

Functional Interaction between Phosducin-like Protein 2 and Cytosolic Chaperonin Is Essential for Cytoskeletal Protein Function and Cell Cycle Progression

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The Chaperonin Containing *Tcp1* (CCT) maintains cellular protein folding homeostasis in the eukaryotic cytosol by assisting the biogenesis of many proteins, including actins, tubulins, and regulators of the cell cycle. Here, we demonstrate that the essential and conserved eukaryotic phosducin-like protein 2 (PhLP2/PLP2) physically interacts with CCT and modulates its folding activity. Consistent with this functional interaction, temperature-sensitive alleles of *Saccharomyces cerevisiae* *PLP2* exhibit cytoskeletal and cell cycle defects. We uncovered several high-copy suppressors of the *plp2* alleles, all of which are associated with G1/S cell cycle progression but which do not appreciably affect cytoskeletal protein function or fully rescue the growth defects. Our data support a model in which Plp2p modulates the biogenesis of several CCT substrates relating to cell cycle and cytoskeletal function, which together contribute to the essential function of *PLP2*.

INTRODUCTION

Phosducin-like proteins (PhLPs) are a conserved family of small thioredoxin-like proteins that were originally identified as modulators of heterotrimeric G protein signaling in the retina (Schroder and Lohse, 1996). Subsequently, they were shown to have roles in G protein signaling in other cell types as well as having G protein-independent functions (Flanary *et al.*, 2000; Blaauw *et al.*, 2003). One of the other functions of PhLPs is the regulation of the eukaryotic protein-folding machine known as Chaperonin Containing *Tcp1* (CCT; also called *TRiC*-containing Ring Complex [TRiC]) (McLaughlin *et al.*, 2002; Martín-Benito *et al.*, 2004; Lukov *et al.*, 2005, 2006; Stirling *et al.*, 2006).

Chaperonins are oligomeric molecular chaperones that bind nonnative proteins and facilitate their transition to the native state (Hartl and Hayer-Hartl, 2002). These barrel-shaped molecular machines undergo large conformational changes to encapsulate and release bound substrate proteins during their ATP-dependent folding cycle (Spiess *et al.*, 2004). In the eukaryotic cytosol, the chaperonin CCT ensures the correct folding and assembly of a variety of proteins. The best-characterized substrates of CCT are actins and tubulins,

although several recent studies have extended the number of known CCT substrates, including several cell cycle proteins (Thulasiraman *et al.*, 1999; Camasses *et al.*, 2003; Siegers *et al.*, 2003; for review, see Spiess *et al.*, 2004). CCT cooperates with another chaperone called Prefoldin (PFD) in the folding of actins and tubulins (Geissler *et al.*, 1998; Vainberg *et al.*, 1998). PFD uses six long coiled-coil “tentacles” to stabilize substrate proteins at the opening of its jellyfish-shaped cavity before their delivery to the open CCT cavity (Vainberg *et al.*, 1998; Siegers *et al.*, 1999; Siegert *et al.*, 2000; Lundin *et al.*, 2004). Together, PFD and CCT compose a folding pathway for cytoskeletal proteins, which, along with PhLPs, control the folding of actin and tubulin (Siegers *et al.*, 1999; Lacefield and Solomon, 2003; Stirling *et al.*, 2006).

PhLPs can be subdivided into three homologous families, called PhLP1, PhLP2, and PhLP3, that share an N-terminal helical domain, a central thioredoxin-like fold, and a charged C-terminal extension (Blaauw *et al.*, 2003). PhLP1 proteins are the best characterized and function both in G protein signaling and the regulation of CCT (Schroder and Lohse, 1996; McLaughlin *et al.*, 2002; Martín-Benito *et al.*, 2004; Lukov *et al.*, 2005, 2006). PhLP3 proteins act as modulators of CCT and play roles in actin and tubulin biogenesis in yeast and microtubule function in *Caenorhabditis elegans* (Lacefield and Solomon, 2003; Ogawa *et al.*, 2004; Stirling *et al.*, 2006). PhLP2 proteins are the least characterized PhLP isoforms, but they are essential in *Dictyostelium discoideum* and possibly also *Saccharomyces cerevisiae* (Flanary *et al.*, 2000; Blaauw *et al.*, 2003). PhLP2 may act as a regulator of apoptosis in mammalian cells, but the significance and mechanism of this function is unclear (Wilkinson *et al.*, 2004).

Yeast possess homologues of PhLP3 and PhLP2, which are encoded by *PLP1* and *PLP2*, respectively (Blaauw *et al.*, 2003). *PLP1/PhLP3* has been implicated in CCT-mediated

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Abbreviations used: CCT, Chaperonin Containing *Tcp1*; DAPI, 4',6-diamidino-2-phenylindole; FACS, fluorescence activated cell sorting; PFD, prefoldin; PhLP, phosducin-like protein.

folding of actin and tubulin, and it is thought to work at the level of CCT to regulate the ATP-hydrolysis-dependent turnover of CCT-substrate complexes (Lacefield and Solomon, 2003; Stirling *et al.*, 2006). Although *PLP1* has an important function in regulating actin and tubulin folding, *plp1Δ* cells have no apparent growth defects. Indeed, most *plp1Δ* phenotypes are only detected in strains that also lack a functional PFD complex (Lacefield and Solomon, 2003; Stirling *et al.*, 2006). Conversely, *PLP2* is seemingly essential for growth, but its cellular function is thus far unknown (Flanary *et al.*, 2000; Lopez *et al.*, 2003).

To elucidate the essential function of *PLP2*, we undertook studies of yeast and human PhLP2 homologues. Yeast *PLP2* binds to CCT as suggested by proteome-wide studies (Gavin *et al.*, 2006). In this regard, we show that *in vitro*, human PhLP2A inhibits actin folding and forms ternary complexes with CCT and actin. We also show in yeast that temperature-sensitive (*ts*) alleles of *PLP2* are defective in CCT-regulated processes such as actin and tubulin function, and cell cycle progression. *plp2-ts* alleles do not, however, exhibit altered sensitivity or resistance to α -factor, supporting the notion that regulating G protein signaling is not part of the essential function of Plp2p, as might be the case for certain mammalian phosphatases (Schroder and Lohse, 1996). Finally, we identify high-copy suppressors of *plp2-ts* alleles that indicate an essential function for *PLP2* during G1/S phase cell cycle progression. Our data support a model in which Plp2p modulates the biogenesis of several CCT substrates, which together contribute to the essential function of *PLP2*.

MATERIALS AND METHODS

Purification of GST-PhLP2A, and CCT

Human PhLP2A (accession no. AF110511) and PhLP3 (accession no. NM_005783) were cloned into the glutathione S-transferase (GST) fusion vector pGEX-6p, and expressed in BL21[DE3]pLysS as described previously (Stirling *et al.*, 2006). GST-fusion proteins were purified with glutathione-Sepharose 4B as per manufacturer's instructions (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). GST alone, used as a control, was expressed and purified exactly as for GST-PhLP2A. CCT was purified from rabbit reticulocyte lysate as described previously (Gao *et al.*, 1992; Melki *et al.*, 1997).

In Vitro Translation and Folding Assays

Actin and tubulin were translated in the T7 quick coupled translation reaction (Promega, Madison, WI) according to manufacturer's instructions. Actin was also translated in *Escherichia coli* lysate by using the EcoPro system (Novagen, Madison, WI). Recombinant GST or GST-PhLP2A was added at ~100 times excess to endogenous levels of CCT before translation (Cowan, 1998). At various time points, aliquots of the reaction were frozen in native gel running buffer and then thawed on ice and analyzed by native gel electrophoresis (Leroux, 2000). GST-pull-downs were performed with glutathione-Sepharose 4B beads according to manufacturer's instructions (GE Healthcare). For GST pull-downs from reticulocyte lysate and *E. coli* lysate translation reactions (Figure 6, B and C), the translation reaction (with or without exogenous CCT) was diluted 100 times into phosphate-buffered saline containing GST or GST-PhLP2A before precipitating the GST-fusions as described above. Mammalian Tcp1 was detected using a rat monoclonal antibody directed against a C-terminal fragment of mouse Tcp1 α (Assay Designs, Ann Arbor, MI).

Yeast Strains, Media, and Growth Assays

For yeast strains and plasmids, see Supplemental Tables S1 and S2. Yeast were grown on YEPD, or synthetic complete media as required (Adams *et al.*, 1997). 5'-Fluoroarotic acid plates were also made as described previously (Adams *et al.*, 1997). For temperature-sensitive growth assays, log-phase cells were serially diluted 10-fold, spotted on the appropriate media, and cultured at the temperatures indicated. To assess the reversibility of the *ts*-alleles, the indicated strains were cultured at the restrictive temperature for the times shown, and plating efficiency was observed directly under the microdissection microscope as described previously (Amberg *et al.*, 2005).

Plp2p, CCT Coimmunoprecipitation

For immunoprecipitation-immunoblotting experiments, a strain bearing integrated *myc*-tagged *PLP2* and hemagglutinin (HA)-tagged CCT2 (AHY994),

or control strains lacking the tagged *PLP2*, or both tagged proteins, were grown to mid-log phase. Extracts were prepared as described previously (Zachariae *et al.*, 1998) from 2×10^6 cells in 0.4 ml of buffer B70 (number indicates millimolar potassium acetate). Cleared extracts (0.35 ml or 4 mg) were incubated for 60 min with antibodies, which were captured with 40 μ l of protein A-Sepharose for 60 min as described previously (Camasses *et al.*, 2003). Beads were washed with the buffers B70 plus bovine serum albumin (1 mg/ml), B70, B150, B200, and B70 followed by immunoblot analysis.

Drug and Mating Factor Sensitivity Assays

Latrunculin B (LatB; Sigma-Aldrich, St. Louis, MO) sensitivity assays were performed as described previously (Ayscough *et al.*, 1997). Benomyl or mating factor sensitivity assays were performed essentially as described for LatB. Briefly, sterile filter paper disks were soaked with the drug or pheromone at the concentration indicated and placed on a soft agar overlay containing a particular yeast strain. The radius of clearance around the disk was measured after 2 d of growth at 30°C. Congenic control strains carrying wild-type copies of the genes under examination were included for each experiment.

Generation of Temperature-sensitive Alleles of PLP2

A *plp2* null strain was made by *HIS3* gene insertion into the *PLP2* coding region. To generate the *plp2-ts* mutants, the *PLP2* gene (including 1 kb of the upstream promoter and 120 base pairs after the stop codon) was cloned into the pRS405 Leu vector. *plp2* mutations were generated within the coding region by error-prone polymerase chain reaction (PCR) (0.3 mM MnCl₂, 0.5 mM dCTP, 0.5 mM dTTP, 0.1 mM dGTP, 0.1 mM dATP, and wild-type *Taq* polymerase for 28 cycles) and the wild-type *PLP2* opening reading frame was replaced via gap-repair. Mutagenized plasmids able to rescue the *plp2* null strain were then screened for temperature sensitivity. Plasmids from temperature-sensitive transformants were sequenced, retransformed, and integrated into AHY955 with a *LEU2* marker before counterselection of the *URA3*-marked *PLP2* plasmid with 5'-fluoroarotic acid (Supplemental Table S1). The *plp2-1* strain contained the mutations: R24/S24, Q91/L91, Q149/R149, V191/A191, L207/Q207, and S259/T259. The *plp2-2* strain contained the mutations: Q91/R91, D132/V132, F164/S164, N175/D175, and E183/G183.

Microscopy

Yeast strains were grown to mid-log phase in YPD or SC media before direct imaging or fixing and staining. Tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma-Aldrich) and 4',6-diamidino-2-phenylindole (DAPI) were used, according to manufacturer's instructions, to stain formaldehyde fixed yeast cells as described previously (Adams *et al.*, 1997). Calcofluor white staining was carried out as described in Amberg *et al.* (2005). Actin immunofluorescence was performed as described by Adams *et al.* (1997). For cell sizing, at least 100 cell diameters were measured in Openlab 5.0.2 (Improvision, Coventry, United Kingdom) for each strain perpendicular to the mother-bud axis at the largest point. Cellular defects were statistically validated using an independent variable *t* test or chi-square analysis, as appropriate.

High-Copy Suppression Screen

A YEp24-based library of *URA3*-marked plasmids containing ~10-kb fragments of *S. cerevisiae* genomic DNA (Carlson and Botstein, 1982) was transformed into *plp2-1* and *plp2-2* cells. Half of the cells were plated at a permissive temperature (29°C) to calculate transformation efficiency and half were plated at the nonpermissive temperature (37°C) to identify suppressing clones. Colonies which grew at 37°C after 48 h were cultivated and the plasmid DNA was isolated and screened by PCR to determine whether it contained a copy of *PLP2*. Plasmid inserts lacking *PLP2* were sequenced at both ends and genes therein were identified using the yeast genome database (www.yeastgenome.org). Plasmids bearing some of the individual genes identified were a kind gift from Dr. Joaquín Ariño (Universidad Autònoma de Barcelona, Barcelona, Spain) (Muñoz *et al.*, 2003).

Cell Synchronization

For fluorescence activated cell sorting (FACS) analysis, cells were synchronized as small G1 cells by centrifugal elutriation as described by Schwob and Nasmyth (1993). After elutriation, samples were incubated at 37°C, and, at 15-min intervals, stained for DNA content with propidium iodide. All samples were analyzed on a FACScan (BD Biosciences, San Jose, CA) as described previously (Epstein and Cross, 1992). For rebudding analyses, cells were synchronized with α -factor according to the low pH method (Amberg *et al.*, 2005). Synchronized cells were shifted to 37°C for 1 h before release into 37°C media. Time points were taken after release and the budding index of the culture scored.

RESULTS

PLP2 Is an Essential CCT-binding Protein

Previously, we found that Plp1p, the yeast PhLP3 homologue, cooperates with CCT and PFD to modulate actin and

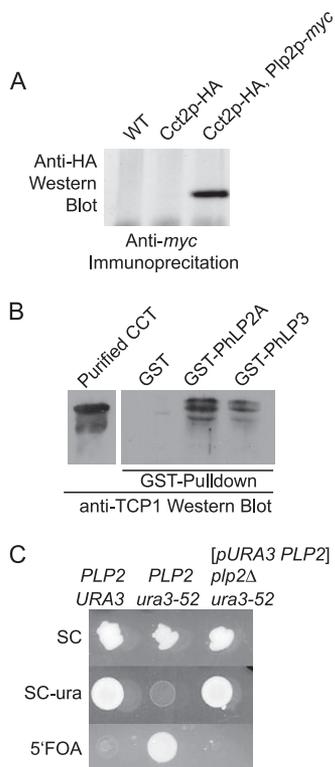


Figure 1. PhLP2/PLP2 is an essential CCT-binding protein. (A) CCT2-HA is immunoprecipitated using an anti-*myc* antibody from *S. cerevisiae* cells coexpressing CCT-HA and *plp2-myc* but not from control wild-type (WT) or CCT-HA-expressing cells. HA-tagged CCT is detected by Western blot analysis by using an anti-HA antibody. (B) Western blot showing CCT coprecipitating in vitro with GST-PhLP2A or GST-PhLP3 in a GST pull-down, but not the GST-alone control. Purified CCT was run as a control to show that the antibody recognizes multiple CCT subunits (left). (C) Counterselection of a *URA3*-marked plasmid carrying *PLP2* ([pURA3 *PLP2*]) in *plp2* Δ cells with 5'-fluoroorotic acid (5'FOA) shows that *PLP2* is an essential gene. Cells prototrophic (*PLP2 URA3*) and auxotrophic (*PLP2 ura3-52*) for uracil (*ura*) were included as controls. SC, synthetic complete media.

tubulin function in vivo (Stirling *et al.*, 2006). We initially hypothesized that the only other yeast phosducin-like protein, Plp2p, might function similarly to modulate CCT activity. However, several lines of evidence suggest that the Plp1 and Plp2 proteins are unlikely to have identical functions in vivo. Yeast lacking *PLP1* are viable, whereas yeast lacking PhLP2 (*PLP2*) do not survive. In addition, overexpression of *PLP1* cannot complement the deletion of *PLP2* (Flanary *et al.*, 2000).

To determine whether *S. cerevisiae* Plp2p influences CCT-mediated protein folding, we first tested whether Plp2p interacts with CCT in vivo. When *myc*-tagged Plp2p was coexpressed with HA-tagged Cct2p, Cct2p coprecipitated with *myc*-Plp2p by using an anti-*myc* antibody, indicating a potentially direct physical interaction between the two proteins (Figure 1A). This result is consistent with previous proteome-scale TAP-tagging studies that identified an interaction between six of eight CCT subunits and Plp2p, suggesting that the entire CCT holocomplex interacts with Plp2p (Gavin *et al.*, 2006). To assess whether this interaction is conserved in mammalian cells, we purified a GST-tagged human PhLP2A fusion protein for use in in vitro experiments. Similar to PhLP3, purified CCT coprecipitated with

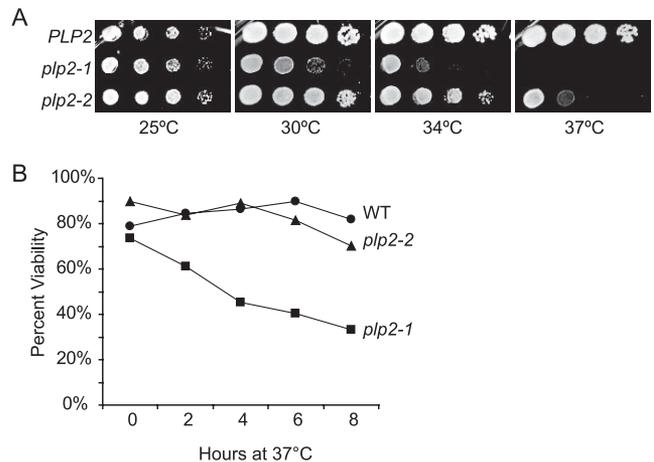


Figure 2. Generation and analysis of temperature-sensitive *plp2* alleles. (A) Wild-type yeast (*PLP2*), or two temperature-sensitive alleles of *PLP2*, namely, *plp2-1* and *plp2-2*, were spotted on YPD media and grown at the temperatures shown for 48 h. *plp2-1* displays a more severe *ts* phenotype than *plp2-2*. (B) Reversibility of the *plp2-ts* alleles was assessed after growth at 37°C for the times shown. Shifted cells were plated at a permissive temperature and the number of microcolony forming cells was assessed under a microdissection microscope. *plp2-2* temperature sensitivity is largely reversible compared with the more severe *plp2-1* allele.

the human GST-fused PhLP2A, but not GST alone, indicating that PhLP2A and CCT form a complex (Figure 1B; Stirling *et al.*, 2006). Given that both native PhLP3 and PhLP1 form complexes with CCT (McLaughlin *et al.*, 2002; Stirling *et al.*, 2006), our results with Plp2p/PhLP2A now confirm that all known phosducin-like proteins (except phosducin itself) interact with CCT and do so not as substrates, but rather as native binding partners (McLaughlin *et al.*, 2002).

There has been some dispute as to whether *PLP2* is truly essential for growth or is instead required for spore germination (Flanary *et al.*, 2000; Lopez *et al.*, 2003). To clarify this discrepancy, we generated a haploid yeast strain lacking the chromosomal copy of *PLP2*, and we transformed it with a *URA3*-marked plasmid containing *PLP2*. When cultured on medium containing the drug 5'-fluoroorotic acid, which selects against cells carrying a functional copy of the *URA3* gene, no growth was observed (Figure 1C). Although it is still possible that *PLP2* is essential in only some strain backgrounds, this experiment demonstrates that some yeast cannot survive without *PLP2*.

Generation of *PLP2* Temperature-sensitive Alleles

To study the effects of *PLP2* loss-of-function, we generated temperature-sensitive alleles of *PLP2* by error-prone PCR mutagenesis of the wild-type gene. Two of the alleles identified were chosen for further characterization, namely, *plp2-1*, the most severe allele, and *plp2-2*, a less severe allele (Figure 2A). The *plp2-1* mutant exhibits growth defects at 30°C, whereas *plp2-2* cells are only slightly impaired for growth at 34°C. Both mutant alleles cause lethality at 37°C. The two alleles contain multiple sequence changes (see *Materials and Methods*), but interestingly, both are mutated at Q91, which aligns to the region at the C terminus of helix 3 between the N-terminal and the thioredoxin domains (Gaudet *et al.*, 1996; Blaauw *et al.*, 2003). To determine whether the *ts*-phenotype is reversible, we cultured wild-type, *plp2-1*, and *plp2-2* cells at 37°C, and then we microdissected arrested cells onto solid medium and incubated them

at 25°C, a permissive temperature of growth for all strains. Figure 2B shows that the temperature sensitivity is not reversible for *plp2-1* cells after 4 h at 37°C, because >50% of the cells are inviable. In contrast, the temperature sensitivity of *plp2-2* cells is largely reversible, because even after 8 h at 37°C, 70% of the cells form colonies after shifting to the permissive temperature (Figure 2B). Thus, phenotypes observed in *plp2-2* cells at 37°C do not reflect general necrotic defects, but rather they indicate specific cellular defects caused by *PLP2* loss-of-function.

plp2-ts Alleles Exhibit Cytoskeletal but Not G Protein-related Defects

Given the putative role of yeast phosducin-like proteins as negative regulators of heterotrimeric G protein signaling, we tested the sensitivity of *plp2-ts* strains to mating pheromone, which is a readout of yeast G protein activation. Consistent with previous data (Flanary *et al.*, 2000), we saw no additional sensitivity to pheromone compared with yeast carrying wild-type *PLP2* (Supplemental Figure S1). Although it is possible that more subtle effects on pheromone signaling were overlooked, these data support an essential role for Plp2p outside of mating pheromone signaling and possibly relating to CCT function (Flanary *et al.*, 2000; Gavin *et al.*, 2006).

Although CCT impacts a variety of substrate proteins, its most abundant and best characterized substrates are the cytoskeletal proteins actin and tubulin, and, accordingly, yeast *ts* alleles of CCT display various actin- and tubulin-related cytoskeletal defects (Gao *et al.*, 1992; Ursic *et al.*, 1994; Vinh and Drubin, 1994; Siegers *et al.*, 1999; Spiess *et al.*, 2004). We therefore predicted that if Plp2p modulates the function of CCT, by way of direct physical interaction (Figure 1, A and B), the *plp2-ts* alleles might also exhibit cytoskeletal anomalies. To investigate this possibility, we first tested the sensitivity of *plp2-ts* alleles to actin and microtubule-disrupting drugs. Figure 3A shows that *plp2-ts* alleles exhibit sensitivity to both latrunculin and benomyl, indicating microfilament and microtubule defects, respectively. Similar phenotypes are also observable in strains carrying *ts* or cold-sensitive (*cs*) alleles of several CCT genes (our unpublished observations; Ursic *et al.*, 1994; Vinh and Drubin, 1994).

Under the microscope, *plp2-ts* cells also exhibited morphological phenotypes consistent with actin and tubulin cytoskeleton defects. When cultured at the nonpermissive temperature of 37°C, *plp2-ts* cells became larger (Figure 3B), a possible indication of defects in actin filament organization (Drubin *et al.*, 1993). Indeed, *plp2-1* cells were significantly larger than their wild-type counterparts even at permissive temperatures (Figure 3B). Moreover, after 8 h of growth at 37°C, the *plp2-ts* cells exhibited budding defects such as multiple buds (0% wild type, 7% *plp2-1*, 30% *plp2-2*; $p < 0.01$; Supplemental Table S3) and a thickening of the bud-neck junction between mother and daughter cells (0% wild-type, 11% *plp2-1*, 26% *plp2-2*; $p < 0.001$; Supplemental Table S4). These types of budding defects are also consistent with disrupted actin function or organization (Drubin *et al.*, 1993).

Finally, we observed chitin organization defects in *plp2-ts* cells as well as in several CCT mutant strains as assessed by calcofluor white staining (Supplemental Figure S2). Chitin defects have been observed before for specific actin mutants, consistent with cytoskeletal defects in our mutant strains (Drubin *et al.*, 1993). Interestingly, no such defects were observed in deletions of either the prefoldin subunit gene *PAC10* or the phosducin-like gene *PLP1*, suggesting a CCT/*PLP2*-specific defect (Supplemental Figure S2).

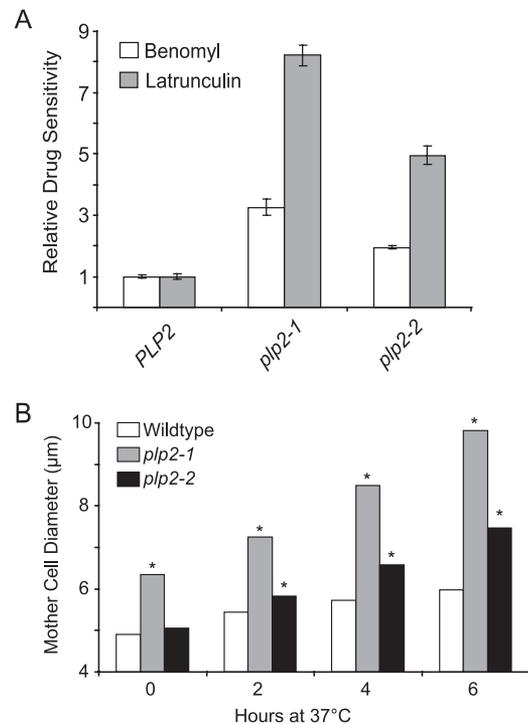


Figure 3. *plp2-ts* cells have increased sensitivity to cytoskeletal-destabilizing drugs and are larger than wild-type cells. (A) Benomyl and latrunculin sensitivity of *plp2-1* and *plp2-2* mutants relative to wild-type (*PLP2*) cells, as determined by relative clearance caused by drug-inoculated paper discs when grown at the semipermissive temperature of 30°C. (B) Cell sizes of the indicated strains were measured perpendicular to the mother-daughter axis for at least 100 cells. An asterisk (*) indicates that, relative to wild type, the cells were significantly larger ($p < 0.01$) as determined by an independent variable *t* test.

Microtubule and Nuclear Defects in *plp2-ts* Alleles

Microtubules are essential for orienting the mitotic spindle and for proper segregation of chromosomes during anaphase. The improper segregation of a nucleus to the daughter bud, therefore, can be an indication of microtubule defects. Such a phenotype can be observed, for example, by abrogating prefoldin or CCT function (Ursic *et al.*, 1994; Lacefield and Solomon, 2003; Stirling *et al.*, 2006). We stained nuclei in *plp2-ts* cells with DAPI to examine possible defects in nuclear segregation. Compared with wild-type cells, a significant ($p < 0.01$) number of unbudded (11%), small-budded (11%), and large-budded (22%) *plp2-1* cells contained multiple nuclei when incubated at the restrictive temperature for 4 h (Figure 4A and Supplemental Table S5). Similarly, the multinucleate phenotype was found to be significant ($p < 0.001$) in small- and large-budded *plp2-2* cells (16 and 23% respectively) at 37°C for 4 h. A statistically significant number of unbudded *plp2-1* cells were also anucleate at both permissive and nonpermissive temperatures, although the defect was more penetrant at high temperature (6 and 28%, respectively) (Figure 4A and Supplemental Table S5). Quantification of the number of cells going through anaphase revealed that both *plp2-1* and *plp2-2* mutant cells had significantly fewer cells in anaphase than wild-type cells at the nonpermissive temperature (37% wild-type, 22% *plp2-1*, and 17% *plp2-2* large-budded cells in anaphase; Supplemental Table S6). This type of defect is consistent with the activation of a cell cycle checkpoint, possibly in response to incorrect anaphase spindle positioning.

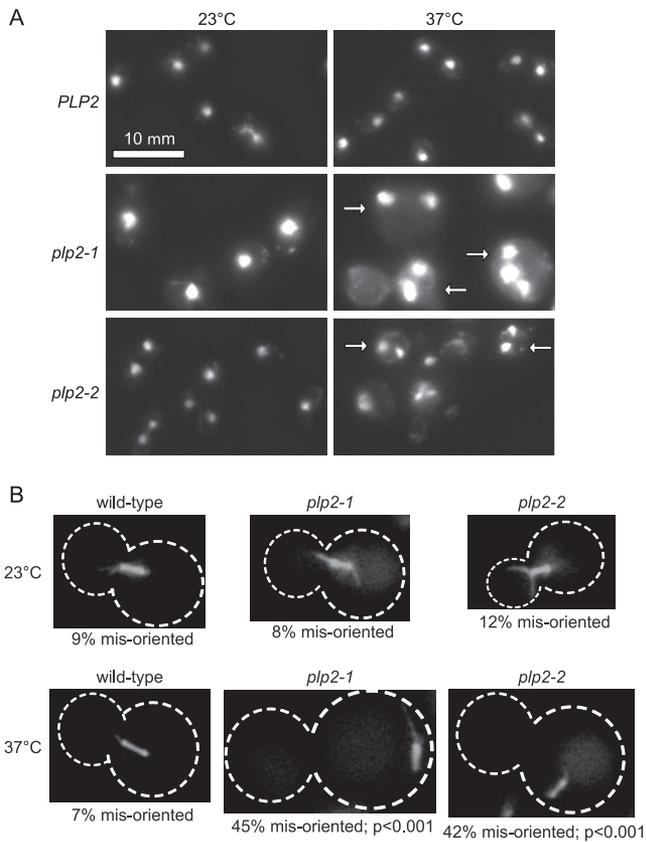


Figure 4. *plp2-ts* cells exhibit aberrant nuclear segregation and spindle orientation. (A) Images of DAPI-stained *PLP2*, *plp2-1* and *plp2-2* cells grown at permissive (23°C) and nonpermissive (37°C) temperatures. Arrows indicate multinucleate cells. (B) GFP- α -tubulin-expressing *PLP2*, *plp2-1*, and *plp2-2* cells were visualized and scored for spindle orientation with respect to the mother-daughter axis. Cells are outlined in white and the percentages of misoriented spindles are indicated below each panel. Representative images are shown of normal spindles for wild-type and *plp2-1* and *plp2-2* cells at 23°C and of misoriented spindles in *plp2-1* and *plp2-2* at 37°C.

One possible explanation for the observed benomyl sensitivity and DNA segregation defects of the *plp2-ts* alleles is a deficiency in tubulin cytoskeleton function. We therefore expressed a green fluorescent protein (GFP)- α -tubulin (Tub1p) fusion protein in wild-type, *plp2-1*, and *plp2-2* cells to assess the integrity of microtubules. Interestingly, we observed superficially normal microtubules in the *plp2* mutant cells incubated at the nonpermissive temperature of 37°C for 4 h (data not shown). Considering that aberrant spindle positioning could also lead to segregation defects, we speculated that the benomyl sensitivity and defects in nuclear segregation may result from errors in spindle positioning, and not spindle assembly or tubulin defects per se. When we examined the orientation of spindles with respect to the mother-bud axis, we found that 45 and 42% ($p < 0.001$) of *plp2-1* and *plp2-2* cells, respectively, had clearly misoriented mitotic spindles (Figure 4B and Supplemental Table S7). Because actin function is required for establishing the correct orientation of the mitotic spindle in the G1 phase of the cell cycle (Theesfeld *et al.*, 1999), these data could in principle explain both the latrunculin and benomyl sensitivity observed (Figure 3A). Whether these defects relate solely to actin dysfunction or whether tubulin also plays a role remains unclear.

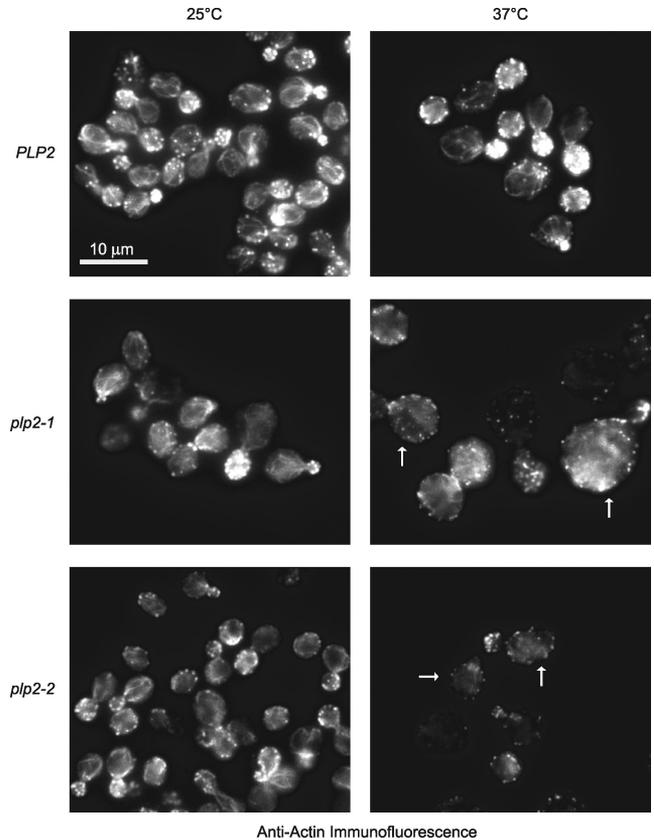


Figure 5. Actin filament organization defects in *plp2-ts* cells. (A) *PLP2*, *plp2-1*, and *plp2-2* cells grown at permissive temperature (25°C) or nonpermissive temperature (37°C) were stained with anti-actin antibodies. Arrows indicate examples of cells lacking clear actin cables and having poorly polarized actin patches.

Actin Polarization Defects in *plp2-ts* Alleles

To better understand any actin cytoskeletal defects in cells with defects in *PLP2* function, we stained the actin cytoskeleton with anti-actin antibodies (see *Materials and Methods*; Figure 5). When examined by immunofluorescence, actin localization in the *plp2-ts* mutants was essentially normal at 25°C, but after shifting to the restrictive temperature of 37°C for 4 h, severe defects were observed in both *plp2-1* and *plp2-2* cells (Figure 5 and Supplemental Table S8). After incubation at 37°C, actin cables were absent in a very large proportion of unbudded *plp2-1* (93%) and *plp2-2* cells (86%), small-budded *plp2-1* (72%) and *plp2-2* cells (94%) and large-budded *plp2-1* (90%) and *plp2-2* cells (91%) (Figure 5 and Supplemental Table S8). In *plp2-1* and *plp2-2* cells, the polarization of the actin cytoskeleton was also defective (Figure 5 and Supplemental Table S8). In wild-type cells, cortical actin patches are polarized to the bud site in unbudded cells and toward the bud tip in small-budded cells. However, at 37°C, normal actin patch polarization in *plp2-1* cells was observed in only 12% unbudded, 36% small-budded, and 10% large-budded cells. In *plp2-2* cells at 37°C, normal actin patch polarization was observed in only 6% unbudded, 30% small-budded, and 7% large-budded cells. A significant proportion of unbudded *plp2-1* cells also exhibited abnormal actin patch polarization even at 25°C (Supplemental Table S8). Importantly, these data also support our observed functional cooperation between Plp2p and CCT in actin folding, because mutations in CCT or PFD subunits show similar

actin organization defects (Ursic *et al.*, 1994; Vinh and Drubin, 1994; Geissler *et al.*, 1998; Vainberg *et al.*, 1998).

Mammalian PhLP2A Inhibits Actin Folding In Vitro and Binds CCT-Actin Complexes

Because disruption of *PLP2* function seems to strongly affect the actin cytoskeleton in vivo, we exploited the robust in vitro actin folding system developed using reticulocyte lysate (Cowan, 1998; Vainberg *et al.*, 1998; Leroux, 2000) to examine whether PhLP2 modulates the folding function of CCT. In this system, nascent [³⁵S]methionine-radiolabeled actin is produced, and actin folding is examined on a native polyacrylamide gel (Leroux, 2000). Because mammalian actin, CCT, and other mammalian components have been well tested in this system, we used purified GST-fused human PhLP2A protein to test for its effect on actin folding. Figure 6A shows that in the presence of excess GST-PhLP2A, the production of native actin was greatly inhibited at all time points assayed, compared to GST alone. The presence of GST-

PhLP2A also led to an increase in the amount of radiolabeled actin associated with both the CCT and PFD complexes (Figure 6A), whose positions on native gels and actin-binding activities are well established (Cowan, 1998; Vainberg *et al.*, 1998; Leroux, 2000). Quantification of the native actin and chaperone:actin bands confirmed our qualitative conclusions that inhibition of folding was occurring and that nonnative actin was accumulating on CCT and PFD (Supplemental Figure S3). This result is consistent with PhLP2A slowing the reaction cycle or inhibiting the activity of CCT; this inhibition could potentially be taking place in a ternary complex with CCT and substrate, as reported for PhLP3, leading to a backlog of unfolded substrate that ends up bound to PFD, and, in this case, also CCT (Figure 6A; Lukov *et al.*, 2006; Stirling *et al.*, 2006).

We therefore examined whether, similar to that observed for PhLP3, PhLP2A could form ternary complexes with CCT and substrate (e.g., CCT-PhLP3-actin or CCT-PhLP3-tubulin; Stirling *et al.*, 2006). To this end, we performed GST pull-downs of in vitro translation reactions of actin or tubulin containing exogenous GST-PhLP2A or GST alone as a negative control. Figure 6B shows in vitro translations of actin and β -tubulin in reticulocyte lysate, which contains endogenous CCT, that have been precipitated with glutathione-conjugated beads. GST-PhLP2A effectively precipitates both actin and β -tubulin, whereas GST alone has no such effect. To determine whether the interaction between PhLP2A and a cytoskeletal protein (actin) depends on CCT (as opposed to PhLP2A binding the substrate directly), we performed a similar experiment with an *E. coli* lysate translation system, which lacks CCT. We found that GST-PhLP2A only precipitates actin when exogenous, purified CCT was added (Figure 6C). Under these conditions, GST-PhLP2A is able to specifically recover CCT in the pull-down (data not shown). Together, these data suggest that PhLP2A negatively modulates CCT function in a manner that may be similar to that of the mammalian PhLP3 protein (Stirling *et al.*, 2006).

High-Copy Suppression of *plp2-ts* Alleles Reveals Links to the G1/S Phase Transition

Our finding that Plp2p influences actin microfilament and tubulin microtubule function in *S. cerevisiae* is consistent with the fact that Plp2p associates with and affects the function of CCT, a chaperone essential for actin and tubulin folding. However, CCT has been implicated in the biogenesis of several other proteins, some of which are linked to the cell cycle. To take an unbiased approach to understanding the essential function(s) of *PLP2*, which may be CCT-dependent or (potentially) independent, we executed a high-copy suppression screen of the *plp2-ts* alleles.

A high-copy plasmid library carrying ~10-kb fragments of yeast genomic DNA (Carlson and Botstein, 1982) was transformed into the *plp2-1* strain, and colonies were plated at 37°C. Plasmids isolated from colonies that grew well at 37°C were screened by PCR to exclude those carrying the *PLP2* gene, a predictable suppressor. Plasmids that did not contain *PLP2* were sequenced to identify the gene(s) present in the suppressing plasmid. One high-copy fragment we isolated contained *PLC1*, a yeast phospholipase C homologue. We confirmed that *PLC1* was a suppressor because a galactose-inducible *PLC1* construct could partially suppress both *plp2-1* and *plp2-2* alleles (Supplemental Figure S4). Another suppressing plasmid encoded two genes, *VHS1* and *PAM1*. These were expressed individually in *plp2-1* cells, and, surprisingly, both were found to partially suppress the temperature-sensitive defect (Figure 7A). In the same screen

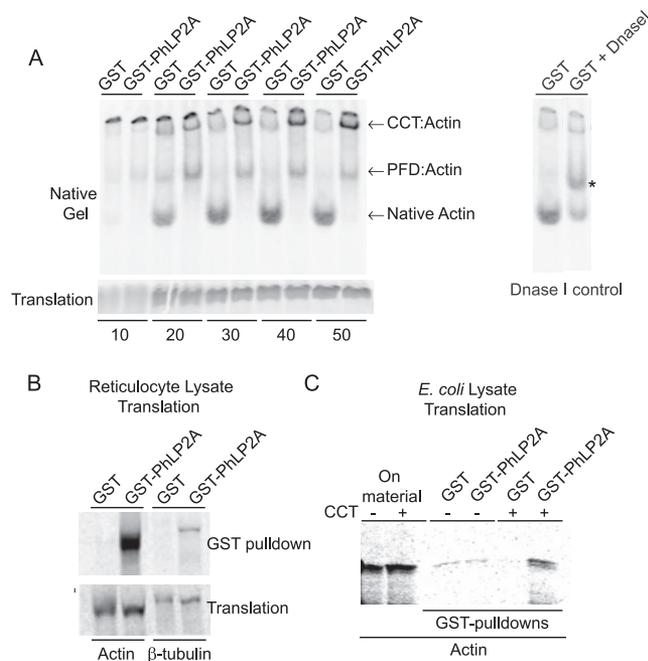


Figure 6. Mammalian PhLP2A modulates CCT-mediated actin folding in vitro. (A) In vitro folding reactions of nascent ³⁵S-labeled actin in the presence of GST or GST-PhLP2A and separated on a nondenaturing gel at the time points (minutes) indicated. CCT:actin and PFD:actin binary complexes, as well as native actin, are indicated. The identity of the fast-migrating band as native monomeric actin was verified by Dnase I-shifting on the native gel (right). Asterisk (*) indicates Dnase I bound native actin. Note the reduction in the intensity of the bottom band. Note the decrease in native actin production over time in the presence of GST-PhLP2A but not GST alone (see Supplemental Figure S3 for quantitation of band intensities). An SDS-gel illustrating the relative amounts of translation products is shown (bottom). (B) Coprecipitation of ³⁵S-labeled actin or β -tubulin with GST-PhLP2A in a reticulocyte translation reaction. The lower panel shows that the levels of translation were comparable. (C) Coprecipitation of ³⁵S-labeled actin produced in *E. coli* lysate takes place only in the presence of GST-PhLP2A and exogenously added CCT, indicating the formation of a ternary complex. Actin cDNA was translated in *E. coli* lysate with or without the addition of purified rabbit CCT, and the relative levels of the translation products are shown on the left. GST-PhLP2A coprecipitated the newly-made actin only in reactions to which CCT had been added, whereas GST alone had no such effect as shown on the right.

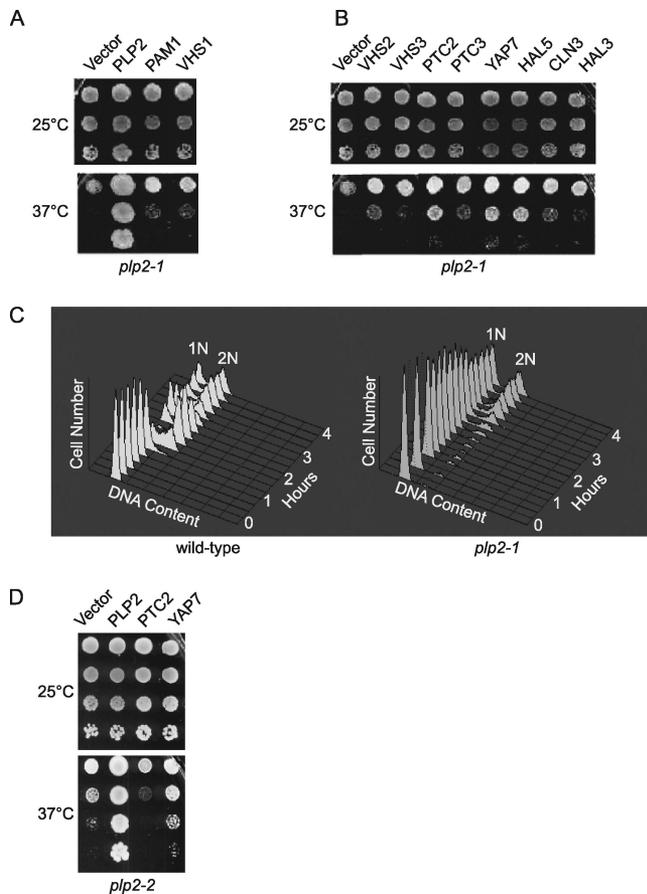


Figure 7. High-copy suppressors of *plp2-1* indicate a role for *PLP2* in cell cycle progression. Partial suppressors of *plp2-1* temperature sensitivity identified in a high-copy screen (*PLC1* suppression is shown in Supplemental Figure S4) (A) and suppressors identified in the literature (B). *plp2-1* cells carrying the indicated plasmids were grown to log phase and serially diluted on SC-Ura media and grown for 2–3 d at permissive (25°C) or restrictive (37°C) temperatures before imaging. (C) A delay in DNA replication is observed in synchronized *plp2-1* cells. Graphs indicate FACS analysis of cells stained for DNA content over time. (D) Allele specific suppression of *plp2-ts* alleles. Serially diluted *plp2-2* cultures carrying the indicated plasmids were grown for 2–3 d on SC-Ura as in A and B before imaging.

from which *VHS1* was previously identified, several other genes, including protein phosphatase subunits, *PAM1* and other genes active in the G1/S phase transition, were found to suppress loss of *SIT4* and *HAL3* (a phosphatase subunit and regulator, respectively). To try and identify additional *plp2-1* suppressors, we tested some of the *sit4-hal3* suppressors found in the screen by Muñoz *et al.* (2003) (Figure 7B). Remarkably, the genes uncovered, *VHS2*, *VHS3*, *PTC2*, *PTC3*, *YAP7*, *HAL5*, *HAL3*, and *CLN3*, were also able to partially suppress temperature-sensitive growth defects of *plp2-1* cells. This apparent lack of specificity suggests that driving cells to continue through the cell cycle helps to somewhat alleviate the *plp2*-based temperature sensitivity. The extensive overlap between suppressors of *plp2-ts* alleles and suppressors of *sit4-hal3* mutants (Muñoz *et al.*, 2003) suggests either that Plp2p directly affects Sit4p and/or Hal3p or that Plp2p works in a parallel pathway to that of Sit4p and Hal3p. The absence of suppressors directly related to cytoskeleton functions may imply that these defects cannot be suppressed by overexpression of any one particular

gene or that any suppression was too weak and thus overlooked.

Although the partial suppressors of *plp2-1* have some diverse functions, many are involved in protein phosphorylation/dephosphorylation and they all play roles in promoting the G1/S phase transition of the cell cycle (Muñoz *et al.*, 2003). These data therefore suggest that *PLP2* also has a role in G1 progression. Indeed, we observed an accumulation of unbudded *plp2-1* cells after 2 h at 37°C (50% unbudded, $p < 0.001$; Supplemental Table S9), although this could also be related to the actin defects reported above. Also, the observation that *plp2-ts* cells become larger than wild-type cells at 37°C is consistent with a pause in progression from G1 to S phase. To test these suppositions, we examined the entry of synchronized *plp2-1* cells into S phase. Synchronized 1N cell populations were prepared by elutriation, grown at 37°C, stained at various time points for DNA content, and subjected to FACS. Figure 7C shows that *plp2-1* cells are delayed in their entry into S phase compared with their wild-type counterparts. Even after 4 h, there is still a greater proportion of *plp2-1* cells in G1 than G2, whereas approximately half of the wild-type cells are found in G2 (Figure 7C). This delay in cell cycle progression is consistent with the G1/S function of the *plp2-1* suppressors we identified, and the accumulation of unbudded cells in asynchronous *plp2-1* cell populations at restrictive temperatures (Supplemental Table S9). Consistent with these observations, we also observed a delay in budding when α -factor-synchronized *plp2-ts* cells were released in 37°C media (Supplemental Figure S5). Importantly, entry into S-phase seems to be independent of actin function (McMillan *et al.*, 1998), and we found that the actin cytoskeleton appears similarly defective in *plp2-1* and *plp2-2* cells carrying high-copy empty vector, or the suppressing *PTC2*- or *YAP7*-plasmids (our unpublished observations). Our data therefore suggest an additional, actin-independent role for *PLP2* in the cell cycle.

To explore the possibility of allele-specific suppression, we tested two of the stronger suppressors of *plp2-1* (*YAP7* and *PTC2*) in *plp2-2* cells (Figure 7A). Although high-copy production of *YAP7* suppresses *plp2-2* temperature sensitivity, *PTC2* overexpression actually inhibits the growth of *plp2-2* cells (Figure 7D). Also consistent with functional differences between the *plp2* alleles, unbudded cells did not accumulate at the restrictive temperature for *plp2-2* as they did for *plp2-1* (Supplemental Table S9). Finally, we have observed some semidominant growth defects for *plp2-1* alleles not found in the *plp2-2* strain (data not shown). Together, the observed allele specificity supports a role for *PLP2* in the cell cycle because the cytoskeletal phenotypes of *plp2-1* and *plp2-2* cells are nearly identical (Figures 3–5). Indeed, if the observed cell cycle defects were somehow an indirect effect of cytoskeletal defects, the two *plp2* alleles would be expected to behave similarly.

DISCUSSION

In recent years, the phosphatase-like proteins PhLP1 and PhLP3 have emerged as modulators of cytosolic chaperonin function in eukaryotes (McLaughlin *et al.*, 2002; Lacefield and Solomon, 2003; Martín-Benito *et al.*, 2004; Lukov *et al.*, 2006; Stirling *et al.*, 2006). We now report in vitro and in vivo studies demonstrating that the PhLP2 subgroup also modulates CCT function. Our data suggest that PhLP2 exerts similar effects on CCT as does PhLP3, in that PhLP2 binds CCT, and when in excess, slows protein folding in a ternary complex with substrates (Figures 1 and 6). This functional mechanism may also extend to PhLP1 proteins, which have

recently been shown to form ternary complexes with CCT and G β to facilitate heterotrimeric G protein assembly (Lukov *et al.*, 2006). Significantly, this is the first study to characterize a PhLP2 protein with respect to CCT function, confirming that members of all three subgroups of phosducin-like proteins cooperate with the chaperonin. This study also describes loss-of-function alleles of *PLP2* for the first time and reveals both cytoskeletal and cell cycle phenotypes consistent with a role in modulating CCT activity *in vivo*. Together these data contribute to the emerging picture of phosducin-like proteins as bona fide CCT cofactors.

Although phosducin-like proteins have generally been implicated in heterotrimeric G protein signaling (Schroder and Lohse, 1996), here we establish that *PLP2* is unlikely to play a critical role in heterotrimeric G protein signaling (Supplemental Figure S1). Instead, the function of Plp2p seems closely aligned with that of CCT; Plp2p binds CCT in yeast and *ts*-alleles of *PLP2* and CCT subunits exhibit similar phenotypes, consistent with a functional cooperation between the CCT chaperonin and Plp2p (Figures 1–7; Ursic *et al.*, 1994; Vinh and Drubin, 1994; Siegers *et al.*, 1999; Camasses *et al.*, 2003). Importantly, and similar to CCT (Ursic *et al.*, 1994; Vinh and Drubin, 1994), we conclusively demonstrate that *PLP2* is essential for viability in *S. cerevisiae* (Figure 1).

Cytoskeletal Phenotypes in *plp2* Loss-of-Function Cells

The reason for the essential nature of *PLP2* is not entirely clear, although we establish cytoskeletal phenotypes as a profound cellular defect of cells lacking functional Plp2p. Cells carrying *plp2-ts* alleles are sensitive to the drugs latrunculin and benomyl, which disrupt actin and tubulin filaments, respectively (Figure 3). The *plp2-ts* strains also exhibit a host of other phenotypes, all of which can be indirect indicators of cytoskeletal defects (Figure 4, Supplemental Tables S3–S6, and Supplemental Figure S2). Moreover, the mitotic spindles of *plp2-1* and *plp2-2* cells become misoriented with respect to the mother-daughter axis when the cells are cultured at high temperature (Figure 4 and Supplemental Table S7). The *plp2-ts* strains also show weakened polarization of actin filaments and a nearly complete loss of actin cables (Figure 5 and Supplemental Table S8). Together, these data support a role for Plp2p in actin and tubulin function, and they are consistent with previous findings that a reduction in *PLP2* expression by using a doxycycline-repressible promoter allele leads to synthetic growth defects in *BNI1* or *ARP2* mutant backgrounds (Mnaimneh *et al.*, 2004; Davierwala *et al.*, 2005). Bni1p (formin) and the actin-related Arp2p protein are involved in the formation of actin cables and actin patches, respectively (for review, see Evangelista *et al.*, 2003).

The interaction between Plp2p and CCT (Figure 1) strongly supports a role for Plp2p in the production of functional actin and tubulin. Interestingly, our data also suggest that actin-based functions may be more sensitive to Plp2p disruption than are tubulin-based functions. This may indicate that the cellular demand for properly folded actin in the budding cell is higher than it is for tubulin, or that actin-based phenotypes manifest before tubulin-specific defects. Our studies provide unequivocal evidence that both actin and tubulin functions are compromised when Plp2p function is perturbed. Furthermore, the effects of Plp2p are likely mediated via a direct functional interaction with CCT alone, or with CCT-substrate protein complexes (Figures 1 and 6).

Cell Cycle Phenotypes in *plp2* Loss-of-Function Cells

Although our characterization of the *plp2-ts* alleles showed many cytoskeletal phenotypes consistent with a functional interaction with CCT, an unbiased high-copy suppression screen revealed a link to the cell cycle, especially the progression out of G1 phase into S phase. When we examined cell cycle progression by DNA content in *plp2-1* cells, we found a delay in DNA replication (Figure 7). Moreover, we observed an excess of unbudded cells when the mutant strains were cultured at high temperature (Supplemental Table S9). Although it is possible that some of these defects relate to actin dysfunction, which can delay budding (as we observed in Supplemental Figure S5), it is likely that Plp2p also affects one or more noncytoskeletal, cell cycle-related CCT substrate(s), as we elaborate below.

Other groups have shown that CCT has an important role in cell cycle progression, because of its effect on the biogenesis of the anaphase promoting complex (APC) regulators Cdc20p and Cdh1p as well as the protein phosphatase subunit Cdc55p, any one of which could be regulated by Plp2p (Camasses *et al.*, 2003; Siegers *et al.*, 2003). Because these particular CCT substrates contain WD-repeats, a role for Plp2p seems plausible in light of the known role for other phosducin-like proteins in WD-repeat folding/assembly by CCT (Lukov *et al.*, 2005, 2006). For example, impaired CCT function leads to precocious entry into S phase because of loss of Cdh1-APC activity, which normally inhibits entry into S phase (Harper *et al.*, 2002; Camasses *et al.*, 2003). If Plp2p were acting to negatively regulate CCT-mediated Cdh1p-APC assembly, then *plp2-1* alleles may accumulate excess Cdh1p-APC, thus slowing entry into S phase.

An alternative connection supported by the literature is one between CCT and type 2A protein phosphatases (PP2As). CCT is known to assist the folding of the yeast PP2A regulator Cdc55p, and physical interactions have been reported between CCT and the yeast phosphatase components Sit4p, Pph21p, Pph22p, Pph3p, and Tap42p (Ho *et al.*, 2002; Siegers *et al.*, 2003; Gavin *et al.*, 2006). The finding that *PAM1*, which bypasses a loss of PP2A activity (Hu and Ronne, 1994), can weakly suppress *plp2-1* and *plp2-2* alleles (Figure 7A; data not shown) is consistent with a role for Plp2p and CCT in the folding of PP2A components. Indeed, all the suppressors we identified were also identified in a screen for suppression of lethality at the G1/S phase transition in a strain lacking the phosphatase *SIT4* and the phosphatase regulator *HAL3* (Muñoz *et al.*, 2003). Intriguingly, the toxicity associated with overexpression of *SIT4* and *SAP155* is suppressed by increased *CCT6* copy number in a manner that is not understood (Kabir *et al.*, 2005).

These putative connections of *PLP2* function to the cell cycle would help to explain the diverse and weak G1-related suppressors we identified as well as the polarization, cytoskeletal, and cell cycle defects resulting from *plp2* mutations. Also of importance is the finding that, in mammalian cells, CCT itself was shown to be up-regulated at the G1/S phase transition and CCT depletion caused arrest of cells at the G1/S phase transition (Yokota *et al.*, 1999; Grantham *et al.*, 2006). These observations suggest an increased folding requirement for some CCT substrates at this cell cycle stage, consistent with our findings.

CONCLUSIONS

Given the literature and the data presented here, we propose a model wherein Plp2p functions to regulate the folding of several CCT substrates that together control cytoskeletal

morphogenesis and cell cycle progression. Based on the phenotypes we observed in *plp2-ts* alleles, candidates for these critical substrates include actin and actin-related proteins, tubulin, Cdc20p, Cdc55p, Cdh1p, and/or as-yet-unconfirmed CCT substrate(s) such as Sit4p. In this model, the additive effects of their altered folding by CCT contribute to the complex phenotypes and eventual lethality associated with loss of *PLP2* function. Alternatively, there could be one critical substrate whose biogenesis wholly depends on Plp2p, although we favor the former option because of the *PLP2* mutant phenotypes. Actin seems to be particularly affected by *PLP2* mutations, suggesting an important role in controlling actin biogenesis and/or the function of an actin regulator such as Bni1p/Arp2p. The conserved nature of PhLP-CCT cooperation and the diversity of substrates likely to be impacted by PhLPs reveal the importance of understanding PhLPs to gain a complete picture of cytosolic chaperonin function in eukaryotes.

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