

# PRR5 regulates phosphorylation, nuclear import and subnuclear localization of TOC1 in the Arabidopsis circadian clock

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Many core oscillator components of the circadian clock are nuclear localized but how the phase and rate of their entry contribute to clock function is unknown. TOC1/PRR1, a pseudoresponse regulator (PRR) protein, is a central element in one of the feedback loops of the Arabidopsis clock, but how it functions is unknown. Both TOC1 and a closely related protein, PRR5, are nuclear localized, expressed in the same phase, and shorten period when deficient, but their molecular relationship is unclear. Here, we find that both proteins interact *in vitro* and *in vivo* through their conserved N-termini. TOC1–PRR5 oligomerization enhances TOC1 nuclear accumulation two-fold, most likely through enhanced nuclear import. In addition, PRR5 recruits TOC1 to large subnuclear foci and promotes phosphorylation of the TOC1 N-terminus. Our results show that nuclear TOC1 is essential for normal clock function and reveal a mechanism to enhance phase-specific TOC1 nuclear accumulation. Interestingly, this process of regulated nuclear import is reminiscent of similar oligomeric pairings in animal clock systems (e.g. timeless/period and clock/cycle), suggesting evolutionary convergence of a conserved mechanism across kingdoms.

The EMBO Journal (2010) 29, 1903–1915. doi:10.1038/emboj.2010.76; Published online 20 April 2010

Subject Categories: signal transduction; plant biology

Keywords: *Arabidopsis thaliana*; circadian clock; nuclear import; oligomerization; phosphorylation

## Introduction

The timing of many physiological and developmental processes in most eukaryotes is under the control of a circadian clock. This endogenous, self-sustaining oscillator maintains a rhythm of ca. 24 h in processes as diverse as human sleep/wake cycles (Ebisawa, 2007), insect pupal eclosion (Pittendrigh, 1981), fungal sporulation (Bell-Pedersen *et al*, 2005) and the movement of plant leaves (Sweeney, 1987).

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Received: 30 November 2009; accepted: 30 March 2010; published online: 20 April 2010

The apparent adaptive significance of a circadian clock lies in the ability to anticipate the regular changes in the environment (light/dark; warm/cold) that arise from the 24-h rotation of the earth, and to adjust its physiology and development accordingly (Ouyang *et al*, 1998; Dodd *et al*, 2005; Johnson and Kyriacou, 2005).

Classical and molecular genetics have helped to identify genes in *Drosophila*, *Arabidopsis*, *Neurospora* and mammals (clock genes) that alter clock function and a common molecular mechanism has emerged. In all systems, both the mRNA and protein products of key genes cycle in abundance and/or activity, creating a transcription–translation feedback loop. The 24-h delay occurs, in part, through the time it takes for the entry of the protein product(s) to the nucleus, where they contribute to the repression of their own transcription, and/or in the time required for these proteins to degrade in the nucleus. More recently, the importance of post-translational control has emerged, particularly the function of protein phosphorylation, and three or more interlocked loops are now apparent in all known eukaryotic clocks (Hardin, 2005; Ueda, 2007; de Paula *et al*, 2007; Harmer, 2009).

In *Arabidopsis*, >20 genes are known to affect circadian cycling (Gardner *et al*, 2006; McClung, 2006; Harmer, 2009). Current models posit three or more interlocked loops that contain both negative and positive regulatory elements that control gene transcription, but the majority of loci known to affect circadian period have not been incorporated into these schemes, either conceptually or functionally (Gardner *et al*, 2006; Locke *et al*, 2006; Zeilinger *et al*, 2006; Harmer, 2009). Those components that have been successfully linked largely belong to two gene families, the closely related MYB transcription factors CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and the five-member PSEUDO-RESPONSE REGULATOR (PRR) gene family (TOC1/PRR1, PRR3, PRR5, PRR7 and PRR9) (Schaffer *et al*, 1998; Wang and Tobin, 1998; Matsushika *et al*, 2000; Strayer *et al*, 2000). The first ‘loop’ to be established involved the upregulation of CCA1 and LHY by TOC1, whose transcription is negatively regulated by CCA1 and LHY (Alabadi *et al*, 2001). Subsequent work tied CCA1 and LHY as positive regulators of PRR7 and PRR9 transcription, which act to negatively regulate CCA1/LHY (Farre *et al*, 2005), and effectively interlink the two different loops. Other components, some postulated and some demonstrated, have been incorporated to comprise a third, transcription-based loop (Locke *et al*, 2006; Zeilinger *et al*, 2006).

In addition, a tremendous explosion of new work in eukaryotic clock systems has shown the fundamental importance of post-transcriptional processes in circadian clocks, particularly proteolysis and phosphorylation (Bell-Pedersen *et al*, 2005; Cha *et al*, 2007; Gallego and Virshup, 2007; Somers *et al*, 2007; Vanselow and Kramer, 2007). In *Arabidopsis*, the F-box protein ZEITLUPE (ZTL) controls

circadian period through the targeted proteasome-dependent degradation of TOC1 and PRR5 (Mas *et al*, 2003b; Kiba *et al*, 2007; Fujiwara *et al*, 2008). In addition, ZTL itself undergoes clock-controlled phase-specific proteolysis, regulated by the circadian cycling of GIGANTEA (GI), which acts as a blue-light enhanced stabilizer of ZTL (Kim *et al*, 2003, 2007; Somers and Fujiwara, 2009). The SCF<sup>ZTL</sup> substrate, TOC1, exhibits circadian phase-specific variation in both abundance and phosphorylation (Fujiwara *et al*, 2008). Although clock control of *TOC1* transcription contributes to much of TOC1 protein cycling, oligomerization with PRR3 shields TOC1 from ZTL-mediated degradation and boosts overall TOC1 levels (Para *et al*, 2007; Fujiwara *et al*, 2008). Phosphorylation of both TOC1 and PRR3 is necessary for their optimal binding and this interaction exemplifies one mechanism by which oligomerization controls protein abundance in the plant clock (Fujiwara *et al*, 2008).

In this study, we report a second, related, mechanism. We show that PRR5 and TOC1 oligomerize through their N-terminal PRR domains. Through this interaction, PRR5 promotes TOC1 nuclear import and phosphorylation, and directs TOC1 subnuclear localization to PRR5-type nuclear foci. Consistent with these results, TOC1 total protein accumulation is significantly decreased and nuclear accumulation is strongly reduced in the *prp5* loss-of-function mutant. Our findings indicate that the PRR5–TOC1 interaction increases TOC1 steady-state levels, similar to the effect of the PRR3–TOC1 interaction. However, PRR3 achieves this as a competitive inhibitor of the cytosolic TOC1–ZTL interaction, whereas PRR5 acts primarily to enhance TOC1 nuclear accumulation, thereby avoiding ZTL-dependent degradation. This PRR5-mediated nuclear import contributes to enhanced TOC1 levels in a manner similar to that observed in other clock systems, indicating a well-conserved mechanism across kingdoms.

## Results

### **PRR5 and TOC1 interact through their N-termini**

Recent studies have shown that TOC1 protein levels can be altered by the presence of other PRR proteins, notably PRR3, through direct protein–protein interactions (Para *et al*, 2007; Fujiwara *et al*, 2008). We expanded on this idea by testing TOC1 interactions with the remaining PRR family members. As the TOC1–PRR3 interaction is mediated through the two N-terminal PRR domains (Fujiwara *et al*, 2008), we next tested whether the N-terminal domain (aa 1–242) of TOC1 can interact with full-length TOC1, PRR5, PRR7 and PRR9 *in vitro* and *in vivo*.

TAP-TOC1<sub>1–242</sub> (TAP-TOC1NT) was expressed in *Nicotiana benthamiana* and resin-bound TOC1NT was incubated with extracts from Arabidopsis plants expressing specific *PRRn::PRRn-GFP* genes (Fujiwara *et al*, 2008). Only PRR5 and PRR9 protein were pulled down, with the PRR5–TOC1 interaction markedly stronger than PRR9–TOC1 (Figure 1A). When specific deletions of TOC1 were further tested with full-length PRR5, only full-length and N-terminal TOC1 were able to pull down PRR5, both *in vitro* and when transiently co-expressed in *N. benthamiana* (Figure 1B).

Reciprocal *in vivo* co-IP experiments using full-length, N-terminal and C-terminal deletions of TAP-PRR5 co-expressed with TOC1-YFP in *N. benthamiana* showed that

the N-terminal domain of PRR5 is sufficient to mediate their interaction (Figure 1C). Taken together, these data show that TOC1 and PRR5 oligomerize through their N-termini.

### **Specific TOC1 short-period alleles diminish interaction with PRR5 and ZTL**

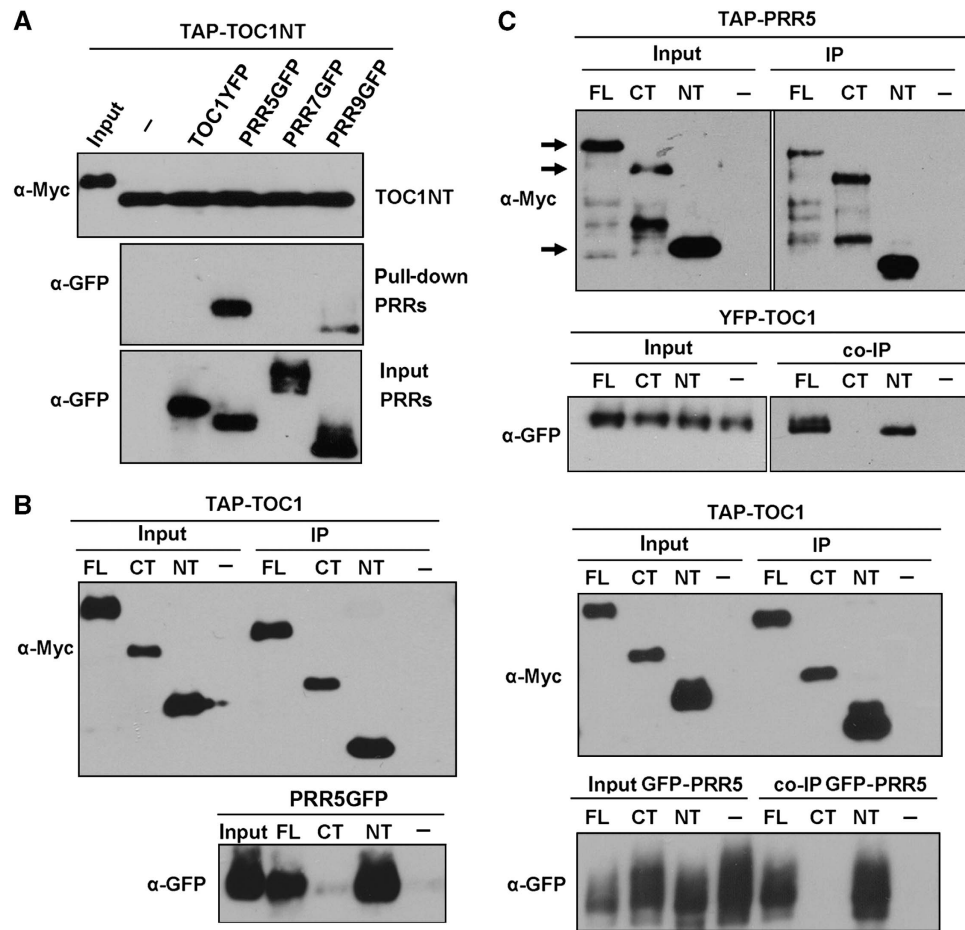
We took advantage of specific *toc1* mutant alleles to further probe the nature of the PRR5–TOC1 interaction. Two short-period point mutations (*toc1-5* and *toc1-8*) alter two different amino-acid residues in the TOC1 PRR domain at proline 96 (P96L) and proline 124 (P124L), respectively (Hazen *et al*, 2005; Kevei *et al*, 2006). Both mutations severely diminish the TOC1–PRR5 interaction three-fold or greater based on co-immunoprecipitation in a transient expression assay (Figure 2A and B). Mutations in highly conserved residues in the C-terminal CCT domain that affect either TOC1 activity (*toc1-1*) or CONSTANS (CO) activity (*co-5* and *co-7*) were also tested (Strayer *et al*, 2000; Robson *et al*, 2001). These mutations had no effect on the TOC1–PRR5 interaction (Figure 2A and B). Taken together, these data support the notion that the PRR domain is essential for the TOC1–PRR5 interaction and suggest an explanation for the short period of the PRR-domain *toc1* alleles.

However, we further tested the effect of these mutations on the TOC1–ZTL interaction, knowing that the N-terminus of TOC1 is also essential for its interaction with ZTL (Fujiwara *et al*, 2008). Interestingly, the same PRR domain mutations strongly reduced the TOC1–ZTL interaction, whereas the introduced CCT domain mutations had no effect on the interaction (Figure 2C and D). As reduced accessibility of TOC1 to ZTL is predicted to mimic a *ztl* mutant and result in a long period, it is likely that the short period of the *toc1-5* and *toc1-8* alleles results instead from the diminished TOC1–PRR5 interaction.

### **PRR5 stabilizes TOC1 post-transcriptionally and independent of phosphorylation state**

The significance of the TOC1–PRR5 interaction was first sought by observing the level of TOC1 in the Arabidopsis *prp5* mutant. The TOC-YFP minigene (TMG) (Mas *et al*, 2003a) was crossed into *prp5-1* and the TMG/TMG *prp5/prp5* genotype was isolated. Total protein extracts from the WT and *prp5* mutant were collected every 4 h from plants grown in 12/12 h light/dark cycles and TMG levels were determined in total protein extracts. Consistently lower levels of TMG were observed during the entire time of detectable expression (Figure 3A and B). RT-PCR analysis indicated that *TOC1* message levels are not significantly different in the two backgrounds, indicating post-transcriptional regulation of TOC1 expression (Figure 3C and D).

We next sought to understand how TOC1 protein levels are sustained by PRR5. Earlier reports showed that all the PRR proteins are phosphorylated *in vivo*, and that the TOC1–PRR3 interaction is highly enhanced by phosphorylation of both proteins (Fujiwara *et al*, 2008). We tested this requirement for TOC1 and PRR5. We bound TAP-TOC1 expressed in *N. benthamiana* to IgG resin and tested whether TOC1 phosphorylation affects the *in vitro* binding of PRR5-GFP from Arabidopsis seedling extracts. PRR5-GFP is able to bind both forms of TOC1 equally well (Supplementary Figure 1A, lower panel, lanes 3 and 4). Similarly, when PRR5-GFP extracts were mock or phosphatase treated, both forms of



**Figure 1** PRR5 interacts with TOC1 *in vitro* and *in vivo*. (A) Test of TOC1 interactions with Arabidopsis extracts expressing specific PRR clock-related proteins using TAP-tagged amino-terminal TOC1 (TAP-TOC1NT, aa 1–242). (B) The TOC1 N-terminus is necessary and sufficient to interact with PRR5 *in vitro* and *in vivo*. Only full length and amino-terminal TOC1 resin are able to pull down PRR5 *in vitro* (left panel). TOC1 interaction with PRR5 *in vivo* was detected by co-immunoprecipitation after transient co-expression of PRR5-GFP with full-length TOC1 (FL) or N-terminal (NT) or C-terminal (CT) TOC1 in *N. benthamiana* (right panel). (C) The PRR5 N-terminus (NT, aa 1–171) is necessary and sufficient to interact with TOC1 *in vivo*. Assay performed as in (B) except full length and deleted forms of TAP-PRR5 (NT, CT) were co-expressed with full-length YFP-TOC1 in *N. benthamiana*. Arrows indicate the migration position of the three forms of PRR5-TAP proteins (FL, CT and NT) with the vertical order of the arrows corresponding, from left to right, to the running position of TAP-tagged PRR5-FL, CT and NT, respectively. The lower bands in some lanes in the upper portion of the panel may be due to partial degradation of PRR5.

PRR5-GFP bound TOC1 similarly (Supplementary Figure 1B, lower panel, lanes 5 and 6). These data indicate that the TOC1-PRR5 interaction is not affected by the phosphorylation status of either of the proteins.

#### PRR5 enhances the proportion of phosphorylated TOC1

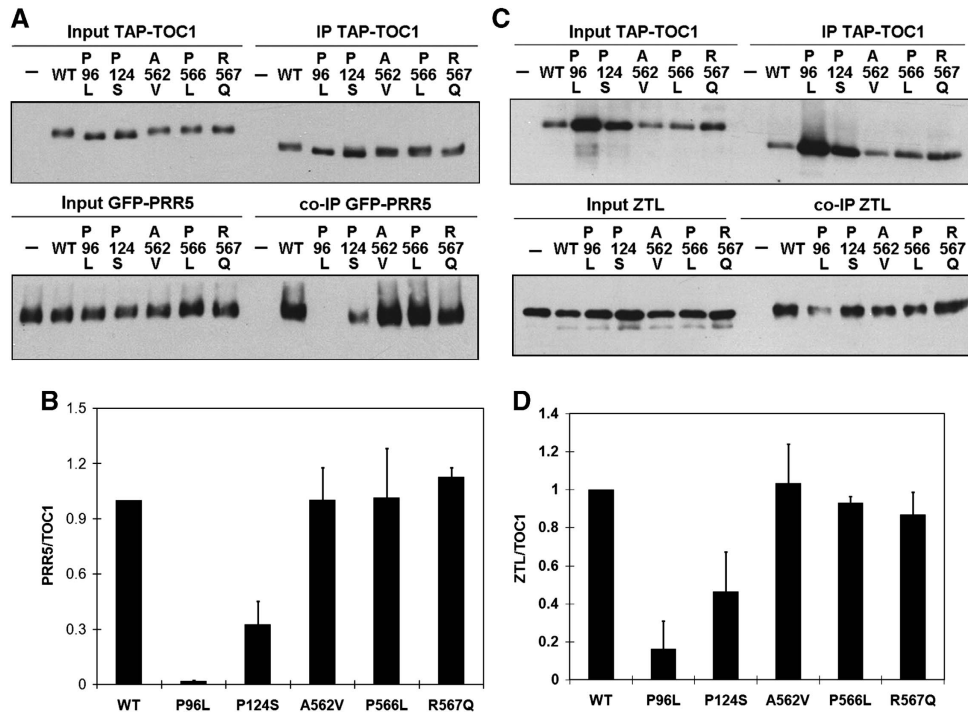
Although the phosphorylation state of PRR5 does not affect binding to TOC1, we observed that the migration of TOC1 was more rapid when the TOC1-PRR5 interaction was diminished by the P96L and P124L mutations in the TOC1 PRR domain (Figure 2A). This suggested that a TOC1-PRR5 interaction might facilitate TOC1 phosphorylation *in vivo*. We tested this idea by co-expressing both proteins at different abundance ratios in *N. benthamiana* and then observed the relative abundance of upper (phosphorylated) and lower (unphosphorylated) forms of TOC1 in total protein extracts.

We adjusted the relative concentrations of two Agrobacterium strains expressing either PRR5-GFP or TAP-TOC1 to obtain different amounts of PRR5 expressed relative to TOC1 in *N. benthamiana* (Figure 4). As the relative abundance

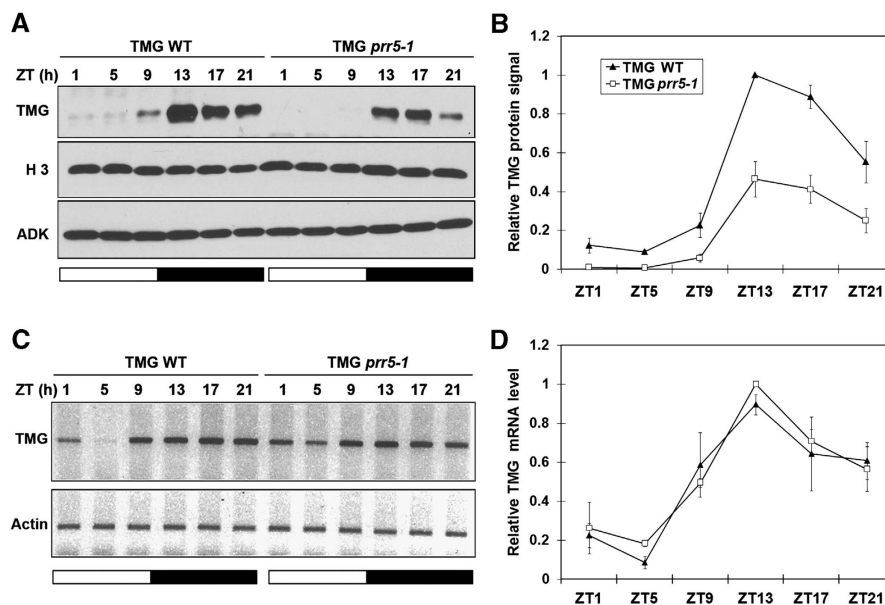
of PRR5-GFP was increased, we observed a concomitant increase in the phosphorylated form of TOC1, relative to the unphosphorylated form using both full-length TOC1 and the TOC1 N-terminus alone (Figure 4A and B; Supplementary Figure S2). Similar experiments conducted with PRR9-GFP had no effect on TOC1 phosphorylation, indicating a specific function for PRR5 (Figure 4A). When the N-terminal and C-terminal domains of PRR5 were co-expressed separately with TAP-TOC1, we did not observe a shift to a greater proportion of phosphorylated TOC1, indicating the need for full-length PRR5 (Supplementary Figure S3A). We observed a similar enhancement of TOC1 phosphorylation in Arabidopsis using ectopically expressed full-length PRR5 (PRR5 FL OX), but no effect when N-terminal PRR5 (PRR5 NT OX) was used (Supplementary Figure S3B).

#### PRR5 promotes TOC1 nuclear accumulation and phosphorylation

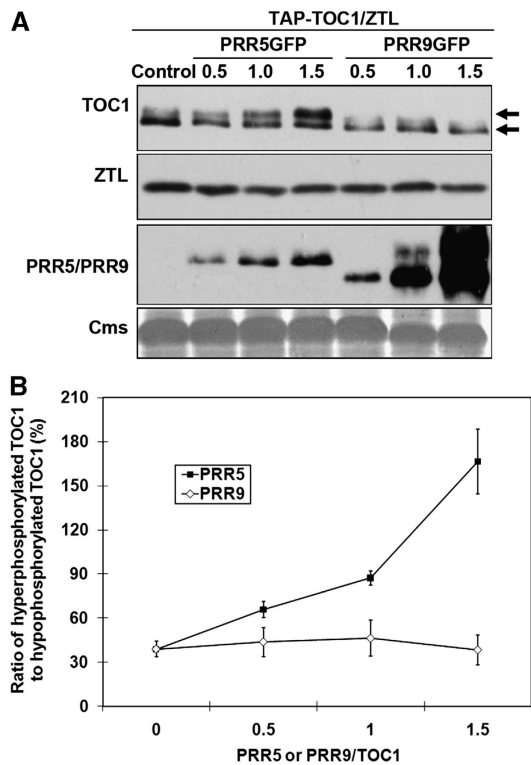
Studies in other circadian systems have indicated that phosphorylation often enhances or facilitates nuclear import



**Figure 2** Interactions between TOC1 and PRR5 and ZTL are disrupted by specific PRR domain point mutations in TOC1. (A) Interactions between TOC1 and PRR5 *in vivo* were detected by co-immunoprecipitation after transient co-expression in *N. benthamiana* of PRR5-GFP with wild-type (WT) and various single amino-acid mutants of TOC1 in the PRR domain (P96L, P124S) and C-terminal region (A562V, P566L and R567Q). After resin binding and enzymatic release, TAP-TOC1 was detected by anti-myc and PRR5 detected by anti-GFP antibodies, respectively. (B) Quantification of the densitometric ratio of co-immunoprecipitated (co-IP) PRR5 to immunoprecipitated TOC1 in (A). Error bars indicate s.d. ( $n = 3$ ). (C) Co-expression and co-IP experiments conducted as in (A) but with ZTL as the test partner for interaction with TOC1. Western blots as in (A) using an anti-ZTL antibody. (D) Quantification of densitometric ratio of co-immunoprecipitated ZTL to immunoprecipitated TOC1 in (C). Error bars indicate s.d. ( $n = 3$ ).



**Figure 3** PRR5 promotes post-transcriptional accumulation of TOC1. (A, B) Immunodetection of TOC1-YFP in *TOC1::TOC1-YFP* (TMG) wild-type and *prrr5-1* mutant plants. TOC1 protein levels are significantly decreased in the *prrr5-1* mutant. Representative data are shown in (A). Quantitation of (A) shown in (B) with values normalized to the maximum expression level (ZT13). Histone H3 and ADK were used as loading controls for nuclear and cytosolic compartments, respectively. (C, D) TOC1 mRNA levels are not significantly affected by the absence of PRR5. Semi-quantitative RT-PCR experiments were repeated three times from the same tissue used in (A). Representative data are shown in (C). Quantitation of (C) are shown in (D) with values normalized to the maximum expression level (ZT13). All error bars are s.d. ( $n = 3$ ). Seedlings were entrained in 12-h light/12-h dark cycles and harvested at the indicated times (ZT; Zeitgeber time indicating the number of hours since lights-on.). White and black bars indicate light and dark periods, respectively.



**Figure 4** PRR5 enhances accumulation of hyperphosphorylated TOC1. (A) Full-length PRR5 and PRR9 were co-expressed transiently in *N. benthamiana* with TOC1 and ZTL in a dosage series. Representative of three independent trials. Numbers indicate the ratio of initial *Agrobacterium* volume of PRR5 or PRR9 to that of TOC1 used for infiltration. (B) The densitometric determination of hyperphosphorylated TOC1 to hypophosphorylated TOC1 in (A). Error bars indicate s.d. ( $n = 3$ ). Cms: Coomassie-stained membrane indicating protein loading. Arrows indicate mobility shifts. ZTL was co-expressed to help enhance the detection of both phosphorylated and unphosphorylated TOC1.

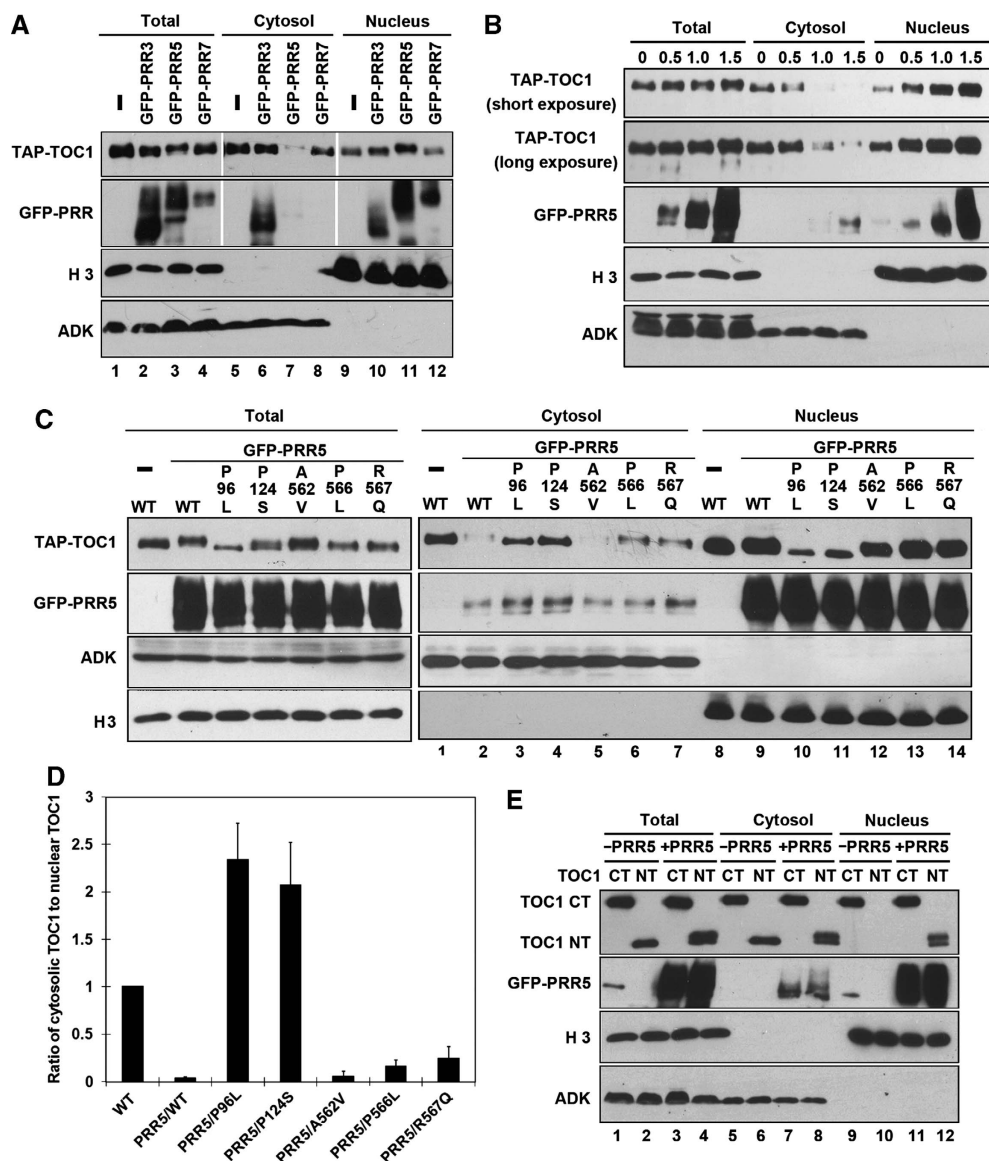
(Blau, 2008; Diernfellner *et al*, 2009). We tested this notion in plants by first observing how co-expression of different PRR proteins effects TOC1 nucleocytoplasmic partitioning. When transiently expressed alone in *N. benthamiana*, TAP-TOC1 clearly partitions in both the cytoplasm and nucleus (Figure 5A, lanes 5 and 9). Co-expression of TAP-TOC1 with GFP-PRR3, a demonstrated TOC1 interactor (Para *et al*, 2007; Fujiwara *et al*, 2008), has no detectable effect on this partitioning of TAP-TOC1 (Figure 5A, lanes 6 and 10). Similarly, co-expression of TAP-TOC1 with GFP-PRR7 has little effect on TOC1 subcellular localization (Figure 5A, lanes 8 and 12). It is notable that neither PRR3 nor PRR7 affect TOC1 localization, despite the differences between these two in their own relative nucleocytoplasmic partitioning. PRR3 is present in both compartments, whereas PRR7 is found largely in the nucleus (Figure 5A; Supplementary Figure S4). Strikingly, GFP-PRR5 co-expression results in near exclusive nuclear localization of TOC1-TAP, with little protein detectable in the cytoplasm (Figure 5A, lanes 7 and 11). In addition, this effect depends on PRR5 levels, with increasing amounts of co-expressed PRR5 resulting in a corresponding increase in the proportion of nuclear TOC1 (Figure 5B). This localization requires full-length PRR5 (Supplementary Figure S5) and depends on TOC1-PRR5 oligomerization, as the same PRR mutations that diminish

their interaction strongly reduce the enhanced nuclear presence of TOC1 (Figure 5C, compare lanes 2, 3, 4 with 9, 10, 11; Figure 5D). These results implicate PRR5 as a strong facilitator of TOC1 nuclear localization. In contrast, under these conditions, TOC1 co-expression has no effect on the localization of PRR3, PRR5 or PRR7 (compare Figure 5A with Supplementary Figure S4).

As the TOC1-PRR5 interaction only requires the N-terminus of TOC1 (Figure 1B), we further tested the coupling of PRR5-mediated nuclear import and phosphorylation of TOC1. We co-expressed the TAP-TOC1 N-terminus and C-terminus with full-length GFP-PRR5 in *N. benthamiana* and examined the partitioning and phosphorylation state of these deletions. When expressed in the absence of PRR5, TOC1 NT remains in the cytosol (Figure 5E, lanes 6 and 10), whereas TOC1 CT is found in both the cytosol and nucleus (Figure 5E, lanes 5 and 9), as expected given that the putative NLS is in the CCT domain (Strayer *et al*, 2000). When co-expressed with GFP-PRR5 TOC1 NT is both mobilized to the nucleus and phosphorylated (Figure 5E, lane 12), and cytosolic TOC1 NT is also phosphorylated (Figure 5E, lane 8). As there is no detectable nuclear TOC1 NT in the absence of PRR5 (Figure 5E, lane 10), these results reinforce the notion that PRR5 facilitates the nuclear import of TOC1, rather than causes an increase in the stability of nuclear TOC1 or a diminished nuclear export. Coupled with PRR5-mediated nuclear import is the phosphorylation of TOC1 NT, although it is not known whether this occurs in the cytosol before nuclear import or whether the cytosolic fraction of phosphorylated TOC1 NT results from the export of nuclear phosphorylated TOC1 NT.

These data predict that TOC1 in the *prr5* mutant should be strongly depleted in the nucleus and enhanced in the cytosol. We tested the nuclear and cytosolic fractions of a TMG *prr5* line by sampling during the times of maximum TOC1 accumulation from plants grown under 12/12 h light/dark cycles. TOC1 in the nucleus is nearly two-fold lower in the *prr5* background relative to WT, whereas reciprocally two-fold higher in the cytosol (Figure 6A and B). Conversely, TOC1-YFP nuclear accumulation in Arabidopsis is significantly enhanced, relative to WT, in a strong PRR5 ectopic expressor (Supplementary Figure S6). These data support the results from the transient assays, indicating that PRR5 significantly enhances the nuclear accumulation of TOC1 in Arabidopsis. Similar experiments in the TMG *prr3* background show a reduction in overall TOC1 levels as reported earlier (Para *et al*, 2007), but no change in relative nucleocytoplasmic localization (Figure 6C and D).

We also examined the relative cytosolic and nuclear accumulation of TOC1 and PRR5 under free-running constant light conditions in backgrounds with different levels and phases of PRR5 expression. During the second day in constant light, cytosolic levels of TOC1 in wild-type Arabidopsis were highest near the end of the subjective night. In contrast, over the same time course, no TOC1 at all is detectable in the cytosol in the PRR5 OX background, and the levels in the *prr5* mutant are comparatively much higher over WT late in the subjective day (Figure 7A). In the nucleus, TOC1 levels in the wild type are relatively high early in the subjective day and drop rapidly until rising slowly to high levels again late in the subjective night. In the *prr5* mutant, nuclear TOC1 is consistently lower at all these time points, relative to wild type.



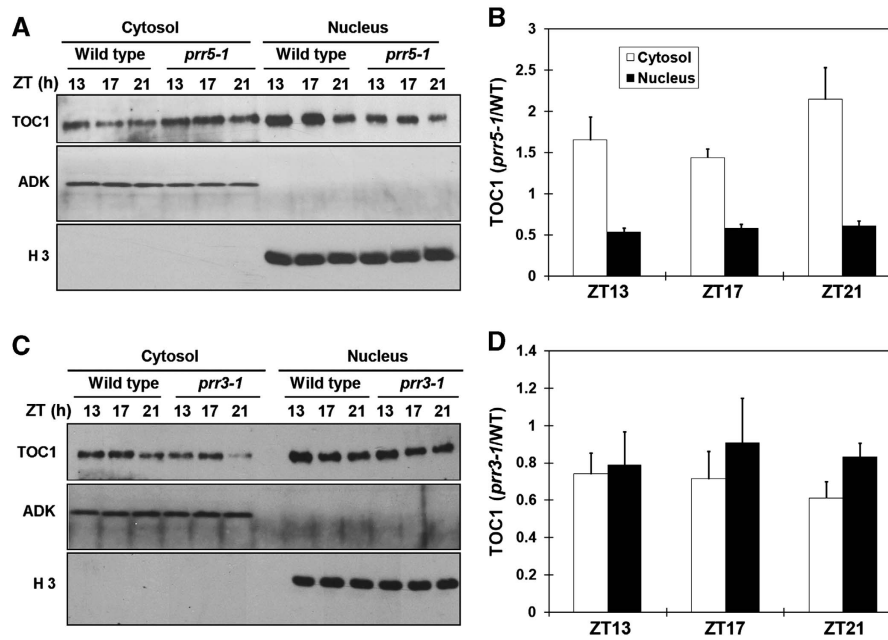
**Figure 5** PRR5 promotes TOC1 cytosolic depletion and nuclear accumulation. (A) PRR5 alone is able to alter the TOC1 nucleocytoplasmic distribution. GFP-PRR3, GFP-PRR5 and GFP-PRR7 were transiently co-expressed with TAP-TOC1 in *N. benthamiana* and tissues were processed for cytosolic and nuclear protein fractions at ZT13 2 days after infiltration. Representative of three trials. (B) PRR5 promotion of TOC1 cytosolic depletion and nuclear accumulation is dose dependent. Numbers indicate the ratio of initial Agrobacteria infiltration volume of PRR5 to that of TOC1. (C) Point mutations in the TOC1 PRR domain specifically reduce TOC1 nuclear accumulation. Left panel shows the total protein expression for each infiltration combination; right panel shows TOC1 and PRR5 accumulation in cytosolic and nuclear fractions. (D) Quantitation of relative cytosolic-to-nuclear TOC1 levels from (C). See Materials and methods for explanation. Error bars indicate s.d. ( $n = 2$ ). (E) TOC1 N-terminus is nuclear localized by PRR5. TOC1 NT and CT as in Figure 1. Histone H3 and ADK antibodies used are as in Figure 3. All immunoblots performed with anti-myc (TAP-TOC1) and anti-GFP (PRR5) antibodies.

Constitutive high expression of PRR5 causes TOC1 to accumulate in the nucleus at times not normally detectable in the wild type (e.g. at 33 h), though levels early in the subjective day (25 h) are unexpectedly low (Figure 7A). Overall, these findings can be explained by the strong phase-specific expression of PRR5 in the wild type where peak accumulation of PRR5 precedes that of TOC1 in both the cytosol and nucleus (compare Figure 7A and B) (Fujiwara *et al*, 2008). This earlier timing and the effect of the PRR5 overexpression and absence on TOC1 levels are consistent with PRR5 acting phase specifically to facilitate TOC1 nuclear accumulation and cytosolic depletion through enhanced nuclear import.

Taken together, our data indicate that PRR5 enhances both the nuclear accumulation and phosphorylation of TOC1.

#### Co-localization of TOC1 and PRR5 in the nucleus

Our initial demonstration of a TOC1-PRR5 interaction did not distinguish between nuclear and cytoplasmic compartments. We tested for nuclear oligomers by sonic lysis of the nuclear fraction followed by an IgG pull down of TAP-TOC1. GFP-PRR5 is detected in this bound fraction, indicating that the interaction occurs in the nucleus (Supplementary Figure S7). We then investigated the subcellular *in vivo* interaction by observing the nuclear localization of both TOC1-mCherry



**Figure 6** Nucleocytoplasmic ratio of TOC1 distribution is altered in *prp5-1*. (A) Subcellular determination of cytosolic and nuclear TOC1-YFP (TOC1) in Arabidopsis wild-type and *prp5-1* mutant. Anti-GFP antibody was used for immunodetection of TOC1-YFP. Histone H3 and ADK antibodies used are as in Figure 3. (B) *prp5-1*/WT ratio of quantitated cytosolic and nuclear TOC1 from (A). A value of 1.0 would indicate identical TOC1 levels in both backgrounds. (C) Subcellular determination of cytosolic and nuclear TOC1-YFP (TOC1) in Arabidopsis wild-type and *prp3-1* mutant. (D) *prp3-1*/WT ratio of quantitated cytosolic and nuclear TOC1 from (C). Error bars indicate s.d. ( $n = 3$ ). Seedlings were entrained in 12-h light/12-h dark cycles and harvested at the indicated ZT times.

and PRR5-GFP when transiently expressed separately or together in *N. benthamiana* (Figure 8). When expressed alone, TOC1-mCHERRY exhibits a pattern of small, dispersed nuclear foci (Figure 8A), very similar to an earlier report describing YFP-TOC1 expression (Strayer *et al*, 2000). In contrast, PRR5-GFP occurs in distinctly larger foci dispersed within the nucleus (Figure 8B), consistent with an earlier report (Matsushika *et al*, 2007). When both proteins are co-expressed, numerous medium-sized PRR5-like nuclear inclusions are evident with TOC1 now co-localized with PRR5 (Figure 8C). These data indicate a co-residence of both proteins, and strongly suggest that TOC1 is recruited by PRR5 to a particular class of nuclear foci.

#### LKP2 contributes to the degradation of PRR5

Although the post-translational effects of PRR5 on TOC1 nuclear localization and phosphorylation strongly affect TOC1 function, PRR5 is also linked to TOC1 through a shared degradation mechanism. Earlier reports have shown that both TOC1 and PRR5 are targeted for degradation by the SCF<sup>ZTL</sup> (Kiba *et al*, 2007; Fujiwara *et al*, 2008). However, the ZTL family also includes the closely related LKP2 and FKF1 proteins (Kiyosue and Wada, 2000; Nelson *et al*, 2000; Somers *et al*, 2000). Whereas FKF1 is strongly linked to photoperiodic timing in Arabidopsis, until now there has been no demonstrated function for LKP2 in the plant clock or flowering time, apart from the effects of strong ectopic expression, which has effects on periodicity, hypocotyl length and flowering time identical to ZTL overexpression (Kiyosue and Wada, 2000; Schultz *et al*, 2001; Somers *et al*, 2004).

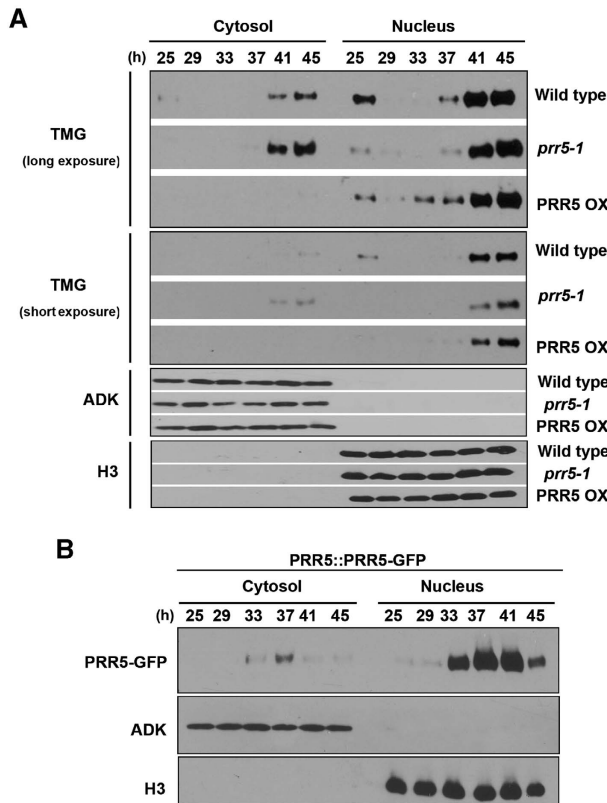
We transformed the *lkp2-1*, *ztl-1* and the *ztl-3 lkp2-1* double mutants with *PRR5::PRR5-GFP* and identified and characterized plants expressing PRR5-GFP in each of the

mutant genotypes. As reported earlier, PRR5 levels are strongly stabilized in the *ztl* mutant under light/dark cycles (Figure 9A) (Kiba *et al*, 2007; Fujiwara *et al*, 2008). In contrast, we observed the same strongly cycling PRR5 steady-state levels in the *lkp2-1* mutant as in the WT Col background (Figure 9A). Oscillation of PRR5 in the *ztl-3 lkp2-1* appeared slightly less robust compared with the *ztl* single mutant, so we tested the effects of cyclohexamide on PRR5 stability in all the backgrounds. We reasoned that if activity of SCF<sup>ZTL</sup> was further compromised by the absence of LKP2, the half life of PRR5 would be longer in the *ztl lkp2* double mutant than in the *ztl* single mutant. PRR5 half-life in the *lkp2-1* mutant was indistinguishable from WT, consistent with no distinguishable differences in free-running period between these backgrounds (Figure 9B and C; Supplementary Figure S8). In contrast, there was a significant increase in the PRR5 half-life in the *ztl lkp2* double mutant, relative to *ztl-3* (Figure 9B and C). A similar stabilization of PRR5 has been observed in the *ztl lkp2 fkf1* triple mutant (Takato Imaizumi, personal communication). These data indicate that LKP2 contributes to the proteasome-dependent degradation of PRR5.

## Discussion

### PRR5 controls the nucleocytoplasmic and subnuclear distribution of TOC1

The five-member family of PRR proteins is intricately linked to the control of circadian period, but the specific function of each has remained unclear. Genetic and molecular evidence implicates most of them in the transcriptional control of a number of core clock genes, but how this is effected is obscure. Our results now show a novel function for PRR5



**Figure 7** Phase-specific effect of PRR5 expression on TOC1 nucleocytoplasmic distribution. **(A)** Cytosolic and nuclear TOC1-YFP (TMG) levels in Arabidopsis wild-type, *pr $r5-1$*  and PRR5 OX backgrounds under free-running constant light conditions. **(B)** Cytosolic and nuclear PRR5-GFP levels in Arabidopsis wild type under free-running constant light conditions. Seedlings were entrained in 12-h light/12-h dark cycles then maintained in constant white light for the number of hours indicated until harvested. Different immunoblot exposure levels are shown for TMG (anti-GFP) panels in **(A)** to allow comparisons between earlier and later time points within and between genotypes. SDS-PAGE fractionated protein extracts from all genotypes and time points within each TMG-probed panel were immunoblotted and probed together and are directly comparable, relative to the cytosolic and nuclear loading controls. Direct comparisons of abundance between TMG and PRR5-GFP panels are not appropriate. All data are representative of two independent trials. Histone H3 and ADK antibodies used are as in Figure 3.

through a specific oligomerization with TOC1 that appears, in part, unrelated to a direct function in control of transcription. The nearly two-fold decrease in steady-state nuclear TOC1 abundance in the *pr $r5$*  mutant concomitant with a similar increase in TOC1 cytoplasmic levels demonstrates a critical function for PRR5 in the control of TOC1 nuclear accumulation and localization. These findings imply that the short period of the *pr $r5$*  mutant is at least partially due to the diminished levels of nuclear TOC1. Similarly, the decreased ability of the *toc1-5* and *toc1-8* proteins to interact with PRR5 explains the short period of those two mutant alleles not as a result of diminished activities but from lower accumulations in the nucleus.

Consideration of earlier studies of *pr $r5$*  and *toc1* single mutants and ectopic expression work reveals that manipulating the levels of both genes affects the clock in similar ways. In addition to the similarly shortened circadian period in the single mutants, the effects on the gene expression of a

number of clock genes in *toc1* and *pr $r5$*  backgrounds are very similar. Of the genes examined, the *toc1* mutant invariably causes a more rapid damping of mRNA rhythms than *pr $r5$* , and is largely epistatic to *pr $r5$*  when compared with the *toc1 pr $r5$*  double mutant (Ito *et al*, 2008). There is a slight degree of additivity apparent, but these results are consistent with TOC1 as the primary effector and PRR5 as an enhancer of TOC1 activity. Similarly, in the *PRR5* and *TOC1* overexpressors morning genes such as *LHY* and *CCA1* both damp to low expression, whereas expression of *PRR9* and *GI* become undetectable very quickly in constant light (LL) (Makino *et al*, 2002; Sato *et al*, 2002). The similarity of these molecular phenotypes, together with data presented here, is consistent with the idea that PRR5 promotes TOC1 function, at least in part, by enhancing nuclear accumulation and subnuclear localization.

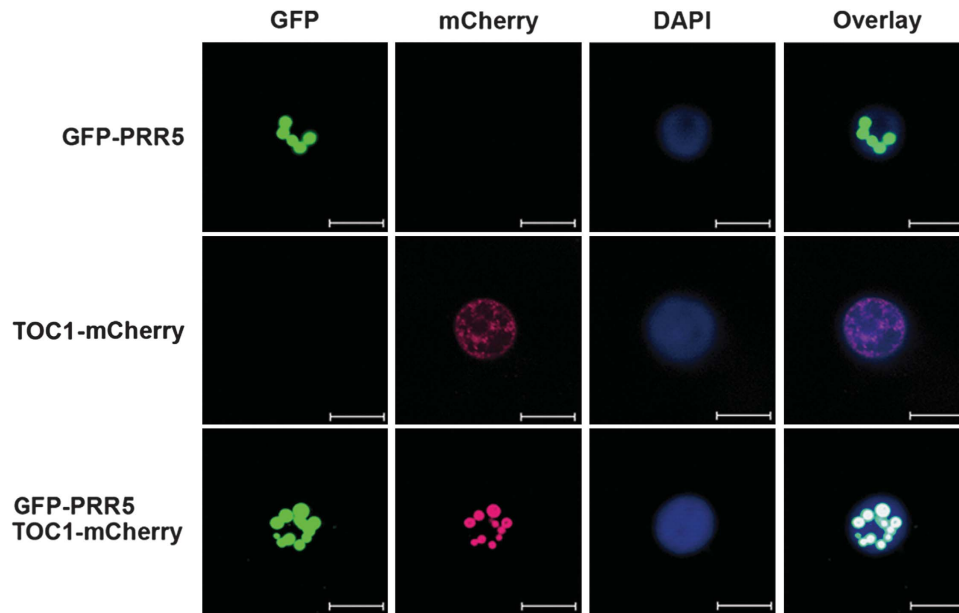
Overexpression of the PRR5 receiver domain (PRR5OX NT) causes a long period in Arabidopsis, very different from the strongly damped of rhythms of overexpressed full-length PRR5 (PRR5OX FL) (Matsushika *et al*, 2007). These data now can be re-interpreted in view of the altered nuclear localization of TOC1 in these two backgrounds (Supplementary Figure S5). The higher accumulation of cytosolic TOC1 in the PRR5OX NT line likely arises from a competitive inhibition of the TOC1-ZTL interaction by PRR5, resulting in an accumulation of TOC1, and probably also endogenous PRR5. Together, the increased level of both may account for the longer period in PRR5OX NT plants (Matsushika *et al*, 2007). At the same time, the much higher levels of nuclear TOC1 in the PRR5OX FL (Supplementary Figure S4) can explain the damped rhythms reported by Matsushika *et al* (2007) and alterations in clock gene expressions (see above), which are similar to those reported when TOC1 levels are strongly elevated (Makino *et al*, 2002; Mas *et al*, 2003a). These results are consistent with the notion that the PRR5 overexpression phenotypes arise from effects on TOC1 nucleocytoplasmic partitioning. In addition, the above findings, along with recent results that identify TOC1 in association with the *CCA1* and *ABAR1* promoters, support the notion that a nuclear population of TOC1 is necessary and a limiting factor in the control of circadian period (Legnaioli *et al*, 2009; Pruneda-Paz *et al*, 2009).

Further support for this idea comes from the re-positioning of TOC1 subnuclear localization to the larger PRR5-type inclusions when the two are expressed together. The specific function of these foci remains unclear, but it is plausible and testable that PRR5 may facilitate TOC1 binding to the E-box of the *CCA1* and *ABAR1* promoters. As neither protein bears a recognizable DNA-binding domain, it is likely that they are part of a larger multi-protein complex at these promoters. However, as the period of the *pr $r5$  toc1* double mutant is considerably shorter than the *toc1* mutant alone (Fujiwara *et al*, 2008; Ito *et al*, 2008), it is likely that PRR5 has an additional function in the clock separate from its effects on TOC1.

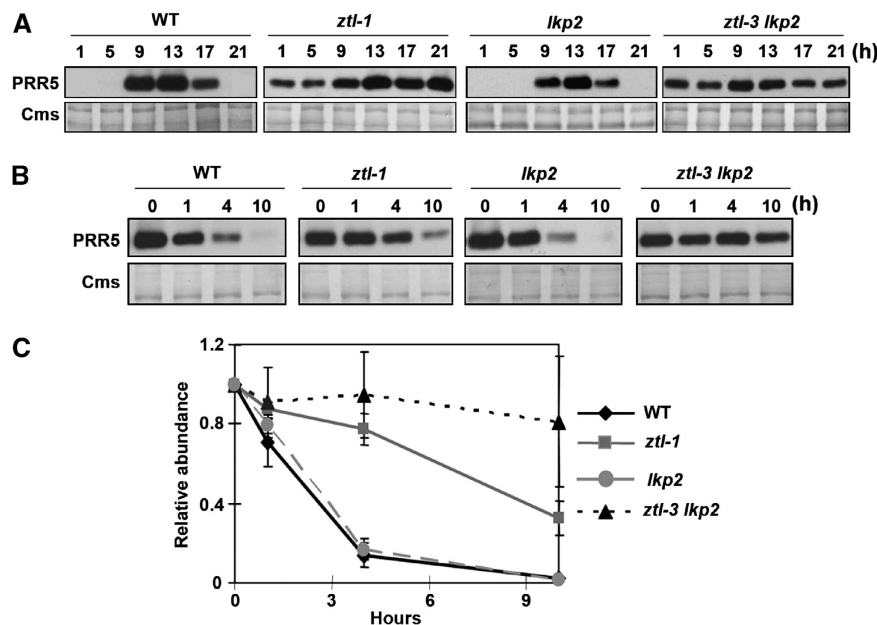
#### Complex dynamics: PRR5, TOC1, PRR3, ZTL and LKP2

The PRR5-TOC1 dimerization domain maps to the same N-terminal receiver domain that facilitates the TOC1-ZTL interaction. The *toc1-5* and *toc1-8* mutations that diminish the TOC1-ZTL interaction similarly reduce TOC1-PRR5 binding. These results imply a complex dynamic between TOC1,





**Figure 8** PRR5 and TOC1 co-localize in the nucleus. 35S::TOC1-mCherry and 35S::GFP-PRR5 were individually or co-expressed in *N. bentamiana* leaves as indicated. Signals from GFP, mCherry, 4',6-diamidino-2-phenylindole (DAPI), and the merged signals (overlay) are shown. Bars = 10  $\mu$ m.



**Figure 9** LKP2 contributes to PRR5 turnover. (A) Diurnal oscillation of PRR5 is further diminished in the *ztl lkp2* background. Time course of PRR5GFP protein levels in 10-day-old plants grown under 12/12L/D cycles probed with anti-GFP antibodies. Coomassie-stained regions (Cms) shown as loading controls. Blots are representative of more than three trials. (B) PRR5 stability regulated by both ZTL and LKP2. Plant growth and tissue processing as in (A) with plants transferred to continuous light at ZT0 and treated with 100  $\mu$ M cyclohexamide (CHX) after 19 h. Plants were harvested at 0, 1, 4 and 10 h after adding CHX. PRR5GFP protein was detected by anti-GFP antibodies. (C) Quantitation relative to Coomassie-stained regions and normalized to time 0. Blots representative of three trials. Means of three trials  $\pm$  s.e.m. are shown.

PRR5 and ZTL in view of the fact that PRR5 is also a proteolytic target of ZTL (Kiba *et al*, 2007; Fujiwara *et al*, 2008). However, although PRR5 might also act as a competitive inhibitor of the ZTL–TOC1 interaction, our data indicate a more potent function as a facilitator of TOC1 nuclear import, where it would escape proteolysis by the SCF<sup>ZTL</sup>, which is undetectable in the nucleus (Kim *et al*, 2007). In this

way, PRR5 has a function similar to PRR3 in that both facilitate an increase in TOC1 accumulation. However, PRR3 achieves this as a competitive inhibitor of the cytosolic TOC1–ZTL interaction, whereas PRR5 acts primarily to enhance TOC1 nuclear accumulation. Thus, through two different mechanisms PRR3 and PRR5 contribute to post-translationally enhancing TOC1 levels.

In addition, LKP2 can now also be added to the interplay between these four proteins. Although strong ectopic expression of LKP2 can disrupt clock function (Schultz *et al*, 2001), *lkp2* and *lkp2 ztl* double mutants show no detectable effect on circadian period (Supplementary Figure S6). These findings initially suggested that normally LKP2 either acts outside the clock or that its contribution to period is minor or not easily detectable. The observed increase in PRR5 stability in the *ztl lkp2* double mutant indicates the latter case; that LKP2 does contribute to the proteolytic degradation of a target protein previously ascribed entirely to the SCF<sup>ZTL</sup> (Kiba *et al*, 2007; Fujiwara *et al*, 2008), but effects on period are subtle. This finding does not exclude the possibility that LKP2 may have effects on other proteolyses apart from its effect on PRR5.

One possible explanation for the additivity between ZTL and LKP2 is heterodimerization or multimerization among the three ZTL family members that might enhance SCF activity (Yasuhara *et al*, 2004). For example, the WD 40 class of F-box proteins contain a short conserved repeat (D domain) directly adjacent to the F-box that is essential for their function by mediating homodimerization and/or heterodimerization between closely related forms (Zhang and Koepp, 2006; Tang *et al*, 2007). The ZTL F-box protein family is of a different class, and although there is a region directly upstream of the F-box in that is highly conserved among the three, it is not known whether this functions as an interaction domain.

#### **TOC1 phosphorylation: multiple functions?**

It is likely that the phosphorylation of TOC1 is critical to its function in a number of ways. As the TOC1–PRR3 interaction is strongly enhanced by the phosphorylated form of both proteins (Fujiwara *et al*, 2008), and the TOC1–PRR3 interaction sequesters some fraction of TOC1 from degradation by SCF<sup>ZTL</sup> (Para *et al*, 2007; Fujiwara *et al*, 2008) at least one function of phosphorylation is to stabilize TOC1. At the same time, the TOC1–ZTL interaction is also enhanced by TOC1 phosphorylation (Fujiwara *et al*, 2008), typical of F-box protein–substrate interactions (Willems *et al*, 2004). Thus, TOC1 phosphorylation facilitates both its degradation and its stabilization, depending on the relative abundances of ZTL and phosphorylated PRR3 (Fujiwara *et al*, 2008).

Our results indicate that in contrast to the PRR3–TOC1 interaction, PRR5 and TOC1 oligomerize independent of their phosphorylation states. Rather, the PRR5–TOC1 interaction can facilitate a kinase activity that phosphorylates the N-terminus of TOC1, although it is not known whether that kinase activity is responsible for all TOC1 phosphorylations. Unlike the strong TOC1 mobility shifts seen when PRR5 is strongly co-expressed, we were unable to detect changes in the TOC1 mobility in the *prr5* mutant (data not shown). This suggests either that the PRR5-mediated kinase activity contributes to only a portion of TOC1 phosphorylation, and a reduced phosphorylation level is not detectable by mobility shift, or that other phosphorylation mechanisms may substitute in the absence of a PRR5-based kinase recruitment platform. It is well known that multiple, often sequential, phosphorylations resulting from the action of multiple kinases are involved in the post-translational control of clock proteins in other systems (Huang *et al*, 2007; Virshup *et al*, 2007; Gallego and Virshup, 2007; Baker *et al*, 2009; Tang *et al*, 2009).

It is possible that the marked increase in the proportion of hyperphosphorylated TOC1 late in the circadian cycle

(Fujiwara *et al*, 2008) is tied to its nuclear function, possibly related to interaction with partners as part of a transcription complex (Pruneda-Paz *et al*, 2009). Alternatively, PRR5-mediated phosphorylation may only be important in facilitating the cytoplasmic interaction of TOC1 and PRR3. The dual functions of PRR5 in enhancing both TOC1 phosphorylation and subnuclear relocalization may be causal or separable: PRR5-mediated phosphorylation may facilitate the re-positioning of nuclear TOC1 or the two processes depend on PRR5 independent of each other. Clearly, further identification of interaction partners along with the phosphorylation sites and the kinases involved will be necessary for a full understanding of the function of phosphorylation in TOC1 function.

#### **Conserved mechanism of post-translational control of nuclear import of core clock components**

The transcription–translation feedback loop that forms the backbone of all eukaryotic oscillators also relies heavily on post-translational mechanisms, and phosphorylation is one of the best characterized (Gallego and Virshup, 2007; Cha *et al*, 2008; Chiu *et al*, 2008; Diernfellner *et al*, 2009). Two well-studied heterodimerizations in the *Drosophila* clock are between timeless (TIM) and period (PER), and clock (CLK) and cycle (CYC) (Benito *et al*, 2007; Saez *et al*, 2007; Maurer *et al*, 2009). Both pairs act to control transcription, with the TIM–PER dimer repressing gene activations initiated by the binding of the CLK–CYC dimer to the E-box of numerous clock-controlled promoters, including *per* and *tim* (Benito *et al*, 2007). As well, both pairs of proteins undergo phosphorylations that can affect their turnover, nuclear localization and regulatory activity (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). In the former case, the TIM–PER interaction specifically protects PER from proteasome-dependent degradation, and facilitates PER phosphorylation. TIM-dependent enhancement of PER import is also likely, although maintenance of the interaction during passage through the nuclear pore is not clear (Meyer *et al*, 2006). Similarly, CLK and CYC proteins interact both in the cytoplasm and the nucleus, and heterodimerization of CLK with CYC promotes both the phosphorylation and nuclear import of CLK (Maurer *et al*, 2009). Thus, our findings here show that in *Arabidopsis*, PRR5 has a similar function to TOC1 as TIM does to PER and CYC to CLK. Interestingly, none of these three pairs is structurally related to each other. Taken together, these findings show that clock-regulated nuclear import through heterodimer formation is fundamental to the molecular circuitry of circadian clocks. Indeed, in the mammalian clock as well, the period2 (PER2)–cryptochrome (CRY) interaction facilitates nuclear import of CRY in mammalian cells (Miyazaki *et al*, 2001).

One notable common feature of these examples is that the transcription of one or both of the dimeric partners is clock regulated. This permits a phase-dependent accentuation of nuclear accumulation of one or both proteins, and may be the reason such a mechanism is found across such disparate circadian systems, which otherwise do not share homologous components. In the present case, PRR5 nuclear accumulation is very similar regardless of the level of TOC1 (Supplementary Figures S2 and S6), suggesting that PRR5 is the key regulatory element in this partnership. With its very strong nuclear localization tendency, it is possible that PRR5 also partners

with other clock components, including PRR3, 7 or 9, to act as a phase-specific nuclear shuttle. With the large number of still uncharacterized proteins in the Arabidopsis clock, it is likely that this type of nuclear transport mechanism will be more commonly found.

## Materials and methods

### Plant material and growth conditions

Construction of TMG (*TOC1::TOC1-YFP* minigene) in Arabidopsis (Col-0 accession) has been described earlier (Mas *et al*, 2003a,b). Generation of TMG *prp5-1* and TMG *prp3-1*, *PRR5::PRR5-GFP* in wild type, *ztl-1*, *lkp2* and *ztl-1 lkp2* is described in detail in Supplementary data. Arabidopsis seedlings were entrained under 12-h white fluorescent light ( $50\text{--}60\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ )/12 h dark cycles for 8–12 days on MS plates with 3% sucrose and 1% agar before harvesting. The tissues were harvested at the indicated time points. For transient expression experiments, 3–4-week-old *N. benthamiana* plants were infiltrated with *Agrobacterium* as described earlier (Fujiwara *et al*, 2008). Tissues were harvested on the third day at ZT12 (16 h light/8 h dark).

### Plasmids

Constructs of TAP-tagged TOC1 full-length, N-terminus (1–242) and C-terminus (243–618) have been described earlier (Fujiwara *et al*, 2008). Constructs of TAP-tagged PRR5 and its deletions, *35S::TOC1-mCherry* and TAP-tagged TOC1 point mutations are described in the Supplementary data.

### Protein extraction and immunoblot analyses

Total protein extraction was carried out as described earlier (Kim *et al*, 2007; Fujiwara *et al*, 2008). For the immunoblot analysis, proteins were size fractionated by 8% SDS–PAGE (acrylamide:bisacrylamide, 149:1) for PRRs proteins or 12% SDS–PAGE (acrylamide:bisacrylamide, 37.5:1) for histone H3 and ADK, then analysed by immunoblot. Immunoblotting was performed using 1:1000 dilution anti-PAP antibodies (Sigma-Aldrich P-1291) or primary polyclonal anti-GFP antibody (Abcam ab6556) or primary anti-histone H3 antibody (Abcam 1791) and 1:4000 polyclonal ADK primary antibodies (gift from Dr David Bisaro) followed by ECL detection using anti-rabbit IgG with horseradish peroxidase-linked whole antibody (GE healthcare NA934V). For immunoblot of primary anti-Myc antibody (Santa Cruz, sc40, 1:300 dilution), 1:1000 HRP-linked anti-mouse IgG (Sigma-Aldrich, A0198) was used as secondary antibody. Chemoluminescence reactions were performed with Supersignal West Pico Chemiluminescent Substrates from Pierce. Quantity One 4.1.1 software was adopted for calculation and quantification of protein signal intensity. In Figure 5D, the cytosolic and nuclear TOC1 signals for WT TOC1 (no PRR5 co-infiltration) were first normalized to the respective cytosolic (ADK) or nuclear (H3) loading controls. The ratios of these values were determined (cytosolic TOC1 to nuclear TOC1) and multiplied by its reciprocal to obtain a reference value of 1. This value was then used as a multiplier for remaining ratios of normalized cytosolic TOC1 to normalized nuclear TOC1 with PRR5 co-infiltrated with the different TOC1 point mutations.

### Fractionation of cytosolic and nuclear proteins

The cytosolic and nuclear protein extractions were carried out with CELLYTPN1 CelLytic PN isolation/Extraction Kit (Sigma-Aldrich) according to the manufacturer's instruction. Briefly, the tissues were ground in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  before use. The ground tissues were gently resuspended with  $1 \times$  nuclei isolation buffer supplemented with 1 mM DTT, 2 mM  $\text{Na}_3\text{VO}_4$ , 2 mM NaF and 25 mM phenylmethylsulfonyl fluoride (PMSF), and were filtered through five layers Miracloth (Calbiochem, San Diego, CA) by

centrifugation at  $4^{\circ}\text{C}$ . The pellet was resuspended with  $1 \times$  nuclei isolation buffer supplemented with 0.3% Triton X-100, 1 mM DTT, 2 mM  $\text{Na}_3\text{VO}_4$ , 2 mM NaF and 25 mM PMSF, 2.5  $\mu\text{g/ml}$  antipain, 2.5  $\mu\text{g/ml}$  chymostatin, 1  $\mu\text{g/ml}$  pepstatin, 5  $\mu\text{g/ml}$  leupeptin, 5  $\mu\text{g/ml}$  aprotinin, and 50  $\mu\text{M}$  MG132, 50  $\mu\text{M}$  MG115, 50  $\mu\text{M}$  ALLN. After incubation on ice (5 min), extracts were centrifuged at 6800 r.p.m. for 5 min. The resulting supernatant was transferred to a new tube containing SDS buffer and designated as the cytosolic fraction. The pellet was resuspended in the above buffer and centrifuged and resuspended repeatedly until the pellet appeared totally white or slightly gray. The pellet was washed with  $1 \times$  nuclei isolation buffer supplemented with 1 mM DTT, 2 mM  $\text{Na}_3\text{VO}_4$ , 2 mM NaF and 25 mM PMSF, dissolved in SDS buffer and designated as the nuclear compartment. The cytosolic and nuclear compartments were loaded onto SDS–PAGE gels in proportion to the volume used in the initial extractions and the resuspension volumes of each compartment.

### In vitro pull-down assay

*In vitro* pull-down assays were performed as described earlier (Fujiwara *et al*, 2008).

### Co-immunoprecipitation

*Agrobacteria* containing both TOC1 full length and its amino- or carboxyl-deletions by TAP tagged with GFP-PRR5 or PRR5 full length and its deletions tagged by TAP with TOC1-YFP were co-infiltrated. Tissues were harvested at ZT12 after infiltration on the third day. The co-immunoprecipitation was performed as described earlier (Fujiwara *et al*, 2008).

### mRNA expression analysis

Total RNA was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA) from tissue of 10-day-old seedlings grown on MS media. After digestion with DNase I (Amplification grade, Invitrogen, Carlsbad, CA), cDNA synthesis was performed with Oligo-dT primer and SuperscriptIII reverse transcriptase kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instruction. cDNA templates were digested with RNaseH before amplification. Gene-specific primers were used for the quantification of corresponding mRNA levels through semi-quantitative RT–PCR as described earlier (Fujiwara *et al*, 2008).

### Confocal laser scanning microscopy

*Agrobacteria* containing *35S::GFP-PRR5* or *35S::TOC1-mCherry* was singly or co-infiltrated into *N. benthamiana* plants as described earlier. Images were collected using a Zeiss LSM 510 confocal microscope with a Plan-Apochromat  $63 \times /1.4$  oil objective using the multi-track method. For GFP and mCherry confocal microscopy, GFP was imaged using 488 nm laser light and a 505–530 nm BP emission filter, and mCherry was imaged using 543 nm laser light and a 585–615 BP emission filter. For DAPI staining, a high-pressure mercury bulb and Zeiss DAPI filter set were used, and the DAPI emission was detected at 461–525 nm on ChS1.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

## Acknowledgements

We thank Dr D Bisaro for his kind gift of the ADK antibody and Dr T Imaizumi for sharing unpublished results. This work was supported by grants to DES from NSF grants MCB-0544137 and IBN-0748749 and in part by Support for Long-term Visit from the Yamada Science Foundation to SF.

## Conflict of interest

The authors declare that they have no conflict of interest.

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