GFP-PRR5 and GFP-PRR9 was mutated (Fig. S1), the TPL–PRR interactions were strongly diminished (Fig. 1*A*). We next performed yeast two-hybrid interaction tests to establish the sufficiency of TPL and the PRRs for their interaction absent other plant factors. We confirmed that TPL is able to homodimerize via the N terminus (23) and show that all three PRR proteins are able to dimerize with the TPL N terminus, with PRR9 interacting the strongest and PRR7 more weakly. The mutated EAR domain severely diminished TPL–PRR9 and TPL–PRR5 complex formation, further demonstrating the necessity of the EAR domain for the interaction (Fig. 1*B*).

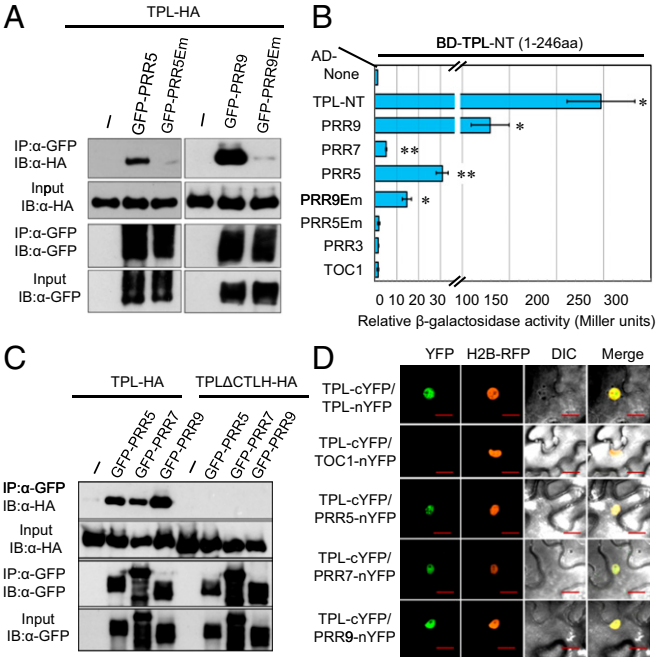

Fig. 1. TPL interacts with PRR5, PRR7, and PRR9 in vitro and in vivo. (*A*) TPL interacts with PRR5 and PRR9 and requires their EAR motifs in planta. *N. benthamiana* plants were grown in 16-h light/8-h dark. Protein extracts from leaves transiently and ectopically expressing TPL-HA and respective GFP-PRR5, GFP-PRR9, and their EAR motif mutations were harvested at ZT10. GFP antibody was used as primary antibody for immunoprecipitations (IP). HA antibody was used to detect TPL immunoblots (IB) and GFP antibody for PRR5, PRR9, and respective EAR motif (Em) mutations in immunoprecipitates. Data are representative of three trials with similar results. (*B*) Liquid LexA yeast two-hybrid assays showing interactions between TPL (N terminus: 1–246 aa) and PRR5, PRR7, and PRR9. The constructs used for this assay are indicated in the figure. Error bars indicate SEM. $*P < 0.05$ and $**P < 0.01$ (*t* test) relative to the AD alone control ($n = 3$). (*C*) CTLH domain of TPL is essential for interaction with PRR5, PRR7, and PRR9 in planta. Protein extraction and immunoprecipitation were performed as in *A* from ectopically coexpressing full-length TPL-HA or full-length TPLΔCTLH-HA (Δ25–91), with respective GFP-PRR5, GFP-PRR7, and GFP-PRR9. Representative data are from three trials with similar results. (*D*) BiFC assay shows TPL interacts with itself and PRR5, PRR7, and PRR9 in the nucleus. N- and C-terminal fragments of yellow fluorescence protein (nYFP and cYFP) were fused to TPL and PRR proteins, respectively. *Agrobacteria* harboring TPL-cYFP and PRRs-nYFP were coinfiltrated into *N. benthamiana* plants. Images were taken from epidermis cell of transfected leaves three days later after infiltration. TPL homodimerization was used as a positive control. Positions of the nuclei were indicated by H2B-RFP. (Scale bars, 20 μm.)

We additionally tested the requirement of the CTLH (C-terminal to LisH) domain within the N terminus of TPL for the interaction with the PRRs. Deletion of the CTLH domain from TPL completely abolished the in vivo TPL–PRR coimmunoprecipitation (Fig. 1*C*), supporting previous two-hybrid reports (23).

Nuclear presence would be required for TPL to act as a transcriptional corepressor with the PRRs. We next used bifluorescence complementation (BiFC) to establish the subcellular localization and in planta interactions between TPL and PRR5, PRR7, and PRR9 by transiently expressing the partner proteins in *N. benthamiana* epidermal cells (Fig. 1*D*). We first determined that TPL can homodimerize in the nucleus (Fig. 1*D*, row 1) and that TOC1 fails to interact with TPL (Fig. 1*D*, row 2). We then observed a strong BiFC nuclear signal when TPL-cYFP (yellow fluorescence protein) was coexpressed with either PRR5, PRR7, or PRR9-nYFP, with a distinctive subnuclear speckle pattern with the TPL-cYFP/PRR5-nYFP pairing (Fig. 1*D*, rows 3–5). The nuclear localization of the TPL and PRR proteins was not caused by their interaction, because GFP-PRR5 with a mutated EAR domain (GFP-PRR5EARmut) was still strongly nuclear-localized (Fig. S2*G*). Taken together, these findings demonstrate a subnuclear interaction between TPL and the PRRs, consistent with TPL acting as a corepressor with PRR5, PRR7, and PRR9.

Depletion of the TOPLESS Family Lengthens Circadian Period. To determine the in vivo significance of these interactions to clock function, we reduced the level of the TPL family expression by targeting artificial microRNA (amiRNA) to the family members by transient expression in *Arabidopsis* leaf protoplasts (24). Two amiRNA species were identified that reduced the levels of *TPL*, *TPR1*, and *TPR4* (amiTPLR14), and *TPR2* and *TPR3* (amiTPR23), respectively (Fig. S3*A*). Each of the two amiRNA species specifically reduced the mRNA levels of the respective genes by 40–60%, with no effect on the levels of the nontargeted family members (Fig. S3*B*). Only cotransfection of both amiRNAs was effective in lengthening circadian period by 2 h, as measured by a *CCA1:LUCIFERASE* (*CCA1:LUC*) reporter, indicating redundancy among this gene family (Fig. 2 *A* and *B*). Similarly, using a *GIGANTEA* (*GI*) promoter-luciferase reporter (*GI:LUC*), only the double amiRNA transfection effectively eliminated rhythmicity, and reduction of two or three of the five family members lengthened the period only slightly (Fig. 2*C* and Fig. S3*C*).The longer period is consistent with a reduced repression (i.e., higher levels) of *CCA1* and *LHY* expression, given that *cca1* and *lhy* mutants are short period (25).

The *prr5 prr7 prr9* mutant is arrhythmic under constant conditions (26); therefore, we reasoned that if the TPL family acts via these three PRRs, we could sensitize the system to a reduction in TPL family levels by testing the effect of the amiRNAs on the *prr5 prr7* mutant. *prr5 prr7* mutants are short-period (27), which we also observed in protoplasts from these plants (Fig. S3 *D* and *E*). When transfected with single amiRNAs, there was little period effect relative to the control, but there is a striking decrease in robustness of the oscillations as indicated by the higher relative amplitude error (RAE) compared with the control (Fig. S3*F*). The double amiRNA transfection most severely reduces circadian amplitude (average RAE = 0.72) to an RAE > 0.6, which is above the value considered for a gene to be rhythmic (Fig. S3*F*). This result phenocopies the arrhythmicity of the *prr5 prr7 prr9* mutant and supports the notion that reduced levels of TPL/TPRs compromise the repressive activities of PRR5, PRR7, and PRR9.

tpl-1 is a temperature-sensitive *TPL* allele that has a dominant-negative effect in reducing the activity of the *TPR* family on plant development (28). We first tested the effects of this allele on circadian period by transient expression in protoplasts. There is significant period lengthening with the *tpl-1* allele, but WT *TPL* has no detectable effect (Fig. S4 *A* and *B*). We also examined the effect of the endogenous *tpl-1* allele on free-running period at the restrictive

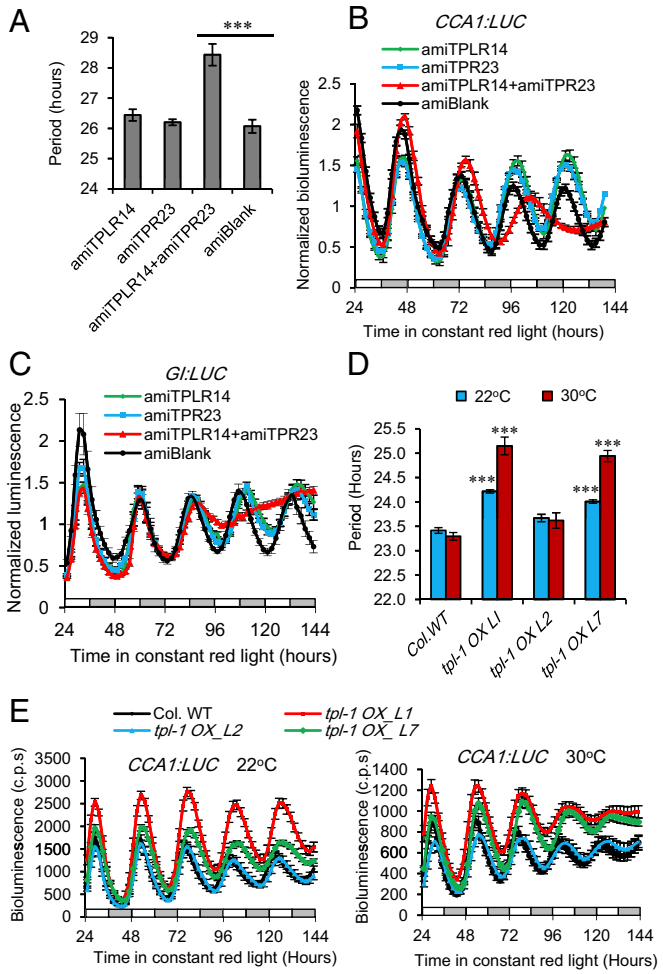

Fig. 2. Depletion or dysfunction of the TPL family lengthens circadian period in *Arabidopsis*. (*A* and *B*) Free-running period and normalized bioluminescence traces (*CCA1:LUC*) from WT protoplast cells transfected with indicated gene-specific artificial microRNA targeting *TPL* family members. Protoplast was isolated from rosette leaves of 4-wk-old *Arabidopsis* Col-0 grown in 12-h light/12-h dark with cellulose and macerozyme digestion. After PEG-4000–mediated transfection, protoplasts were transferred to constant red light at ZT9 for image acquisition with 2-h intervals. Each dataset was normalized to the mean expression level over the 24- to 144-h sampling schedule. Fourier transform-nonlinear least squares was used to calculate free running period. Data represent mean ± SEM ($n = 6$). ***$P < 0.001$ (*t* test) relative to amiBlank control ($n = 6$). White and gray regions indicate subjective light and dark period. Period lengthening was only observed when expression of all five TPL family members was reduced. Similar results were obtained in three independent trials. (*C*) As in *B*, using *GI:LUC* as reporter showing loss of rhythmicity when expression of all five *TPL* members is reduced. Representative data are from two trials with similar results. (*D* and *E*) Overexpression of *tpl-1*, a dominant-negative temperature-sensitive allele of *TPL*, lengthens free running period (*CCA1:LUC*). Overexpression lines are more severely lengthened at the restrictive temperature (30 °C) than at the permissive temperature (22 °C). Seedlings of T2 progeny after germination were grown in 12-h light/12-h dark for 7 d to entrain. Seedlings were transferred to constant red light at ZT1 for image acquisition with 2-h intervals for 1 wk. White and gray regions indicate subjective light and dark period. Free-running periods in *D* were calculated by Fourier transform-nonlinear least squares. Data represent mean ± SEM ($n = 20$). ***$P < 0.001$ (*t* test) relative to the WT control ($n = 20$). Average traces of bioluminescence were shown in *E*. Data represent mean ± SEM ($n = 20$). Data are representative of at least two independent trials with similar results.

temperature (28 °C) and observed a loss in amplitude of the *CCA1-LUC* reporter, higher overall expression of this reporter, and lengthened period (0.8 h) (Fig. S4 *C* and *D*). We next identified three independent transgenic lines constitutively expressing *tpl-1* at very strong (L1 and L7) and weak levels (L2) (Fig. S5*A*). The strong expressors were developmentally stunted relative to WT and the weak expressor, but otherwise were able to propagate normally (Fig. S5*B*). At 22 °C the periods of L1 and L7 were slightly less than 1 h longer than WT and L2, and *CCA1:LUC* luminescence levels were consistently higher as well, despite the smaller leaf area of the individual plants (Fig. 2 *D* and *E*). At the *tpl-1* restrictive temperature (30 °C), WT period was similar to 22 °C, but the L1 and L7 lines showed periods nearly 2 h longer than WT (Fig. 2 *D* and *E*) and ∼1 h longer than the same lines at 22 °C. The longer periods and higher absolute levels of luminescence (Fig. 2*E*) are consistent with increased *CCA1* expression levels, caused by a loss of repression of *CCA1* transcription by the *tpl-1* allele. The stronger circadian phenotypes seen in these lines, compared with the original *tpl-1* allele, is consistent with the extent of the dominant-negative effect being dependent on the expression level of the tpl-1 protein.

TPL Represses *CCA1* and Chromatin Presence Requires PRR5, PRR7, and PRR9. To determine if TPL resides at the same regions of the *CCA1* and *LHY* promoters as previously identified for PRR5, PRR7, and PRR9 (15), we performed ChIP in lines expressing *TPL:TPL-HA*. At ZT6, when PRR9, PRR7, and PRR5 are resident at both promoters, TPL-HA was significantly enriched at the same sites (A2, A3 and A6, A7) (Fig. S6) identified by Nakamichi et al. (15) relative to a region of the *TOC1* promoter and other control sites (Fig. 3 *A* and *B*). No enrichment was found at the *LHY* and *CCA1* promoters in the *prr5 prr7 prr9* background, indicating that these proteins are required for TPL to occupy either promoter (Fig. 3 *A* and *B*). At ZT18, when the protein levels and residence of PRR5, PRR7, and PRR9 at the *CCA1* and *LHY* promoters are very low (15), we found no significant difference in TPL-HA residence between WT and the triple mutant, with occupancy similar to the negative-control chromatin sites (Fig. 3 *C* and *D*). Taken together, these results indicate that the three PRR proteins link TPL to the *LHY* and *CCA1* promoters.

If the TPL/TPR family acts as corepressors of *CCA1* and *LHY*, then expression of both genes should increase when TPL family members are absent or reduced. We tested the mRNA levels of *CCA1* and *LHY* in the *tpl-1* background after light/dark entrainment followed by 4 d in constant white light. Expression of both genes was consistently higher than the WT during normal trough times, as previously reported for the *prr5 prr7 prr9* mutant (26) (Fig. 4*A*). Similarly, transient reduction of TPL family expression in WT protoplasts resulted in an approximately twofold increase in *CCA1:LUC* expression (Fig. S7). Reciprocally, when TPL was transiently overexpressed *CCA1* levels were concomitantly reduced by half (Fig. 4*B*). However, there was no reduction in *CCA1* expression in the *prr5 prr7 prr9* background, confirming that these PRRs are required for TPL/TPR proteins to effect *CCA1* repression (Fig. 4*B*).

TPL Forms an in Vivo Tripartite Complex with PRR9 and HDA6. HDAs, particularly HDA19, have been implicated as additional components in the TPL/TPR repression mechanism (28, 29). We tested such requirements here both pharmacologically and interactively. Trichostatin A (TSA) is an effective inhibitor of HDA (30), and we measured the effect of TSA application on *CCA1* expression in the WT and *tpl-1* backgrounds. During the first 11 h following TSA application, during subjective dawn, TSA treatment was effective in significantly increasing *CCA1:LUC* expression in WT seedlings at most time points, but had no effect in the *tpl-1* background (Fig. 4*C*). Conversely, transient overexpression of *HDA19* and the closely related family member, *HDA6*, significantly reduced *CCA1:LUC* expression (Fig. 4*D*). Both results are consistent with HDA repressing *CCA1* expression through TPL/PRR interactions.

A further prediction is that reduced levels of HDA6 and HDA19 will lengthen period by compromising TPL family function. We used two different amiRNA approaches to test this. The first test

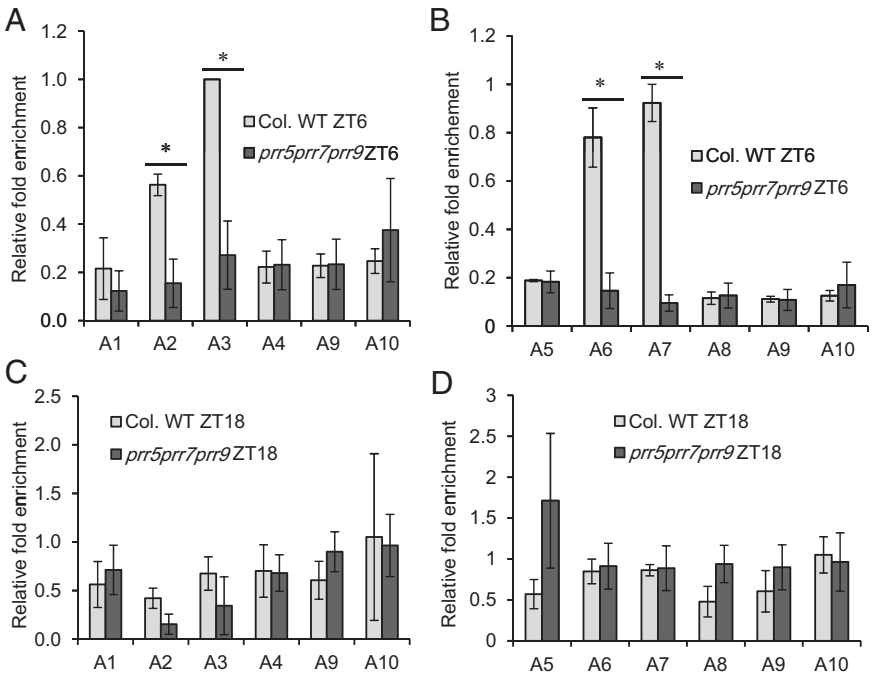

Fig. 3. TPL associates with promoters of *CCA1* and *LHY* in vivo. Two-week-old long-day grown seedlings, which possess *TPLpro:TPL-HA* or not, were harvested at indicated time points (ZT6 or ZT18) and cross-linked with 1% formaldehyde. The nuclei were isolated using the CELLYTPN1 CelLytic PN isolation/Extraction Kit. After sonication and clearance, ChIP assays were performed with anti-HA antibody. The amount of immunoprecipitated DNA was quantified by quantitative PCR (qPCR) using primers specific to each amplicon. Schematic representation of the locations of amplicons for ChIP analysis is in Fig. S6. Samples of Col-0 and *prr5 prr7 prr9* without the *TPLpro:TPL-HA* transgene were used as negative control. Relative fold-enrichment was normalized to its respective negative background control. (*A* and *B*) ChIP_qPCR assay of *TPLpro:TPL-HA* in WT and *prr5 prr7 prr9* backgrounds shows that TPL associates with *CCA1* (*A*) and *LHY* (*B*) promoters at ZT6. Data represent mean ± SEM ($n = 3$ biological replicates). *$P < 0.05$ (*t* test) relative to *prr5 prr7 prr9*. (*C* and *D*) ChIP_qPCR assay of *TPLpro:TPL-HA* in WT and *prr5 prr7 prr9* backgrounds shows that TPL does not significantly bind to *CCA1* (*C*) and *LHY* (*D*) promoters at ZT18. Data represent mean ± SEM ($n = 3$ biological replicates).

was to express individually or together two amiRNAs designed to reduce HDA6 and HDA19 separately, and the second method was to use a single amiRNA (amiHDA6/19) designed to specifically reduce the two HDAs together (Fig S8*A*). AmiHDA6 reduces HDA6 mRNA by approximately 40% and amiHDA19 reduces HDA19 by approximately 60% (Fig S8*B*). When cotransfected, levels of both mRNAs are reduced 60–80%, and amiHDA6/19 reduced both mRNAs by approximately 50% (Fig. S8*B*). Cotransfection of the two single amiRNAs or amiHDA6/19 lengthened period by approximately 1 h; single-target amiRNAs alone had little to no effect on period (Fig. S8 *C* and *D*). These results indicate an additive effect on period lengthening consistent with the notion that these HDAs act through PRR5/7/9-TPL family complexes to control period.

We next tested the in vivo interactability of TPL, PRR9, and HDA6. HDA6-TAP can effectively coimmunoprecipitate TPL-HA (Fig. 4*E*, lane 3), but GFP-PRR9 is not present in HDA6-TAP immunoprecipitates unless TPL-HA is coexpressed with them (Fig. 4*E*, compare lanes 2 and 4). Importantly, when the PRR9 EAR domain is mutated (Fig. 4*E*, lane 5) GFP-PRR9 is absent from HDA6-TAP immunoprecipitates of tissue extracts that have all three proteins expressed. These results demonstrate that an interaction between TPL and PRR9 is required for HDA6 to immunoprecipitate PRR9, identifying TPL as a necessary adaptor between PRR9 and HDA6. Taken together our results indicate the presence of a PRR-TPL-HDA complex at the promoters of the *CCA1* and *LHY* that represses the transcription of both genes (Fig. 4*F*).

Discussion

The extensive role for transcriptional repression in the circadian clock has been highlighted by a series of recent reports that demonstrate diverse mechanisms of action (5, 7–9, 31). For example, CCA1 and LHY repress *TOC1* and likely other evening-phased targets through association with DET1, which acts as a corepressor, possibly in the context of a larger CDD complex (9). However, CCA1 and LHY repression of evening-phased *ELF4* may be through a different mechanism, by early-day suppression of *ELF4* activation by the three transcription factors FAR-RED ELONGATED HYPOCOTYL 3 (FHY3), FAR-RED IMPAIRED RESPONSE 1 (FAR1), and LONG HYPOCOTYL 5 (HY5) (31). Morning-phased genes, such as the *PHYTOCHROME INTERACTING FACTORs* (*PIF*s) and *PRR9*, are repressed through an entirely different set of factors, the evening active ELF3-ELF4-LUX complex (12–14).

Here we have identified a unique transcriptional repression complex by which members of the TOPLESS corepressor family associate the promoter-binding function of three PRR proteins (PRR5, PRR7, and PRR9) with the chromatin-modifying activity of HDAs (e.g., HDA6) to inhibit the expression of two core circadian transcription factors, *CCA1* and *LHY*. The TOPLESS family of proteins has been extensively associated with plant developmental programs, including most hormone-signaling pathways (auxin, jasmonic acid, abscisic acid, and ethylene), meristem maintenance, floral induction, and biotic stress (20–23, 29). In all known cases, TPL members are recruited by transcription factors to repress gene expression in processes that alter development or mediate biotic or abiotic stresses. A role in more short-term, recurrent processes, such as the circadian cycle, was unknown and unanticipated. Additionally, our results strengthen the genetic and interactive data implicating HDAs as essential components in TPL family-mediated repression (28, 29). This finding is consistent with the well-established roles for HDAs in Groucho (*Drosophila*) and

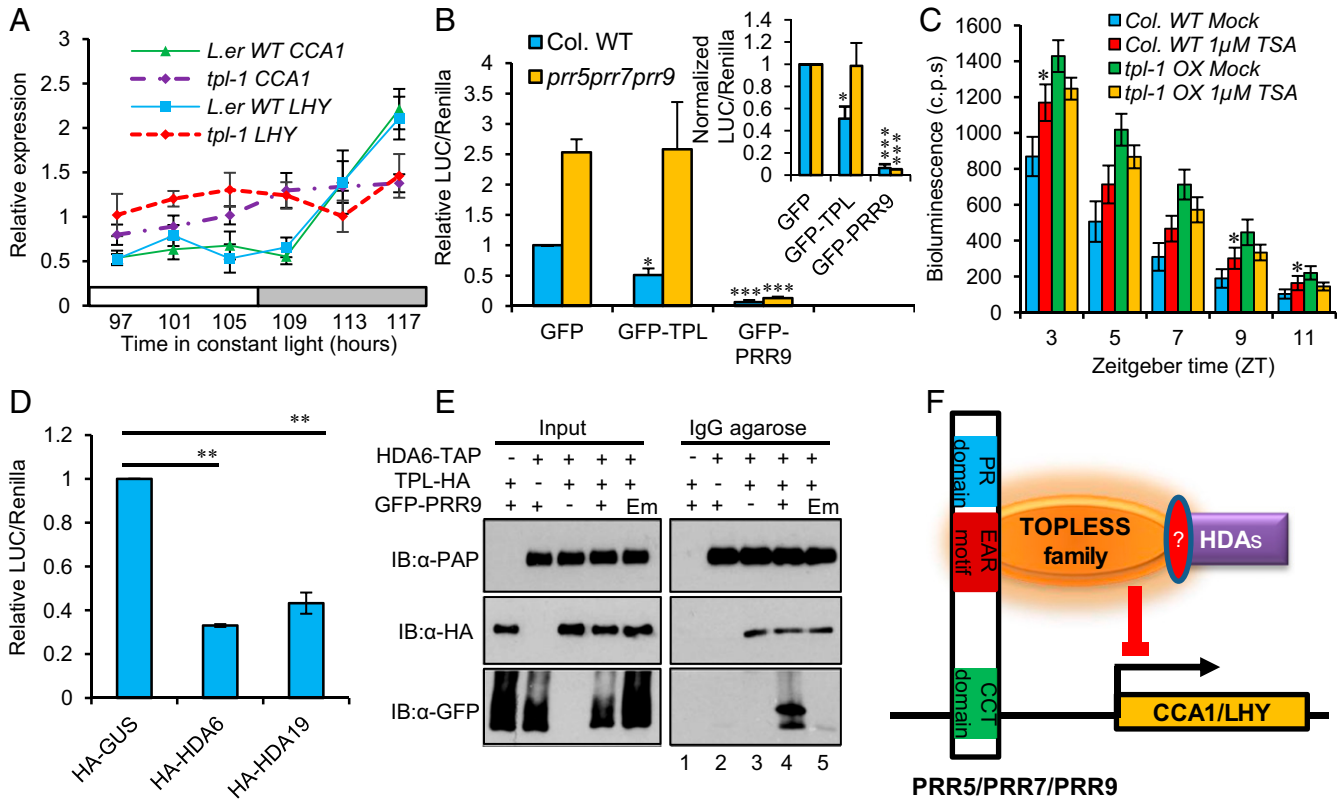

Fig. 4. TPL/TPRs and HDAs repress *CCA1* and *LHY* transcription and form a trimeric complex with PRR9 in vivo. (*A*) Seedlings of homozygous *tpl-1* and Landsberg (*er*) WT after germination were grown in 12-h light/12-h dark for 10 d and then were transferred to low light and 30 °C conditions for additional 4 d. Tissues were harvested at indicated time points. The relative abundance of *CCA1* and *LHY* mRNA to Actin mRNA were measured by RT-qPCR. Data represent mean ± SEM ($n = 3$ independent biological replicates). (*B*) Dual bioluminescence assay shows TPL represses *CCA1* transcription in WT but not in *prr5 prr7 prr9* protoplasts. *CCA1:LUC* reporter and the effectors (GFP alone, GFP-TPL, and GFP-PRR9) were cotransfected into Col-0 protoplast with 35S:Rluc reporter as transformation efficiency control. Data are mean ± SEM ($n = 3$ biological replicates). $^{*}P < 0.05$ and $^{***}P < 0.001$ (*t* test) relative to the GFP control vector ($n = 3$). Embedded graph shows results normalized to GFP control transfections within each genotype. *GFP-PRR9* was used as a positive control. (*C*) TSA derepresses transcription of *CCA1* in Col-0 WT seedlings but not *tpl-1* constitutive expressing seedlings. One-week-old seedlings of Col-0 WT and *tpl-1* overexpressor line1 were transferred to liquid MS medium with mock or 1 μM TSA at ZT1 and released to constant red light at ZT3 for image acquisition with a 2-h interval. Data represent mean ± SEM ($n = 20$). $^{*}P < 0.05$ (*t* test) relative to respective mock-treated control. Data are representative of three independent trials with similar results. (*D*) Dual bioluminescence assay shows HDA6 and HDA19 repress *CCA1* transcription. This assay was conducted as in *B* with HA-HDA6 and HA-HDA19 as effectors. Data represent mean ± SEM ($n = 3$ individual biological replicates). $^{**}P < 0.01$ (*t* test) relative to the HA-GUS control vector ($n = 3$). (*E*) HDA6, TPL, and PRR9 form a trimeric complex in vivo. Protein extracts from *N. benthamiana* leaves ectopically expressing HDA6-TAP, TPL-HA, and GFP-PRR9 or its EAR motif mutation were harvested at ZT6 and cross-linked with 1% formaldehyde. HDA6-TAP immunoprecipitations coprecipitate TPL and PRR9 but not the PRR9 EAR-mutated protein (Em). Data are representative of three trials with similar results. (*F*) Proposed model indicating the primary role of the TPL family of corepressors in the regulation of *CCA1* and *LHY* expression. PRR5, PRR7, and PRR9 directly bind to the promoters of *CCA1* and *LHY* via the C-terminal CCT domain, while recruiting TPL/TPR proteins to the chromatin via their EAR motifs. TPL/TPR proteins directly or indirectly link HDAs to this complex to repress transcription; "?" indicates that additional factors may be involved in the HDA–TPL family interaction.

TUP1-SSN6 (*Saccharomyces cerevisiae*) corepression complexes (32, 33), functional orthologs of the TPL family in plants.

Our results are also consistent with recent findings correlating oscillations in the acetylation state of histone 3 (H3Ac) at the *LHY* and *CCA1* promoters with the circadian expression of both genes (34). H3Ac levels are rising and become highest at both promoters during times of maximum gene expression (34), and are therefore largely antiphasic with times at which PRR9, PRR7, and PRR5 reside at the *CCA1* and *LHY* chromatin (15). Hence, a PRR-TPL-HDA complex likely acts to deacetylate H3 as part of a gene-repression mechanism, and supports the notion of H3Ac as a marker of active transcription. Our results indicate HDA6 and HDA19 as at least two deacetylases involved transcriptional repression of *CCA1* and *LHY*.

As an essential cofactor in various transcriptional repression mechanisms, the TOPLESS protein family might be expected to be permissive rather than instructive in their regulatory function, and their expression might be constitutive. To address this question in the context of the clock, we examined the message levels of the *TPL* family over diurnal and circadian time courses. *TPL* alone exhibits a nearly fourfold diurnal oscillation in mRNA accumulation with maximum levels during late night (Fig. S9*A*). In constant light, rhythms dampen considerably, suggesting little-to-weak circadian control of mRNA levels (Fig. S9*B*). We also tested TPL and TPR1 protein rhythms (Fig. S9 *C*–*E*). TPL-HA protein levels oscillate twofold in long-day with maximum expression near dawn (Fig. S9 *C* and *E*), raising the possibility that phase-dependent change in abundance could feature in TPL-mediated regulation. Post-translational modifications of TPL (35) could amplify the significance of this difference, raising the possibility of interlinking hormonal and developmental pathways with the circadian system. Hence, our results suggest that the daily rhythmic oscillations of the clock may be integrated into the widespread developmental role of this protein family.

TOC1 is closely related to the other PRRs and is phased last in the morning-to-evening sequence of circadian expression of this family: PRR9-PRR7-PRR5-TOC1 (16, 36). Interestingly, the EAR

domain that is essential to the TPL–PRR interaction is lacking in TOC1. The position of the EAR domain in the other three members varies, but it is outside the highly conserved N-terminal PRR domain and C-terminal CCT domain that are the only shared motifs among this PRR protein family. Hence, although our evidence suggests that TOC1 represses *CCA1* and *LHY* through mechanisms different from the other PRRs, it remains possible that other adaptor proteins may link DNA-bound TOC1 and HDA to effect repression. HDA is well-known as a common feature of circadian regulation (37–40) and there are various mechanisms by which platforms for HDA action are established (41, 42). It will be interesting to determine if TOC1 shares any molecular elements of repression with the earlier-phased PRRs.

Recent work in *Neurospora crassa* reported that orthologs of the *S. cerevisiae* TUP1-SSN6 complex, RCO1-RCM1 (21, 43), play a role in the *Neurospora* clock by anchoring a repressor complex that globally inhibits morning expression of a suite of circadian-regulated metabolic genes with evening phases (44). Expression of the repressor, CONIDIAL SEPARATION 1 (CSP1) is clock-controlled with a morning phase, and complexes with the co-repressor RCO1-RCM1 to place lipid metabolism and membrane composition under circadian regulation. With TOPLESS acting as a functional ortholog to the TUP1/RCO1 components of two fungal systems, we now also demonstrate an evolutionarily conserved mechanism of transcriptional repression among circadian systems.

Materials and Methods

Plant Materials and Growth Conditions. Further information on experimental growth conditions, TSA treatments, plasmid constructions, and rhythm analyses used in this study are provided in *SI Materials and Methods*. See also Tables S1 and S2 for a list of oligonucleotides and amiRNA constructs.

Protein Analysis. Details of the ChIP, immunoblot procedures, and yeast two-hybrid techniques are provided in *SI Materials and Methods*.

RNA Extraction, Quantitative PCR, and Dual Bioluminescence Assay. Details describing RNA handling, protoplast isolation, DNA transfection, and luciferase imaging are provided in *SI Materials and Methods*.

BiFC Analysis and Confocal Microscopy. Agrobacteria containing 35S:nYFP$_{(1-158)}$-TPL or 35S:nYFP$_{(1-158)}$-DCL4, and 35S:cYFP$_{(159-238)}$-PRRs were coinfiltrated into *N. benthamiana*. A Nikon Eclipse C90i confocal microscope with minimum or medium aperture was used to image epidermal cells. Samples were excited by 488-nm laser set at 10% power, and emissions were collected at gain settings between 7 and 8. Negative controls were imaged under the same conditions. H2B-RFP was imaged using 543-nm laser light and 585–615 BP emission filter. Images were processed using Nikon NIS-Elements software.

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