Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*

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The underlying mechanism of circadian rhythmicity appears to be conserved among organisms, and is based on negative transcriptional feedback loops forming a cellular oscillator (or 'clock')[1,2]. Circadian changes in protein stability, phosphorylation and subcellular localization also contribute to the generation and maintenance of this clock[1,2]. In plants, several genes have been shown to be closely associated with the circadian system[3,4]. However, the molecular mechanisms proposed to regulate the plant clock are mostly based on regulation at the transcriptional level[3,4]. Here we provide genetic and molecular evidence for a role of ZEITLUPE (ZTL)[5–7] in the targeted degradation of TIMING OF CAB EXPRESSION 1 (TOC1)[8,9] in *Arabidopsis thaliana* (thale cress). The physical interaction of TOC1 with ZTL is abolished by the *ztl-1* mutation, resulting in constitutive levels of TOC1 protein expression. The dark-dependent degradation of TOC1 protein requires functional ZTL, and is prevented by inhibiting the proteosome pathway. Our results show that the TOC1–ZTL interaction is important in the control of TOC1 protein stability, and is probably responsible for the regulation of circadian period by the clock.

TOC1 is involved in a transcriptional feedback loop[10] to control its own expression and that of CCA1 and LHY, two Myb DNA binding proteins associated with the clock[11,12]. TOC1 has also been identified as a link between environmental information and clock outputs, with an essential function in constant darkness and in the integration of red light signals to the clock[13]. Two members of the ZTL protein family (ZTL[5–7] and LKP2[14]) have also been implicated in the circadian clockwork. Sequence similarity of their LOV (light, oxygen and voltage) domains with those of WHITE COLLAR-1 (WC-1) and the phototropins suggests they may bind flavin and function as light sensors in *Arabidopsis*[15,16]. The presence of a Kelch domain and an F-box motif implies their participation in E3 ubiquitin ligase SCF complexes[17,18]. However, no functional evidence has been reported for any member of the ZTL family in targeting specific substrates for degradation.

Our previous studies have shown that a TOC1 RNAi transgene reduces TOC1 messenger RNA levels and shortens the free-running period of circadian gene expression in continuous white light[13], whereas increased TOC1 gene dosage (using a TOC1 minigene (TMG) that expresses TOC1 under its endogenous promoter) delays the pace of the clock[13]. Conversely, lengthening of the free-running period was reported for loss-of-function and null *ztl* mutants[5,6]. We therefore explored the inverse phenotypic correlation between TOC1 and ZTL expression by conducting a genetic study in which *ztl-1* mutant plants were transformed with either the TOC1 RNAi or the TMG constructs[13]. The *ztl-1* mutant plants exhibit circadian and photomorphogenic phenotypes similar to the null *ztl-3*/*ado* mutants[6] (data not shown). Under constant white light (LL), the *ztl-1*/*TOC1* RNAi plants displayed circadian phenotypes similar to those observed in *TOC1* RNAi plants[13], with period lengths 3–4 h shorter than wild type (Fig. 1a, b). These results indicate that the *ztl-1* phenotype is only apparent in the presence of a functional TOC1, and is consistent with TOC1 acting downstream of ZTL. In *ztl-1*/TMG, *CAB2::LUC* expression displayed circadian periods longer and with higher relative amplitudes error (that is, weaker rhythmicity) than those observed in either the single *ztl-1* mutant or TMG plants (Fig. 1c, d). Similar results were obtained with *ztl-3*/TMG plants; *CAB2::LUC* expression was clearly affected, with very long circadian periods and weak rhythms (Supplementary Fig. 1a, b). These results indicate that the long-period phenotype of TMG plants was enhanced by the *ztl* mutations. These findings are consistent with the notion that ZTL might target TOC1 for degradation.

We next examined the possible interaction between TOC1 and ZTL using the yeast two-hybrid system. Our results showed high lacZ activity in yeast colonies co-expressing TOC1 and ZTL, indicating a strong interaction between both proteins. Weaker interactions were detected with the other members of the ZTL

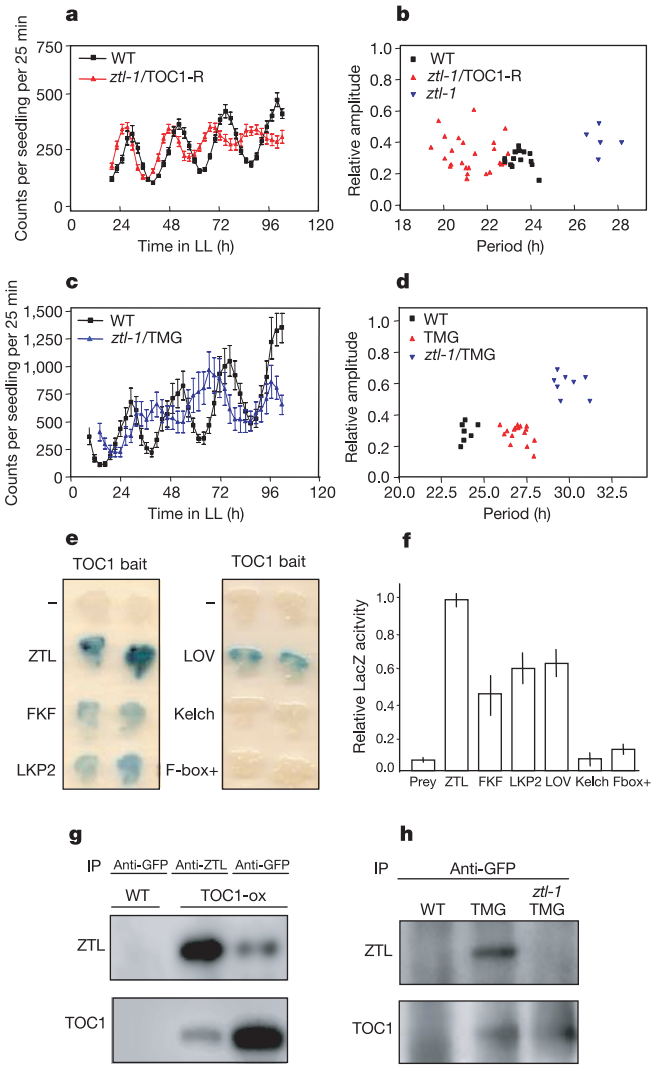

Figure 1 Genetic and molecular interaction between TOC1 and ZTL. **a**, **c**, Bioluminescence analysis of *CAB2::LUC* expression in LL. **b**, **d**, Period estimates of *CAB2::LUC* expression analysed as described[21,22]. Seedlings were grown in LD cycles before transferring to LL. The experiments were repeated three times. WT, wild-type; TOC1-R, TOC1 RNAi; TMG, TOC1 minigene. **e**, TOC1 interaction in yeast with the ZTL family of proteins, and with the indicated domains of ZTL. −, empty prey vector; F-box +, F-box and Kelch domains. **f**, β-galactosidase activities in yeast. Values are the average of three colonies and the error bars represent standard deviations. **g**, **h**, Western blot analysis of immunoprecipitation with anti-GFP antibody and detection with antibody to ZTL[23] (see Methods). Plants were grown in LD cycles and processed at ZT-14.

family, FKF1 and LKP2 (Fig. 1e, f). Using different domains of ZTL, we found that the LOV domain was sufficient for the interaction with TOC1, whereas the Kelch or the F box + Kelch domains failed to interact in yeast (Fig. 1e, f). PAS domains are conserved among circadian systems, and have been identified as protein–protein interaction domains in a number of sensing proteins[19]. The PAS domains mediate interaction between WC-1 and WC-2, and such interactions are essential for circadian function in *Neurospora*[20]. To verify the physical interaction between TOC1 and ZTL *in vivo*, we performed co-immunoprecipitation assays with TOC1–yellow fluorescent protein (YFP) overexpressing plants[13] (TOC1-ox) which express TOC1–YFP under the strong 35S promoter. The YFP was used as a tag owing to the lack of a specific TOC1 antibody. Immunoprecipitation with anti-GFP antibody and subsequent detection of ZTL (Fig. 1g) showed a protein of molecular mass approximately 66 kDa coincident with the size of the protein immunoprecipitated with the ZTL antibody (lane anti-ZTL, Fig. 1g). Detection using the GFP antibody revealed a protein band with a molecular mass of 110 kDa that coincided with the predicted molecular size of the TOC1–YFP fusion protein. The physical interaction between TOC1 and ZTL was also observed in TMG plants (Fig. 1h). The TMG plants express TOC1–YFP under the TOC1 native promoter. Interestingly, we were unable to immunoprecipitate ZTL when *ztl-1*/TMG plants were assayed (Fig. 1h), although mutant *ztl-1* protein is expressed near wild-type levels (data not shown). These results indicate that the physical interaction between TOC1 and ZTL is abolished by the *ztl-1* mutation. Our yeast two-hybrid experiments show that the LOV domain is involved in the interaction of ZTL with TOC1. The *ztl-1* mutation in the Kelch domain[5] may affect the conserved tertiary structure of the protein and disrupt the interaction with TOC1. The lack of interaction between ZTL and TOC1 correlates with, and most probably accounts for, the circadian phenotypes observed in *ztl-1* and in *ztl-1*/TMG plants.

The genetic and molecular interaction between TOC1 and ZTL suggested that TOC1 protein levels might be regulated by ZTL. Time-course analysis in TMG seedlings grown in 12 h:12 h light: dark (LD) cycles revealed a rhythmically expressed TOC1 protein that closely followed the rhythmic pattern of mRNA expression (Fig. 2a, b). In *ztl-1*/TMG plants, the rhythmicity of TOC1 protein abundance was disrupted, with high levels at all time points assayed (compare Fig. 2a and c). Notably, the effects of the *ztl-1* mutation were specific for TOC1 protein and did not alter the overall rhythm of TOC1 mRNA expression (Fig. 2d). Together, these results support the notion that a post-translational mechanism, involving a functional ZTL, regulates the changes in TOC1 protein levels.

TMG seedlings displayed a long-period phenotype of *CAB2::LUC* expression in LL (Fig. 1d). Compared to LD cycles, TOC1 rhythmic accumulation in LL was delayed, with reduced amplitude and higher protein levels at the expected trough in the subjective day (Fig. 3a, b). In *ztl-1*/TMG (Fig. 3c) and *ztl-3*/TMG (Supplementary Fig. 1c), TOC1 protein expression was consistently high, with no rhythmic variations throughout the sampling interval. Interestingly, analysis of different transgenic lines revealed that the severity of the long-period circadian phenotypes correlated with higher levels of TOC1 protein (Fig. 3d). In *ztl-1*/TMG lines expressing very high levels of TOC1 protein, *CAB2::LUC* was arrhythmic and no circadian period (in the range of 15 to 45 h) was obtained after fast-Fourier transform-nonlinear least squares (FFT-NLLS) analysis[21,22]. These results indicate that light/dark transitions and a functional ZTL are important in the control of TOC1 protein abundance.

We next made use of a cell-free assay to examine TOC1 *in vitro*

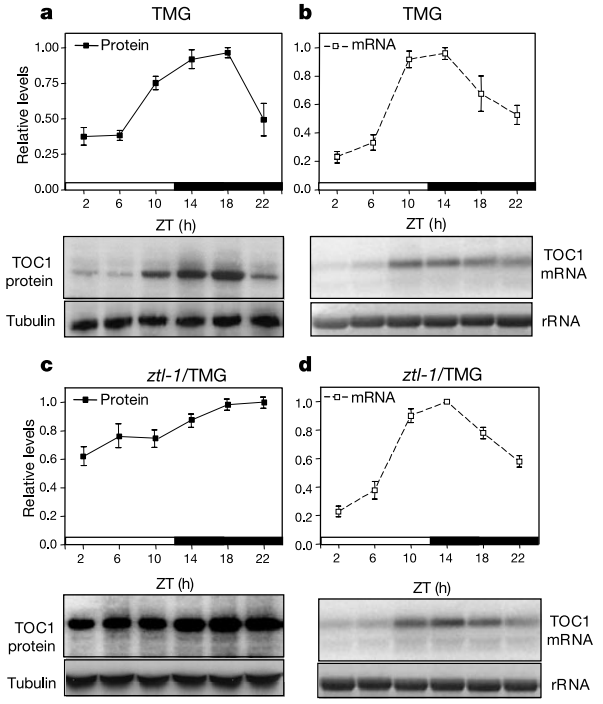

Figure 2 TOC1 protein and RNA expression in LD cycles. **a**, **c**, Immunodetection of TOC1 protein using anti-GFP antibody (see Methods) in TMG (**a**) and *ztl-1*/TMG (**c**) seedlings. Samples were collected at the indicated ZT (ZT-0 is light-on). TOC1 immunoreactivity was quantified relative to the maximum value for each blot after normalization to the tubulin. **b**, **d**, TOC1 mRNA in TMG (**b**) and *ztl-1*/TMG (**d**) plants analysed by northern blot. Samples were processed as described in Methods. rRNA was used as control. Data are mean of at least two independent experiments. White and dark bars represent light and dark period, respectively.

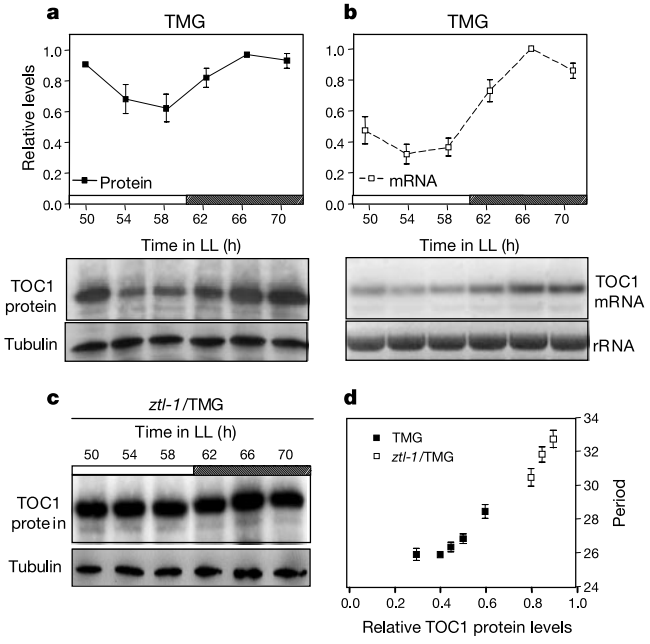

Figure 3 TOC1 RNA and protein expression in LL. **a**, **c**, Immunodetection of TOC1 protein using anti-GFP antibody (see Methods) in TMG (**a**) and *ztl-1*/TMG (**c**) seedlings grown in LD cycles before transferring to LL. Samples were collected at the indicated ZT (ZT-0 is light-on) and processed at the indicated time after the last LD light-off. **b**, TOC1 mRNA in TMG seedlings analysed by northern blot. Samples were processed as described in Methods. rRNA was used as control. Data are mean of at least two independent experiments. White bars represent light period; hatched bars indicate subjective night. **d**, Correlation between circadian period of CAB2::LUC expression and TOC1 protein levels in TMG and *ztl-1*/TMG lines. Seedlings were grown in LD cycles before transferring to LL. Period estimates were analysed as described[21,22].

degradation at the time of maximum (zeitgeber time, ZT-19) and minimum (ZT-7) TOC1 protein expression. In TMG extracts assayed at ZT-19, TOC1 protein levels were significantly reduced within 30 min (Fig. 4a). This degradation was prevented by using proteasome-specific inhibitors (Fig. 4a and Supplementary Fig. 2). The protease inhibitors aprotinin and leupeptin had little effect in preventing TOC1 degradation (Supplementary Fig. 2). Interestingly, in *ztl-1*/TMG extracts, TOC1 protein levels showed little change with no evident effect on protein abundance after adding proteosome (MG115 and MG132) or protease (aprotinin, leupeptin) inhibitors (Fig. 4a and Supplementary Fig. 2). When samples were processed at ZT-7, no degradation was observed in either TMG or *ztl-1*/TMG plants (Fig. 4b). The use of cycloheximide, a protein-synthesis inhibitor, revealed that the rate of TOC1 protein decrease was significantly higher at ZT-19 than at ZT-7 (data not shown), confirming that protein degradation rather than RNA translation is primarily responsible for the differential abundance of TOC1 protein. These results suggest that the rapid decrease in TOC1 protein levels from ZT-19 (Fig. 2a) occurs by degradation via the proteosome pathway and requires a functional ZTL protein.

To investigate a possible light- or dark-dependent TOC1 degradation, protein levels were analysed on transfer to constant darkness (DD, Fig. 5a) or constant light (LL, Fig. 5b). Extended darkness at ZT-24 into the first subjective day caused a clear reduction in TOC1 levels as compared to those observed in LD-grown seedlings (Fig. 5a). In contrast, when the light period was extended into the first subjective night, high levels of the TOC1 protein were maintained at all circadian time (CT) points analysed (Fig. 5b). Upon transfer to LL (not shown) or DD conditions (Fig. 5c, d), no evident changes in TOC1 protein levels were observed in *ztl-1*/TMG or *ztl-3*/TMG seedlings as compared with corresponding levels in LD cycles. These findings indicate that independent of its circadian regulation, TOC1 is preferentially degraded in the dark and ZTL plays a major role in that degradation. ZTL protein levels cycle with peak expression at approximately ZT-10 to ZT-12[23]. The similar phase of TOC1 and ZTL expression suggests that additional factors might be involved in the regulation of TOC1. It is possible that light signals modulate changes in the binding affinity of the LOV domain and/or in the target degradation activity of ZTL. In LD, the light period appears to delay for some hours into the dark period the ability of ZTL to effect TOC1 degradation. In LL, only the rapid degradation mediated by ZTL is affected, so that TOC1 protein oscillates albeit with a lower amplitude. This situation is analogous to that of TIM in *Drosophila*, where TIM is degraded in the light by a CRY-dependent mechanism, but the protein still cycles in DD although with reduced amplitude[2]. We propose that both the circadian control of TOC1 mRNA expression and the ZTL-dependent TOC1 protein degradation act together to establish the rhythmic changes in TOC1 levels.

The alternation of day and night cycles has shaped the circadian clockwork throughout evolution. In plants, transmission of light signals to the core of the oscillator relies on the overlapping activities of different photoreceptors[24,25]. The mechanisms and signalling pathways from this photoreception to the circadian outputs are just beginning to be understood. TOC1 expression is controlled by both transcriptional and post-translational mechanisms. In the former, the morning-expressed CCA1/LHY proteins bind to the evening-element sequence present in the TOC1 promoter, repressing its expression at dawn[10]. Decreasing CCA1/LHY levels relieve that repression, resulting in rising levels of TOC1 mRNA during the day. A second regulation at the post-translational level occurs with the activation of ZTL that targets TOC1 for degradation. Both levels of regulation ensure a narrow peak of TOC1 expression that might be critical in maintaining the robustness and stability of the clock. Evidence showing the involvement of the proteasome pathway at the core of the oscillator in the mammalian system[26] and in *Drosophila*[27,28] suggests that tightly regulated protein degradation by the proteosome might be a

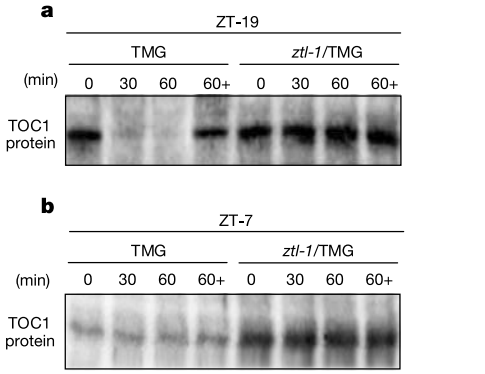

Figure 4 Cell-free TOC1 protein degradation assay. Immunodetection of TOC1 protein in TMG and *ztl-1*/TMG seedlings grown in LD cycles. Samples were collected at ZT-19 (**a**) and ZT-7 (**b**). Protein extracts were incubated in an *in vitro* degradation buffer (see Methods) with or without inhibitors for the indicated time (min). Approximately 80 μg (TMG) and 40 μg (*ztl-1*/TMG) of total proteins were loaded onto gels. 60+ indicates extracts treated with 50 μM MG132.

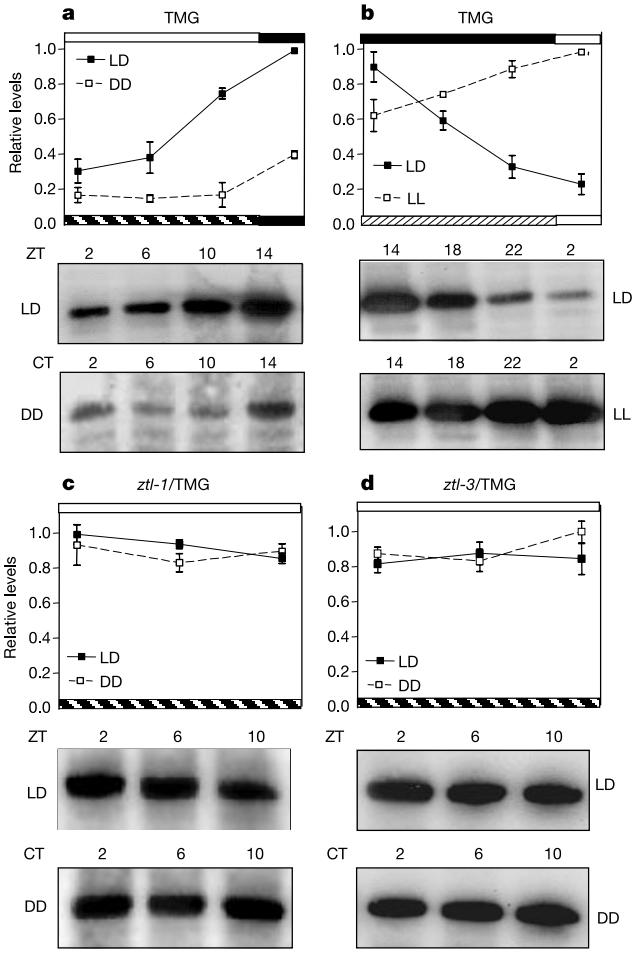

Figure 5 Dark-dependent TOC1 protein degradation. Comparison of TOC1 protein in seedlings grown in LD cycles and on transfer to DD (**a**, **c**, **d**) or LL (**b**). Protein levels were analysed at ZT-2, ZT-6, ZT-10, ZT-14 or their corresponding circadian times (CT). Approximately 80 μg of total protein was loaded in TMG extracts (**a** and **b**) whereas 50 μg was loaded in *ztl-1*/TMG (**c**) and *ztl-3*/TMG (**d**). White and dark bars represent light and dark period, respectively. Hatched bar in **a**, **c** and **d** indicates subjective day; hatched bar in **b** indicates subjective night.

conserved aspect in the regulation of the eukaryotic circadian clocks. □

Methods

Plant growth conditions and bioluminescence analysis

Arabidopsis plants transformed by *Agrobacterium*-mediated DNA transfer were selected in MS-agar, 3% sucrose plates. Seedlings were grown under 12 h:12 h light:dark (LD) cycles with 60 μmol m^{-2} s^{-1} of cool white fluorescent light at 22 °C. Analysis of bioluminescence rhythms under continuous white light was performed as previously described[29,30]. FFT-NLLS analysis was used to estimate period lengths of individual traces[21,22]. *TOC1* minigene (TMG–YFP) plants express the *TOC1* genomic sequence (from 2,354 base pairs (bp) upstream of the ATG to 410 bp downstream of the stop codon) fused to the YFP. The TOC1 RNAi and TOC1-ox constructs were previously described[13].

Yeast two-hybrid assays

The coding region of *TOC1* complementary DNA was cloned into the pBMT116 vector (Clontech) as a translational fusion to the LexA DNA binding protein and used as a bait for the yeast two-hybrid studies. ZTL full-length and the domains LOV/PAS (residues 1–155), Kelch (residues 290–609) and F-box + Kelch (residues 200–609) were cloned into the pACT2 vector (Clontech) and used as prey. The yeast transformation and enzymatic activity on plates and liquid media was performed following manufacturer recommendations (Clontech Yeast Protocols Handbook).

Coimmunoprecipitation experiments

Wild-type, TOC1-ox, TMG and *ztl-1*/TMG plants were grown in 12 h:12 h LD cycles for 15 days and processed for co-immunoprecipitation at ZT-14. TOC1–YFP immunoprecipitation was performed at 4 °C for 4 h using anti-GFP antibody (Molecular Probes) in a buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl; 0.5% NP-40, 1 mM EDTA, 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, 5 μg ml^{-1} antipain, 1 μg ml^{-1} pepstatin, 5 μg ml^{-1} chymostatin and 50 μM MG132. Protein A/agarose beads were used to precipitate the immunoprotein complexes. ZTL detection was performed using anti-ZTL antibody[23].

Western and northern blots

Proteins were extracted from 0.2 g of tissue and ground in 0.5 ml of grinding buffer (50 mM HEPES–KOH pH 7.5, 100 mM KCl, 10% glycerol, 0.1% Triton X-100, 2 mM DTT, 5 mM EDTA, 0.5% polyvinylpolypyrrolidone (PVPP), 50 μM MG132 and Protease Inhibitor Cocktail (Roche)). Protein concentration was determined using the Bradford method (protein assay, Bio-Rad) and approximately 70–80 μg of total protein was loaded per lane. Homogeneous protein transference to nitrocellulose membranes was confirmed by red Ponceau staining. Anti-GFP (Molecular Probes) and anti-ZTL antibodies[23] were used to detect TOC1–YFP and ZTL protein, respectively. As control, membranes were incubated with anti-tubulin antibody (Sigma). For northern blot analysis, RNA from 12-day-old seedlings was isolated using the RNeasy kit (Qiagen) and separated on 1.2% agarose/formaldehyde gels as previously described[13]. Northern blots were performed using radioactively labelled full-length YFP DNA fragment as probe. Quantification of the blots and analysis of the images was performed on a Phosphorimager and using ImageQuant software (Molecular Dynamics) and Scion Image software (Scion Corporation).

Cell-free degradation assays

Seedlings were ground in liquid nitrogen, resuspended in buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT and 5 mM ATP) and clarified by centrifugation. Equal amounts of extracts were transferred to individual tubes and incubated at room temperature with or without inhibitors as indicated. Reactions were stopped by adding protein gel-loading buffer.

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Proteomic characterization of the human centrosome by protein correlation profiling

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The centrosome is the major microtubule-organizing centre of animal cells and through its influence on the cytoskeleton is involved in cell shape, polarity and motility. It also has a crucial function in cell division because it determines the poles of the mitotic spindle that segregates duplicated chromosomes between