Mitochondrial Inheritance Is Delayed in *Saccharomyces cerevisiae* Cells Lacking the Serine/Threonine Phosphatase *PTC1*

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In wild-type yeast mitochondrial inheritance occurs early in the cell cycle concomitant with bud emergence. Cells lacking the *PTC1* gene initially produce buds without a mitochondrial compartment; however, these buds later receive part of the mitochondrial network from the mother cell. Thus, the loss of *PTC1* causes a *delay*, but not a complete block, in mitochondrial transport. *PTC1* encodes a serine/threonine phosphatase in the high-osmolarity glycerol response (HOG) pathway. The mitochondrial inheritance delay in the *ptc1* mutant is not attributable to changes in intracellular glycerol concentrations or defects in the organization of the actin cytoskeleton. Moreover, epistasis experiments with *ptc1Δ* and mutations in HOG pathway kinases reveal that *PTC1* is not acting through the HOG pathway to control the timing of mitochondrial inheritance. Instead, *PTC1* may be acting either directly or through a different signaling pathway to affect the mitochondrial transport machinery in the cell. These studies indicate that the timing of mitochondrial transport in wild-type cells is genetically controlled and provide new evidence that mitochondrial inheritance does not depend on a physical link between the mitochondrial network and the incipient bud site.

INTRODUCTION

Mitochondria are essential organelles that produce the majority of the cellular ATP required for the growth and proliferation of eukaryotic cells. Cytological studies indicate that mitochondrial morphology and distribution can vary in different cell types, ranging from small, spherical organelles to a complex reticulum or network (Bereiter-Hahn, 1990; Bereiter-Hahn and Vöth, 1994). Although it is clear that mitochondria can increase in mass and divide by fission, they cannot be synthesized de novo and must be inherited by daughter cells during division.

In the budding yeast *Saccharomyces cerevisiae*, mitochondria form a network of tubular membranes located at the cell cortex (Hoffman and Avers, 1973). A portion of this mitochondrial network is transported

into the emerging bud very early in the cell cycle (G_1 -S) (Stevens, 1981). Both genetic and biochemical analyses suggest that this polarized transport of mitochondria from mother to bud is an active process requiring functions of the cytoskeleton including actin (Drubin *et al.*, 1993; Lazzarino *et al.*, 1994; Simon *et al.*, 1995; Hermann *et al.*, 1997) and an intermediate filament-like protein (McConnell *et al.*, 1990; McConnell and Yaffe, 1992, 1993; Berger and Yaffe, 1996). A number of studies indicate that transmission of mitochondrial genomes (nucleoids) is also regulated during yeast budding, and genes that affect this process have been isolated (Strausberg and Perlman, 1978; Zinn *et al.*, 1987; Diffley and Stillman, 1991, 1992; Azpiroz and Butow, 1993; Chen *et al.*, 1993; Guan *et al.*, 1993; Campbell *et al.*, 1994; Backer, 1995; Zelenaya-Troitskaya *et al.*, 1995; Nunnari *et al.*, 1997). Genetic screens have also identified molecules that play a role in establishing and/or maintaining mitochondrial morphology (Burgess *et al.*, 1994; Sogo and Yaffe, 1994; Berger and Yaffe, 1996; Berger *et al.*, 1997). In all of the mitochon-

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drial inheritance and morphology mutants described to date, daughter cells that fail to receive a mitochondrial compartment do not separate from the mother cell and are unable to produce buds themselves.

Transmission of the mitochondrial network in wild-type yeast always begins immediately after bud emergence, suggesting that mitochondrial inheritance is tightly coordinated with the cell cycle (Stevens, 1981). This coordination could be achieved by attaching the mitochondrial network to structures at the incipient bud site and passively pulling the organelle into the growing bud. Alternatively (or in addition), molecules that control mitochondrial transport could be regulated in a cell cycle-dependent manner. Although mutations that completely block mitochondrial inheritance have been isolated (see above), genetic analyses performed to date have not uncovered genes that control the "timing" of mitochondrial movement into the bud. Here we report the isolation of a new mutation, *mdm28* (*mdm* = mitochondrial distribution and morphology), that causes a pronounced delay in mitochondrial inheritance. *mdm28*-null cells initially produce buds that lack mitochondria, but these buds eventually receive part of the mitochondrial network from the mother cell. *MDM28* is identical to *PTC1*, a gene encoding a serine/threonine phosphatase that is thought to regulate the high-osmolarity glycerol response (HOG) pathway (Maeda *et al.*, 1994). Our analyses suggest that Ptc1p does not act through the HOG pathway kinases to influence the mitochondrial transport machinery in the cell. Rather, Ptc1p may be acting directly or through an alternative signaling pathway to affect this process. These studies identify a new role for the Ptc1p serine/threonine phosphatase in cells and provide the first evidence that mitochondrial inheritance is not physically linked to bud emergence in yeast.

MATERIALS AND METHODS

Yeast Strains

The *S. cerevisiae* strains used in this study are listed in Table 1. Plasmid *pptc1-Δ1* (provided by E. Benson and G. Payne, University of California at Los Angeles, Los Angeles, CA) was used to construct the *PTC1* disruption in strain JSY118. The plasmid pDHG12 (provided by M. Gustin, Rice University, Houston, TX) was used to disrupt *HOG1* in FY250 to create AMY36. All disruptions were verified by Southern blot analysis. Strains AMY36 and JSY118 were mated to produce AMY38. AMY38 was sporulated to produce the strain AMY43. For some experiments, the strains JSY118 and JSY836 were transformed with the plasmid pOK29 (generously provided by O. Kerscher and R. Jensen, Johns Hopkins Medical School, Baltimore, MD) to allow visualization of the mitochondrial network by Cox4-GFP.

Culture Conditions and Media

The yeast strains were grown and maintained in liquid YP dextrose (YPD) or YP glycerol (YPG) media and on YPD or YPG plates at 25°C. The JSY836 and JSY118 strains containing the pOK29 plasmid

were grown on SG minus histidine plates to select for maintenance of the plasmid. Liquid cultures were grown in Innova 3000 water bath shakers (New Brunswick Scientific) at shaking speeds between 200 and 240 rpm. To induce sporulation, cells were transferred to sporulation plates containing amino acids at 25% of supplemented minimal medium levels (Kaiser *et al.*, 1994) and grown at 25°C.

Cloning and Disruption of *MDM28* (*PTC1*)

Temperature-sensitive mitochondrial inheritance mutants were isolated and back-crossed as described by Hermann *et al.* (1997). The strain containing the *mdm28* mutation was transformed with yeast genomic libraries contained within the YCp50 plasmid (Rose *et al.*, 1987) (obtained from American Type Culture Collection, Rockville, MD) and the p366 plasmid (provided by T. Formosa, University of Utah, Salt Lake City, UT). Colonies were selected for growth at 37°C on SG minus leucine for p366 transformants and SG minus uracil for YCp50 transformants. Three colonies from the YCp50 library and eight colonies from the p366 library were found to contain plasmids that rescued the *mdm28* temperature-sensitive growth defect on glycerol and the mitochondrial inheritance delay phenotype.

Restriction analysis and sequencing indicated that the YCp50 transformants contained three different overlapping inserts between 9.1 and 12.1 kb. These inserts were in a region of the left arm of chromosome IV that had previously been sequenced. The region of DNA responsible for complementing the *mdm28* phenotype was located within the cosmid SCCHRV42 and SC8119, which contained three genes, *PTC1*, *YTA5*, and *D2930*. Standard subcloning procedures were used to show that the *PTC1* gene complemented the mutant phenotypes in *mdm28*. All eight p366 transformants were shown to contain *PTC1* by Southern blotting. *PTC1* (GenBank accession number L14593) was originally identified as the gene *TPD1* in a screen for mutants in tRNA biosynthesis (van Zyl *et al.*, 1989).

A *ptc1* null mutation was generated using the *pptc1-Δ1* plasmid. Briefly, a 1.7-kb fragment was released from *pptc1-Δ1* by digestion with the restriction enzyme *EcoRI*. This fragment was then transformed into the yeast strain JSY836, and TRP⁺ colonies were selected. The *ptc1::TRP1* disruption was verified by Southern blotting of genomic DNA from the TRP⁺ transformants.

Characterization of the *ptc1Δ* Phenotype

To determine the effects of the *PTC1* deletion on growth rate, both wild-type (JSY836) and *ptc1Δ* (JSY118) cells were grown to log phase in liquid YPD or YPG medium at 25°C. Equal numbers of cells were serially diluted (10-fold dilutions) and spotted onto YPG or YPD solid medium. Plated cells were grown at 25, 30, or 37°C for 3 d. Growth rates were also analyzed in liquid medium. Cultures grown overnight in YPD or YPG medium were diluted to 0.1 OD₆₀₀/ml in fresh YPD or YPG medium and grown at 25, 30, or 37°C. The densities of the cultures were measured at the indicated times.

Yeast mitochondrial networks were visualized by growing cells overnight in YPD at 25°C and staining with 100 nM 3,3'-dihexyloxycarbocyanine (DiOC₆; Molecular Probes, Eugene, OR) as described previously (Hermann *et al.*, 1997). Alternatively, JSY836 and JSY118 cells expressing the Cox4-GFP protein (targeted to the mitochondrial matrix; pOK29 plasmid provided by O. Kerscher and R. Jensen) were grown overnight under the same conditions and inspected microscopically. To quantify mitochondrial inheritance, fields of stained, dividing cells were analyzed for the presence or absence of the mitochondrial network in buds (*n* ≥ 100). Comparable results were obtained using both of these staining methods (Table 2), suggesting that the mitochondrial inheritance phenotype observed in *ptc1Δ* cells is not attributable to the presence of the Cox4-GFP fusion protein.

To synchronize cultures, cells grown to log phase in YPD medium at 25, 30, or 37°C were diluted to 0.5 OD₆₀₀/ml, and α -factor (Sigma, St. Louis, MO) was added to a final concentration of 5 μ M. After 3 h

at the appropriate temperature, aliquots of each culture were analyzed microscopically to quantify the extent of synchronization (percentage of unbudded cells). The synchronized cultures were pelleted, washed two times in YPD medium, resuspended in YPD, and incubated at the indicated temperatures. Bud size and mitochondrial inheritance (DiOC₆) were scored at 0.5-h intervals until the first round of budding was nearly completed in the *ptc1Δ* cultures.

Analysis of the Actin Cytoskeleton in *ptc1Δ* Cells by Indirect Immunofluorescence

Indirect immunofluorescence was used to analyze the organization of actin and the distribution of mitochondrial networks in wild-type and *ptc1Δ* cells. Log phase JSY836 and JSY118 cultures grown at 30°C in YPD medium were fixed essentially as described by Pringle *et al.* (1991). The cells were stained simultaneously with the following primary antibodies: 1) goat anti-actin antibody (1:50 dilution; Karpova, *et al.*, 1993); and 2) mouse anti-porin monoclonal antibody (1:75 dilution; Molecular Probes). The primary antibodies were visualized by staining with the following secondary antibodies (applied sequentially): 1) rabbit anti-goat DTAF (1:1000 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); and 2) goat anti-mouse LRSC (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc.). Actin cytoskeletons and mitochondrial networks were visualized on a Zeiss epifluorescence microscope as described previously (Roeder and Shaw, 1996). Both mitochondrial distribution and actin organization were scored in budded cells. For actin organization, categories scored included no actin staining (approximately 1% in wild-type and *ptc1Δ* cells), wild-type actin organization (actin cables oriented toward the bud and actin patches clustered in the bud), or non-wild-type actin organization (absent or incorrect actin patch localization and actin cables absent or incorrectly oriented). For mitochondrial distribution, categories scored included the presence or absence of mitochondrial network in buds.

Analysis of the Effects of High-Osmolarity Medium on Mitochondrial Inheritance

JSY836 and JSY118 cells containing the pOK29 plasmid were grown overnight at 25°C in YPD and resuspended at a cell density of 0.5 OD₆₀₀/ml in YPD containing 0.9 M NaCl to induce intracellular glycerol synthesis. Mitochondrial inheritance by buds was scored at 5 and 24 h after transfer to high-osmolarity medium. In control experiments, intracellular glycerol levels before and after growth in YPD plus 0.9 M NaCl were measured enzymatically as described previously (Jiang *et al.*, 1995). When cells were grown in YPD medium, the glycerol concentration in the *ptc1Δ* strain (JSY118) was 1.3-fold higher than that of the isogenic wild-type strain (JSY836). The glycerol concentration of wild-type cells grown in YPD containing 0.9 M NaCl was 8.8-fold higher (after 5 h) and 6.5-fold higher (after 24 h) than that observed in YPD alone. The glycerol concentration of *ptc1Δ* cells grown in YPD containing 0.9 M NaCl was 6.6-fold higher (after 5 h) and 3.25-fold higher (after 24 h) than that observed in *ptc1Δ* cells grown in YPD alone.

Microscopy

In all experiments, the cells were viewed with a Zeiss Axioplan microscope equipped with differential interference contrast optics, epifluorescence capabilities, and a Zeiss Acroplan-Neofluar 100× (numerical aperture, 1.3) objective. LRSC (rhodamine), DiOC₆/Cox4-GFP/DTAF and DAPI fluorescence were visualized using 546, 450–490, and 365 nm bandpass filters, respectively.

Images were captured using a video camera and assembled into figures as described previously (Roeder and Shaw, 1996). A Tektronix (Wilsonville, OR) Phaser II sdx dye sublimation printer was used to print Figures 1, 2, and 4.

RESULTS

MDM28 Is Required for Mitochondrial Inheritance and Is Identical to the Serine/Threonine Phosphatase PTC1

The *mdm28-1* mutant was originally identified in a screen for temperature-sensitive yeast strains exhibiting defects in mitochondrial inheritance (Hermann *et al.*, 1997). In *mdm28-1* mutant cells, 29.8% (25°C) or 43.9% (37°C) of growing buds failed to inherit mitochondrial networks stained with the potential-dependent fluorescent dye DiOC₆. In contrast, only a small percentage of buds produced in a wild-type culture lack mitochondrial staining (4.9% at 25°C or 2.4% at 37°C). The *mdm28* strain grew slowly compared with wild type on YPD solid medium at 37°C and failed to grow at 37°C on YPG medium (containing the nonfermentable carbon source glycerol). This recessive, temperature-sensitive growth defect on YPG medium segregated 2:2 in back-crosses with a wild-type strain and was linked to the mitochondrial inheritance phenotype observed in the original *mdm28* isolate.

We cloned the *MDM28* gene by complementation of the temperature-sensitive growth defect on glycerol in the *mdm28-1* strain (see MATERIALS AND METHODS). Integrative mapping studies demonstrated that the cloned DNA contained the wild-type *MDM28* gene. A combination of DNA sequence analysis and subcloning indicated that a fragment containing the previously identified gene, *PTC1*, rescued both the mitochondrial inheritance defect and the temperature-sensitive growth defect in the *mdm28-1* mutant strain.

PTC1 encodes a type 2C serine/threonine phosphatase first isolated in a screen for mutants deficient in de novo tRNA biosynthesis (originally called *TPD1*; van Zyl *et al.*, 1989). *PTC1* was independently identified by Maeda *et al.* (1993) based on its synthetic lethal interaction with a tyrosine phosphatase mutant, *ptp2*. Subsequent studies showed that overexpression of Ptc1p could suppress the lethality caused by deletion of *SLN1*, a gene required for regulation of intracellular osmolarity (Maeda *et al.*, 1994). Sln1p is a plasma membrane osmosensor that negatively regulates a downstream MAP kinase cascade in the HOG pathway. In yeast, the HOG pathway plays a critical role in regulating intracellular glycerol concentrations in response to osmotic stress. Both Ptp2p and Ptc1p are proposed to negatively regulate MAP kinases in the HOG pathway (see below).

A previous study showed that *ptc1* null strains exhibited additional phenotypes including reduced sporulation efficiency and temperature-sensitive defects in cell separation during mitotic growth (reported as 80% multiply budded cells at 37°C; Robinson *et al.*, 1994). The mutant also failed to grow at 37°C on YPD and at 28°C on a subset of nonfermentable carbon sources (glycerol, pyruvate, and acetate). When we

Table 1. Yeast strains used in this study

Strain	Genotype	Source
JSY836	<i>Mata</i> , <i>ura3-52</i> , <i>his3Δ200</i> , <i>trp1Δ63</i>	This laboratory
JSY118	<i>Mata</i> , <i>ura3-52</i> , <i>his3Δ200</i> , <i>trp1Δ63</i> , <i>ptc1::TRP1</i>	This study
FY250	<i>Mataα</i> , <i>Mal</i> , <i>Gal2⁺</i> , <i>ura3-52</i> , <i>his3Δ200</i> , <i>trp1Δ63</i> , <i>leu2Δ1</i>	F. Winston (Winston <i>et al.</i> , 1995) ^a
AMY36	<i>Mataα</i> , <i>Mal</i> , <i>Gal2⁺</i> , <i>ura3-52</i> , <i>his3Δ200</i> , <i>trp1Δ63</i> , <i>leu2Δ1</i> <i>hog1::URA3</i>	This study
AMY38	<i>Mata/α</i> , <i>ura3-52/ura3-52</i> , <i>his3Δ200/his3Δ200</i> , <i>trp1Δ63/trp1Δ63</i> , <i>ptc1::TRP1/+</i> , <i>+/leu2Δ1</i> , <i>hog1::URA3/+</i>	This study
AMY43	<i>Mata</i> , <i>ura3-52</i> , <i>his3Δ200</i> , <i>trp1Δ63</i> , <i>ptc1::TRP1</i> , <i>hog1::URA3</i>	This study
SEY6210	<i>Mata</i> , <i>ura3-52</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>lys2-801</i> <i>suc2-Δ9</i> , <i>GAL</i>	S. Emr (Robinson <i>et al.</i> , 1988)
JSY2090	<i>Mata</i> , <i>ura3-52</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>lys2-801</i> <i>suc2-Δ9</i> , <i>GAL</i> , <i>ptc1::URA3</i>	T. Ketela and H. Bussey
JSY2092	<i>Mataα</i> , <i>ura3-52</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>lys2-801</i> <i>suc2-Δ9</i> , <i>GAL</i> , <i>pbs2::KANMX2</i>	T. Ketela and H. Bussey
JSY2093	<i>Mata</i> , <i>ura3-52</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>lys2-801</i> <i>suc2-Δ9</i> , <i>GAL</i> , <i>pbs2::KANMX2</i> , <i>ptc1::URA3</i>	T. Ketela and H. Bussey
JSY2589	<i>Mata</i> , <i>ura3-52</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-Δ200</i> , <i>trp1-Δ901</i> , <i>lys2-801</i> <i>suc2-Δ9</i> , <i>GAL</i> , <i>ptp2::KANMX2</i>	T. Ketela and H. Bussey

^a Same genotype as FY834 but *LYS2*.

generated a disruption of *PTC1* (*ptc1Δ*) in our standard laboratory strain background (see MATERIALS AND METHODS), we observed similar growth defects on nonfermentable carbon sources (glycerol, ethanol, and acetate). In addition, in our strain background, the *ptc1Δ* mutant grew slower than wild type on solid YPG medium at 25 and 30°C and did not form colonies at 37°C (Figure 1D). Although our *ptc1* mutant grew essentially as well as wild type on YPD plates at 25 and 30°C, colonies formed at 37°C were much smaller than wild type (Figure 1C). Similar differences in wild-type and *ptc1Δ* growth rates were also apparent in log phase cells grown in YPD and YPG liquid medium (Figure 1, A and B).

Mitochondrial Inheritance Is Delayed, But Not Blocked, In Cells Lacking PTC1

When a wild-type strain is grown at 25°C (Figure 2, A and B) or 37°C (Roeder, Hermann, Keegan, Thatcher, and Shaw, unpublished observations) in YPD liquid

medium, mitochondrial networks visualized with a targeted form of the green fluorescent protein (Cox4-GFP) were easily detected in growing buds, regardless of bud size. In contrast, *ptc1Δ* mutant cells often produced buds that lacked this mitochondrial staining at 25°C (Figure 2, C and D) and at 37°C (Roeder, Hermann, Keegan, Thatcher, and Shaw, unpublished data). Despite this mitochondrial inheritance defect, the *ptc1* mutant appeared to grow in YPD liquid culture at both temperatures (Figure 1A), suggesting that at least some buds produced by this strain were viable. Quantification of mitochondrial inheritance in the *ptc1Δ* strain revealed that a high percentage of small *ptc1Δ* buds lacked DiOC₆-stained mitochondrial networks (63.0% at 25°C, 66.7% at 30°C, and 66.7% at 37°C). However, by the time buds were half the diameter of the mother cell (large buds), the defect was less severe (14.9% at 25°C, 16.7% at 30°C, and 5.6% at 37°C; Table 3). These results suggested that the timing of mitochondrial transfer to buds might be delayed, rather than blocked, in the *ptc1* mutant strain. To test this possibility, we compared mitochondrial inheritance defects in wild-type and *ptc1* cells during a single round of budding at 25, 30, and 37°C in YPD liquid medium after α -factor arrest and release. (This experiment could not be done in YPG medium, because *ptc1Δ* mutant cells never bud after α -factor arrest and release when glycerol is provided as the sole carbon source). As shown in Tables 4–6 and represented graphically in Figure 3, when wild-type cells arrested in the unbudded stage were released, the majority of newly formed small buds (Figure 3, A, C, and E, open triangles, 1-h time point) contained DiOC₆-stained mitochondrial networks (Figure 3, A, C and E, closed diamonds, 1-h time point). As expected, at later time points the large buds also contained mitochondrial networks (Figure 3, A, C and E, open

Table 2. Comparison of the mitochondrial inheritance delay in DiOC₆ and Cox4-GFP-stained *ptc1Δ* cells

Strain	Temperature (°C)	Mitochondrial label	% of buds without mitochondria (n) ^a
JSY836 (WT)	25	DiOC ₆	1 (201)
	25	Cox4-GFP	1 (400)
JSY118 (<i>ptc1Δ</i>)	25	DiOC ₆	24.9 (205)
	25	Cox4-GFP	27 (400)
JSY836 (WT)	37	DiOC ₆	3 (202)
	37	Cox4-GFP	5.9 (407)
JSY118 (<i>ptc1Δ</i>)	37	DiOC ₆	51.7 (201)
	37	Cox4-GFP	40 (403)

^a n, Number of budded cells scored.

Figure 1. Growth of wild-type (*PTC1*) and *ptc1Δ* cells at 25, 30, and 37°C. The OD₆₀₀ values of *PTC1* and *ptc1Δ* cells grown in YPD (A) or YPG (B) at 25, 30, or 37°C were measured at the indicated time intervals. *PTC1* and *ptc1Δ* strains were serially diluted, spotted onto solid YPD (C) or YPG (D) medium, and grown at 25, 30, and 37°C for 3 d.

circles). In the *ptc1Δ* cells, the majority of small buds produced (Figure 3, B, D, and F, open triangles, see 1-h time point) did not contain mitochondrial networks (Figure 3, B, D, and F, closed diamonds, see 1-h time point). However, the proportion of buds containing mitochondria increased as bud size increased, indicating that mu-

tations in *PTC1* caused a *delay*, rather than a complete block, in mitochondrial inheritance (Figure 3, B, D, and F, open circles and closed diamonds). DAPI staining did not reveal a similar delay in nuclear segregation, suggesting that mutations in *PTC1* do not cause a general delay in cytoplasmic organelle transfer to buds.

Figure 2. *ptc1Δ* cells exhibit defects in mitochondrial inheritance. DIC images (A and C) and Cox4-GFP mitochondrial staining (B and D) of representative wild-type (*PTC1*) and *ptc1Δ* cells grown at 25°C are shown. Bar, 5 μ m.

ptc1Δ cells failed to grow in YPG medium at 37°C (Figure 1, B and D); however, this growth defect was not simply attributable to the failure of *ptc1* buds to inherit mitochondria. Although *ptc1Δ* cells undergo an immediate cell cycle arrest upon transfer to 37°C in YPG medium (Figure 1B), the percentage of budded cells in the arrested cultures that contained DiOC₆-stained mitochondrial networks was comparable to wild type (Table 7; wild type, 7.8%, vs. *ptc1Δ*, 7.0%). In addition, a delay in mitochondrial inheritance cannot completely explain the slow growth observed for *ptc1Δ* cells in YPG medium at 25 and 30°C (Figure 1, B and D). At 25°C, we observed only a slight mitochondrial inheritance delay in the *ptc1* mutant relative to wild type (Table 7, 10.2 and 4.9%, respectively) and no

delay in the *ptc1* mutant relative to wild type at 30°C (Table 7, 7.7 and 10.3%, respectively). Although the doubling times of *ptc1Δ* cells grown in YPD medium at 25°C (2.5 h) and 30°C (2.2 h) are comparable to wild type (2.3 h at 25°C, 2.3 h at 30°C), the *ptc1* mutant cells divide more slowly than wild-type cells in YPG medium (*ptc1Δ*: 18.9 h at 25°C, 22.7 h at 30°C; wild type: 10.1 h at 25°C, 5.9 h at 30°C). Thus, mitochondrial inheritance apparently “catches up” with slowly growing buds when *ptc1* cells are cultured in glycerol at 25 and 30°C, and, as a result, no severe delay phenotype is detected.

Increased Frequency of Petite Generation in *ptc1Δ* Cells

We also observed that *ptc1Δ* cells maintained on solid YPD medium generated petites (lost mitochondrial genome function) at a higher frequency than wild type. At 30°C, 24.4% of *ptc1Δ* cells became petite compared with 1.8% in an isogenic wild-type strain. This increased frequency of petite generation in *ptc1Δ* probably contributes to the growth defects observed for this strain on YPG medium (Figure 1). Although increases in the frequency of petite generation have been observed for other strains defective in mitochondrial distribution and morphology, the molecular basis for this phenomenon is unclear (reviewed in Berger and Yaffe, 1996). An increased frequency of petite formation could be attributable to the fact that buds sometimes inherit DNA-free mitochondrial compartments. To test this possibility for the *ptc1* mutant, aliquots of fixed, wild-type, and *ptc1Δ* cultures (YPD, 30°C) were labeled with anti-porin antibodies to quantify the inheritance of mitochondrial compartments. The samples were simultaneously labeled with DAPI to quantify the inheritance of mitochondrial nucleoids. In wild-type cultures, only 5.3% of small buds and 0% of large buds lacked anti-porin-stained mitochondrial net-

Table 3. Mitochondrial inheritance in *PTC1* and *ptc1Δ* cells grown in YPD

Strain	Temperature (°C)	% of unbudded cells	% of small ^a budded cells	% of small buds without mitochondria	% of large ^b budded cells	% of large buds without mitochondria	% of total buds without mitochondria
JSY836 (WT)	25	24	37	5.4	39	0	2.6
JSY118 (<i>ptc1Δ</i>)	25	26	27	63.0	47	14.9	32.4
JSY836 (WT)	30	39	21	0	40	0	0
JSY118 (<i>ptc1Δ</i>)	30	40	18	66.7	42	16.7	31.7
JSY836 (WT)	37	30	35	11.4	35	0	5.7
JSY118 (<i>ptc1Δ</i>)	37	22	24	66.7	54	5.6	24.4

The data shown correspond to the 6-h time point in Figure 1A. At least 100 cells were scored for each strain at each temperature.
^a Buds less than half the diameter of the mother cell.
^b Buds greater than half the diameter of the mother cell.

Table 4. Mitochondrial inheritance is delayed in *ptc1Δ* cells after α -factor arrest and release at 25°C

Strain	Time after release from α -factor (h)	% of unbudded cells	% of small buds ^a	% of small buds without mitochondria	% of large buds ^b	% of large buds without mitochondria	% of multiply budded cells ^c	% of multiply budded cells without mitochondria in largest bud
WT	0.5	96	4	25	0		0	
<i>ptc1Δ</i>	0.5	99	0	0	1	0	0	
WT	1.0	25	72	9.7	3	0	0	
<i>ptc1Δ</i>	1.0	37	59	93.2	4	25	0	
WT	1.5	25	29	3.4	46	0	0	
<i>ptc1Δ</i>	1.5	32	48	87.5	20	5	0	
WT	2.0	8	20	10	71	1.4	1	0
<i>ptc1Δ</i>	2.0	4	23	78.3	72	2.9	1	0
WT	2.5	16	56	7.1	28	0	0	
<i>ptc1Δ</i>	2.5	13.9	16.8	88.2	60.4	0	8.9	0
WT	3.0	10	56	5.4	34	0	0	
<i>ptc1Δ</i>	3.0	8	11	81.8	26	7.7	55	3.6
WT	3.5	41	14	7.1	45	0	0	
<i>ptc1Δ</i>	3.5	10	10	80	19	0	61	0

At least 100 cells were scored for each strain at each time point.

^a Buds less than half the diameter of the mother cell.

^b Buds greater than half the diameter of the mother cell.

^c An increase in the percentage of multiply budded cells is observed in *ptc1Δ* cells after α -factor treatment. The percentage of unbudded wild-type cells upon α -factor release at 25°C was 99%. The percentage of unbudded *ptc1Δ* cells on α -factor release at 25°C was 90%.

works. Similarly, only 11.4% of small and 0% of large wild-type buds lacked DAPI-stained mitochondrial nucleoids. In *ptc1Δ* cultures, 81.1% of small and 10.3% of large buds lacked anti-porin-stained networks. Again, similar results were observed when *ptc1Δ* cells were stained with DAPI. In this case, 87.2% of small and 3.3% of large *ptc1Δ* buds lacked DAPI-stained nucleoids. These results

suggest that both the mitochondrial compartment and mitochondrial nucleoids are eventually inherited by large *ptc1Δ* buds. Although it seems likely that there is some relationship between the mitochondrial inheritance delay and the high frequency of petite generation in the *ptc1Δ* strain, further studies will be required to determine the mechanism of petite formation.

Table 5. Mitochondrial inheritance is delayed in *ptc1Δ* cells after α -factor arrest and release at 30°C

Strain	Time after release from α -factor (h)	% of unbudded cells	% of small buds ^a	% of small buds without mitochondria	% of large buds ^b	% of large buds without mitochondria	% of multiply budded cells ^c	% of multiply budded cells without mitochondria in largest bud
WT	0.5	78	22	4.5	0		0	
<i>ptc1Δ</i>	0.5	84	16	93.8	0		0	
WT	1.0	19	43	4.7	38	0	0	
<i>ptc1Δ</i>	1.0	26	69	98.6	5	40	0	
WT	1.5	2	9	11.1	86	0	3	0
<i>ptc1Δ</i>	1.5	7	20	80	73	17.8	0	
WT	2.0	6	27	7.4	67	1.5	0	
<i>ptc1Δ</i>	2.0	4	12	91.7	55	18.2	29	3.4
WT	2.5	27	13	7.7	60	0	0	
<i>ptc1Δ</i>	2.5	4	6	83.3	26	7.7	64	9.4

At least 100 cells were scored for each strain at each time point.

^a Buds less than half the diameter of the mother cell.

^b Buds greater than half the diameter of the mother cell.

^c An increase in the percentage of multiply budded cells is observed in *ptc1Δ* cells after α -factor treatment. The percentage of unbudded wild-type cells upon α -factor release at 30°C was 99%. The percentage of unbudded *ptc1Δ* cells on α -factor release at 30°C was 98%.

Table 6. Mitochondrial inheritance is delayed in *ptc1Δ* cells after α -factor arrest and release at 37°C

Strain	Time after release from α -factor (h)	% of unbudded cells	% of small budded cells ^a	% of small buds without mitochondria	% of large budded cells ^b	% of large buds without mitochondria	% of multiply budded cells ^c	% of multiply budded cells without mitochondria in largest bud
WT	0.5	50	48	22.9	2	0	0	
<i>ptc1Δ</i>	0.5	50	45	93.3	5	60	0	
WT	1.0	9	78	6.4	13	0	0	
<i>ptc1Δ</i>	1.0	12	75	88	13	30.8	0	
WT	1.5	34	18	22.2	48	0	0	
<i>ptc1Δ</i>	1.5	15.7	30.4	90.3	53.9	41.8	0	
WT	2.0	34	31	12.9	35	0	0	
<i>ptc1Δ</i>	2.0	15	9	77.8	55	18.2	21	0
WT	2.5	51	35	11.4	14	0	0	
<i>ptc1Δ</i>	2.5	16.8	14.9	60	29.7	46.7	38.6	23.1
WT	3.0	19	56	5.4	24	0	1	0
<i>ptc1Δ</i>	3.0	27.3	21.2	76.2	7.1	14.3	44.4	9.1

At least 100 cells were scored for each strain at each time point.

^a Buds less than half the diameter of the mother cell.

^b Buds greater than half the diameter of the mother cell.

^c An increase in the percentage of multiply budded cells is observed in *ptc1Δ* cells after α -factor treatment. The percentage of unbudded wild-type cells upon α -factor release 37°C was 98%. The percentage of unbudded *ptc1Δ* cells on α -factor release at 37°C was 88%.

The Mitochondrial Inheritance Delay in *ptc1Δ* Cells Is Not Attributable to Changes in Actin Organization

In yeast, filamentous actin (F-actin) is organized into cortical patches and cables (bundles of F-actin) that undergo cell cycle-regulated changes in distribution (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Winsor and Schiebel, 1997). Actin patches are required for cell expansion and are found randomly distributed in unbudded cells, clustered in the growing bud early in division, and assembled at the mother bud neck during cytokinesis. Actin cables are also randomly distributed in unbudded cells but become polarized along the mother bud axis during division. A number of studies suggest that the actin cytoskeleton plays an important role in regulating mitochondrial distribution, morphology, and inheritance, although the exact nature of this role is not yet clear. Mitochondrial membranes have been shown to align along some actin cables in yeast cells, and certain mutant actin alleles exhibit defects in mitochondrial morphology, distribution, and motility (Drubin *et al.*, 1993; Lazzarino *et al.*, 1994; Simon *et al.*, 1995). In addition, cells lacking a novel protein, Mdm20p, completely lack actin cables and display severe defects in mitochondrial inheritance (Hermann *et al.*, 1997). In vitro, yeast mitochondria can bind to phalloidin-stabilized actin filaments and display a myosin-like, actin-based motor activity on their surface (Lazzarino *et al.*, 1994; Simon *et al.*, 1995). This activity may be attributable to a novel motor protein, since mutations in the known *S. cere-*

visiae myosin genes do not block mitochondrial movement in vitro (Simon *et al.*, 1995).

An increase in external osmolarity and activation of the HOG kinase cascade induces yeast to accumulate glycerol (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Posas *et al.*, 1996; see below). Previous work indicated that this accumulation of intracellular glycerol is accompanied by a transient disassembly of the actin cytoskeleton (Brewster and Gustin, 1994). Because mutations in *PTC1* have been shown to activate the *HOG* pathway (Jiang *et al.*, 1995), we examined the possibility that the mitochondrial inheritance delay observed in the *ptc1Δ* strain was the result of a defect in organization of the actin cytoskeleton. After fixing *ptc1* mutant cells grown at 30°C in YPD medium, actin cytoskeleton organization (visualized by indirect immunofluorescence with goat anti-actin antibodies) and mitochondrial distribution (visualized by mouse anti-porin antibody staining) were scored in individual cells. As summarized in Table 8, the organization of actin patches and cables was similar to wild type in dividing *ptc1Δ* cells that contained mitochondrial networks in growing buds (Figure 4, compare A and B with C and D). More importantly, polarized actin cables and clustered bud patches were also present in dividing *ptc1Δ* cells that had not yet transported anti-porin-stained mitochondrial networks into growing buds (Figure 3, E and F). Although we cannot rule out the possibility that subtle changes in actin organization are occurring in *ptc1Δ* cells, these results suggest that the mitochondrial inheritance delay in *ptc1Δ* does not result from global defects in actin organization.

Figure 3. Mitochondrial inheritance is delayed in *ptc1Δ* cells. After release from α -factor arrest, wild-type (A, C, and E) and *ptc1Δ* (B, D, and F) cells were scored for bud size (open squares, triangles, and circles) and mitochondrial inheritance (closed diamonds) by the bud at 25°C (A and B), 30°C (C and D), and 37°C (E and F). The percentages of unbudded wild-type cells upon α -factor release at 25, 30, and 37°C were 99, 99, and 98%, respectively. The percentages of unbudded *ptc1Δ* cells upon α -factor release at 25, 30, and 37°C were 90, 98, and 88%, respectively. Note that at 0.5 h in B, only one budded cell (Table 4; 1%, $n = 100$) was present, and this bud contained mitochondria.

Table 7. Mitochondrial inheritance in *PTC1* and *ptc1Δ* cells grown in YPG

Strain	Temperature (°C)	% of unbudded cells	% of small ^a budded cells	% of small buds without mitochondria	% of large ^b budded cells	% of large buds without mitochondria	% of total buds without mitochondria
JSY836 (WT)	25	39	38	7.9	23	0	4.9
JSY118 (<i>ptc1Δ</i>)	25	51	29	17.2	20	0	10.2
JSY836 (WT)	30	42	33	18.2	25	0	10.3
JSY118 (<i>ptc1Δ</i>)	30	48	30	13.3	22	0	7.7
JSY836 (WT)	37	36	39	12.8	25	0	7.8
JSY118 (<i>ptc1Δ</i>)	37	43	35	11.4	22	0	7.0

The data shown correspond to the 16-h time point in Figure 1B. At least 100 cells were scored for each strain at each temperature.

^a Buds less than half the diameter of the mother cell.

^b Buds greater than half the diameter of the mother cell.

Elevating Intracellular Osmolarity Does Not Cause a Mitochondrial Inheritance Delay

The HOG pathway plays a critical role in the yeast osmopressure response and is composed of a signal transducer (Sln1p, Ypd1p, and Ssk1p) and an MAP kinase cascade (Ssk2p/Ssk22p, Pbs2p, and Hog1p) (Figure 5). Under conditions of normal extracellular osmolarity, the Sln1p histidine kinase is autophosphorylated, and a relay system sequentially transfers the phosphate from Sln1p to Ypd1p and finally to Ssk1p (Posas *et al.*, 1996). Phosphorylated Ssk1p cannot activate the Ssk22p and Ssk2p MAPKKs and, as a result, signaling via the Hog1p kinase is inhibited. When extracellular osmolarity is high, the Sln1p kinase is inhibited, the active, unphosphorylated form of Ssk1p interacts with the Ssk22p and Ssk2p MAPKKs, and the HOG MAP kinase cascade is turned on. Activation of the Hog1p kinase at the end of this cascade results in a variety of cellular responses, including production of intracellular glycerol. Pbs2p (MAPKK) and Hog1p (MAPK) in the pathway are thought to be dephosphorylated and down-regulated by

the serine/threonine phosphatases Ptc1p and Ptc3p (Maeda *et al.*, 1994) and the tyrosine phosphatases Ptp2p and Ptp3p (Maeda *et al.*, 1994; Wurgler-Murphy *et al.*, 1997), as illustrated in Figure 5.

If Ptc1p dephosphorylates and down-regulates Hog1p and/or Pbs2p, then cells lacking *PTC1* should have elevated Hog1p kinase activity. As a result, intracellular glycerol concentrations should be elevated in the mutant even in low extracellular osmolarity (Jiang *et al.*, 1995; see MATERIALS AND METHODS). To determine whether elevated glycerol concentrations account for the mitochondrial inheritance delay observed in the *ptc1Δ* mutant, wild-type and *ptc1Δ* cells expressing the Cox4-GFP protein were grown asynchronously overnight in YPD at

Table 8. Actin distribution is normal in *ptc1Δ* cells

	JSY836 (WT) (n = 200)	JSY118 (<i>ptc1Δ</i>) (n = 200)
% of cells with mitochondria in buds ^a	95.0	54.5
% of cells with mitochondria in buds; wild-type actin organization	98.4	96.3
% of cells without mitochondria in buds; wild-type actin organization	100.0	100.0

Double staining of the actin cytoskeleton and the mitochondrial network was observed in 100% of wild-type cells and 99% of *ptc1Δ* cells in these experiments. n, Number of budded cells scored.

^a Only budded cells were scored.

Figure 4. Actin cables and patches are present and properly localized in *PTC1* and *ptc1Δ* cells that contain mitochondria in buds (C and D) and in cells that have not transported mitochondria into buds (E and F). Wild-type (*PTC1*) and *ptc1Δ* cells grown in YPD medium at 30°C were fixed and stained with both actin (B, D, and F) and porin (A, C, and E) antibodies. Bar, 5 μ m.

Figure 5. Proposed regulatory role of Ptc1p in the HOG response pathway (Meada *et al.*, 1994; Posas *et al.*, 1996). When yeast cells are exposed to conditions of high extracellular osmolarity, the HOG MAP kinase cascade (shown above) is activated, producing a variety of cellular responses, including glycerol production. Under conditions of normal extracellular osmolarity, the kinase cascade is inactivated. Yeast cells also contain a second transmembrane osmosensor, Sho1p (not shown), which activates the Pbs2p kinase directly in response to high osmolarity (Meada *et al.*, 1995; Posas *et al.*, 1996).

25°C and transferred into YPD medium supplemented with 0.9 M NaCl. Previous studies have shown that addition of NaCl to the medium induces glycerol synthesis in wild-type yeast (Edgley and Brown, 1983; Blomberg and Adler, 1989; see MATERIALS AND METHODS). To allow the strains to recover from the initial osmotic shock and resume budding, mitochondrial distributions were scored at 5- and 24-h time points. As summarized in Table 9, very few of the wild-type cells (6%) exhibited a mitochondrial inheritance defect after 5 h at 25°C in the high-osmolarity-producing medium. In contrast, 40.5% of the *ptc1Δ* cells produced buds without mitochondria (Table 9). Even after 24 h of culture, the percentage of wild-type buds without mitochondria remained low (3.5%), although *ptc1Δ* cells still produced buds lacking the organelle (20%). These results indicate that increasing intracellular osmolarity in a wild-type strain does not induce a delay in mitochondrial inheritance.

Ptc1p Is Not Acting Through the HOG Pathway to Affect the Mitochondrial Transport Machinery

Genetic evidence implicates Ptc1p as a negative regulator of the Hog1p and/or Pbs2p kinases in the HOG

Table 9. Mitochondrial inheritance is not affected by culture in high-osmolarity medium

Strain	Growth medium	% of buds without mitochondria ^a	
		5 h (n) ^b	24 h (n) ^b
JSY836 (WT)	YPD + 0.9 M NaCl	6.0 (402)	3.5 (403)
JSY118 (<i>ptc1Δ</i>)	YPD + 0.9 M NaCl	40.5 (400)	20.0 (400)

^a Only budded cells were counted.
^b Hours after transfer to high-osmolarity medium. n, Number of budded cells scored.

pathway (Maeda *et al.*, 1993, 1994). Thus, it was formally possible that hyperactive Hog1p or Pbs2p kinases in this pathway were responsible for the mitochondrial inheritance delay we observed in the *ptc1* mutant. If this model were correct, disruption of the *HOG1* or *PBS2* gene in a *ptc1* strain should abolish (or at least reduce) the mitochondrial inheritance defect observed in the *ptc1* mutant alone. To test this possibility, mitochondrial inheritance was compared in *ptc1 hog1* and *ptc1 pbs2* double mutants and isogenic control strains (wild type, *ptc1Δ*, *hog1Δ*, and *pbs2Δ* single mutant strains) in YPD medium at 25°C. As shown in Table 10, only a small percentage of the wild-type (JSY836) and *hog1Δ* (AMY36) cells produced buds lacking DiOC₆-stained mitochondrial networks (4.7 and 2.3%, respectively). More importantly, the *ptc1Δ hog1Δ* double mutant (AMY43) did not exhibit a less pronounced mitochondrial inheritance defect than that observed in the *ptc1Δ* single mutant (JSY118) (Table 10; 26.9 and 29.6% of buds without mitochondrial

Table 10. Epistasis analysis between *ptc1Δ* and HOG1 pathway mutants

Strain (25°C in YPD)	% of buds without mitochondria (n) ^a
JSY836 (WT)	4.7 (301)
JSY118 (<i>ptc1Δ</i>)	29.6 (301)
AMY36 (<i>hog1Δ</i>)	2.3 (301)
AMY43 (<i>ptc1Δ, hog1Δ</i>)	26.9 (305)
SEY6210 (WT)	2.7 (300)
JSY2090 (<i>ptc1Δ</i>)	33.3 (600)
JSY2092 (<i>pbs2Δ</i>)	1.3 (300)
JSY2093 (<i>ptc1Δ, pbs2Δ</i>)	29.6 (301)
JSY2589 (<i>ptp2Δ</i>)	1.2 (85)
SEY6210 (<i>ptc1Δ, ptp2Δ</i>)	ND ^b

^a Only budded cells were counted. n, Number of budded cells scored.
^b ND, Because of the severity of the synthetic growth defect in the *ptc1Δ, ptp2Δ* double mutant, mitochondrial inheritance could not be analyzed in this strain.

staining, respectively). In addition, a mitochondrial inheritance defect was not observed in the *pbs2Δ* mutant (JSY2092, 1.3%) relative to the wild-type control (SEY6210, 2.7%), and comparable mitochondrial inheritance defects were displayed by the *ptc1Δ* single (JSY2090, 33.3%) and *ptc1Δ pbs2Δ* double (JSY2093, 29.6%) mutant strains (Table 10). These results indicate that: 1) Hog1p and Pbs2p are not required for mitochondrial inheritance in yeast; and 2) the serine/threonine phosphatase Ptc1p is not exerting its effect on the timing of mitochondrial transport through the activities of these two kinases. [Cells lacking Ptp2p, a tyrosine phosphatase known to down-regulate Hog1p activity, did not exhibit a delay in mitochondrial transport either (Table 10).] Whether Ptc1p is affecting the mitochondrial inheritance machinery directly or indirectly through some other cellular pathway remains to be determined.

DISCUSSION

We have shown that cells lacking the serine/threonine phosphatase encoded by *PTC1* exhibit a pronounced delay, but not a complete block, in mitochondrial inheritance during mitotic growth. The mitochondrial inheritance delay in *ptc1Δ* cells is not attributable to defects in the organization of the actin cytoskeleton, which is known to play a role in mitochondrial transport (Lazzarino *et al.*, 1994; Simon *et al.*, 1995; Hermann *et al.*, 1997). Although Ptc1p is thought to be a negative regulator of the HOG pathway, we demonstrated that the mitochondrial inheritance defect in *ptc1Δ* strains does not result from changes in intracellular glycerol concentrations. Furthermore, Ptc1p is not acting through the HOG pathway kinases Hog1p or Pbs2p to control the timing of mitochondrial transfer to buds; the mitochondrial inheritance delay observed in the *ptc1Δ* strain does not change substantially in *ptc1Δ hog1Δ* or *ptc1Δ pbs2Δ* double mutants. Instead, Ptc1p appears to be acting either directly or through a different signaling pathway to affect the mitochondrial transport machinery in the cell.

Ptc1p is the first serine/threonine phosphatase reported to affect mitochondrial transport in yeast. Although it is possible that dephosphorylation by Ptc1p directly regulates the activities of one or more proteins required to move the mitochondrial network into buds, only a few proteins required for mitochondrial inheritance have been isolated to date, and none of them have been shown to be phosphorylated. Alternatively, Ptc1p may be affecting mitochondrial inheritance indirectly through a kinase cascade other than the HOG pathway. The protein kinase C (PKC) pathway is currently the only other signaling cascade proposed to be regulated by Ptc1p (Huang and Symington, 1995). Protein kinase C (Pkc1p) regulates an MAP kinase cascade (*BCK1/SLK1*, *MKK1/2*, and *MPK1/*

SLT2) implicated in cell wall metabolism (Errede and Levin, 1993), DNA metabolism (Huang and Symington, 1994), and response to decreases in extracellular osmolarity (Davenport *et al.*, 1995). Mutations in *PTC1* were shown to suppress temperature-sensitive defects of a *pkc1* allele and were also found to be lethal in combination with mutations in *MPK1*, the terminal MAP kinase in the PKC pathway (Huang and Symington, 1994). Due to the synthetic lethality of the *ptc1 mpk1* double mutant, we were unable to determine whether a hyperactive Mpk1p kinase is responsible for the mitochondrial inheritance delay observed in the *ptc1Δ* mutant. Further analysis is required to determine whether Ptc1p is affecting mitochondrial transport through the upstream *MKK1* and *MKK2* encoded serine/threonine kinases in the PKC pathway or kinases in another signaling pathway.

Although *PTC1* is the first single locus reported to control the timing of mitochondrial inheritance, a similar phenotype may occur in a strain carrying mutations in two different genes (*BRO1* and *CAF1*; Nickas and Yaffe, 1996). Interestingly, *BRO1* encodes a novel protein that also interacts genetically with Mpk1p, the terminal kinase in the PKC pathway. A detailed characterization of mitochondrial phenotypes in the *BRO1* and *CAF1* single and double mutants may provide additional insights regarding the link between the PKC kinase cascade and the temporal control of mitochondrial inheritance.

In principle, mitochondrial inheritance could be accomplished by at least three different mechanisms: 1) the mitochondrial network could diffuse into the bud as it forms; 2) the mitochondrial network could be transported into buds through a cytoskeletal-based motor activity; or 3) the mitochondrial network could attach to the incipient bud site and be passively "pulled" into the expanding daughter cell. Our observation that the mitochondrial network can move into large *ptc1Δ* buds well after they have already formed suggests that mitochondrial attachment to the bud site is not strictly required for inheritance or that multiple mechanisms are operating to transport this organelle into the bud. Although other interpretations are possible, we believe our findings eliminate the attachment model as the primary mechanism of mitochondrial inheritance in yeast.

Studies of living yeast cells suggest that mitochondrial inheritance routinely occurs early in the cell cycle, after inheritance of the endoplasmic reticulum and Golgi apparatus but before the segregation of the nucleus. Our studies of the *ptc1Δ* mutant provide the first genetic evidence that the timing of this mitochondrial transport is actively regulated and coordinated with the cell cycle. It is somewhat surprising that the mitochondrial transport delay in *ptc1Δ* is not accompanied by a more dramatic change in the growth properties of these cells. Apparently, the order in which some es-

sential cytoplasmic organelles are inherited is not critical as long as some part of that compartment is received by the bud before cytokinesis. This flexibility may help ensure that small perturbations in organelle distribution during cell division do not lead to drastic effects on cell division.

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