***PTC1* Is Required for Vacuole Inheritance and Promotes the Association of the Myosin-V Vacuole-specific Receptor Complex**

Yui Jin, P. Taylor Eves, Fusheng Tang,* and Lois S. Weisman

Life Sciences Institute, Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-2216

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Organelle inheritance occurs during cell division. In *Saccharomyces cerevisiae*, inheritance of the vacuole, and the distribution of mitochondria and cortical endoplasmic reticulum are regulated by Ptc1p, a type 2C protein phosphatase. Here we show that *PTC1/VAC10* controls the distribution of additional cargoes moved by a myosin-V motor. These include peroxisomes, secretory vesicles, cargoes of Myo2p, and *ASH1* mRNA, a cargo of Myo4p. We find that Ptc1p is required for the proper distribution of both Myo2p and Myo4p. Surprisingly, *PTC1* is also required to maintain the steady-state levels of organelle-specific receptors, including Vac17p, Inp2p, and Mmr1p, which attach Myo2p to the vacuole, peroxisomes, and mitochondria, respectively. Furthermore, Vac17p fused to the cargo-binding domain of Myo2p suppressed the vacuole inheritance defect in *ptc1Δ* cells. These findings suggest that *PTC1* promotes the association of myosin-V with its organelle-specific adaptor proteins. Moreover, these observations suggest that despite the existence of organelle-specific receptors, there is a higher order regulation that coordinates the movement of diverse cellular components.

INTRODUCTION

During each cell cycle, cytoplasmic organelles are actively distributed between dividing cells to maintain organelle copy number and volume. The yeast *Saccharomyces cerevisiae* is an excellent model system for studying the spatial and temporal control of organelle inheritance. In yeast, several organelles are transmitted from mother to daughter cells. These include the vacuole, mitochondria, the endoplasmic reticulum (ER), late-Golgi elements and peroxisomes (reviewed in Weisman, 2006).

Early in the cell cycle, a portion of each organelle is transported into the emerging bud. The polarized transport of most organelles from the mother to the bud is an active process that requires the actin cytoskeleton, myosin-V motors, and receptor proteins, which physically connect the motor to organelle cargoes (Beach *et al.*, 2000; Yin *et al.*, 2000; Boldogh *et al.*, 2001; Barr, 2002; Bretscher, 2003; Du *et al.*, 2004; Fagarasanu *et al.*, 2006b, 2007; Weisman, 2006). Thus, formation of a complex between the motor and receptor protein is important for polarized organelle transport.

Similar to yeast, in vertebrates, myosin-V motors move cargoes along the actin cytoskeleton. The best studied cargo of vertebrate myosin-V are melanosomes, which are moved in melanocytes by myosin-Va. Melanosomes attach to myosin-Va through Rab27a and melanophilin (Fukuda and Kuroda, 2002; Wu *et al.*, 2002). Rab27a, through geranylgeranylation, attaches to the melanosome membrane,

and melanophilin connects myosin-Va and Rab27a. Myosin-V-based intracellular movement has been analyzed in many other eukaryotes, including frogs, fish, mammals, and plants; plant myosin-XI is the functional homologue of yeast and vertebrate myosin-V (Kinkema and Schiefelbein, 1994; Provance and Mercer, 1999; Tuma and Gelfand, 1999; Desnos *et al.*, 2007; Li and Nebenfuhr, 2007; Sheets *et al.*, 2007; Shimmen, 2007).

Genetic screens have identified several molecules that play a role in organelle inheritance. For example, vacuole inheritance utilizes the actin cytoskeleton and the class-V myosin complex, composed of Myo2p, Vac17p, and Vac8p (Hill *et al.*, 1996; Wang *et al.*, 1998; Ishikawa *et al.*, 2003; Tang *et al.*, 2003). Myo2p is one of two class-V myosin motors in budding yeast. Vac17p links Myo2p to the vacuole. Regulation of *VAC17* likely plays a critical role in the control of vacuole movement. Protein and mRNA levels of *VAC17* are tightly regulated during the cell cycle (Spellman *et al.*, 1998; Zhu *et al.*, 2000; Tang *et al.*, 2003). In addition, phosphorylation of Vac17p plays a role in the initiation of vacuole inheritance (Peng and Weisman, 2008). Increased synthesis of *VAC17* is likely a key event in the initiation of vacuole movement. Likewise, degradation of Vac17p is required for the termination of vacuole inheritance and the detachment of Myo2p from the vacuole membrane (Tang *et al.*, 2003). Vac8p binds directly to Vac17p and attaches to the vacuole membrane via myristoylation and palmitoylation (Wang *et al.*, 1998; Peng *et al.*, 2006; Tang *et al.*, 2006). Although the regulation of *VAC17* contributes to the control of vacuole inheritance, other members of the myosin-V transport complex are also potential targets for the regulation of vacuole movement.

Other yeast organelles are also moved by myosin-V. Mitochondria are moved in part by Myo2p through interaction with Mmr1p and Ypt11p (Itoh *et al.*, 2002, 2004; Boldogh *et al.*, 2004; Altmann *et al.*, 2008; Valiathan and Weisman, 2008).

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* Present address: Department of Biology, University of Arkansas, Little Rock, AR 72204-1099.

Address correspondence to: Lois S. Weisman (lweisman@umich.edu).

Peroxisomes are moved by a complex of Myo2p and the peroxisomal membrane protein, Inp2p (Fagarasanu *et al.*, 2006a). Late-Golgi elements are moved by Myo2p with Ypt11p, a Rab GTPase, and Ret2p, a subunit of the coatomer complex (Rossanese *et al.*, 2001; Arai *et al.*, 2008). Secretory vesicles are moved by Myo2p (Pruyne *et al.*, 1998; Schott *et al.*, 1999) and require the Rab GTPases Ypt31p and Ypt32p (Casavola *et al.*, 2008; Lipatova *et al.*, 2008). Spindle orientation is also regulated by Myo2p and requires Kar9p, which connects Myo2p to Bim1p and subsequently to the ends of cytoplasmic microtubules (Beach *et al.*, 2000; Yin *et al.*, 2000). Similarly, the cortical ER is moved by Myo4p, the other yeast myosin-V, which forms a complex with She3p (Estrada *et al.*, 2003). Several mRNAs are also moved by Myo4p and require both She3p and She2p, proteins whose roles are not well established (Bohl *et al.*, 2000; Long *et al.*, 2000; Takizawa *et al.*, 2000; Takizawa and Vale, 2000; Shepard *et al.*, 2003; Heuck *et al.*, 2007; Hodges *et al.*, 2008). Moreover, rather than attachment to the globular tail, She3p binds the rod of Myo4p. Thus, the mechanism of cargo attachment for Myo4p is likely to be distinct from Myo2p.

Mutations that completely block vacuole inheritance, *myo2-2*, *vac8*, and *vac17*, led to the discovery of the core machinery that links the vacuole to the actin cytoskeleton. However, little is known about the genes that control vacuole movement. One candidate for the control of vacuole movement is *PTC1*. *PTC1*, a gene encoding a type 2C serine/threonine protein phosphatase, is required for the temporal control of the distribution of mitochondria, the cortical ER, and the vacuole (Roeder *et al.*, 1998; Du *et al.*, 2006). However the precise function of Ptc1p in these processes is unknown.

In addition to organelle inheritance, Ptc1p plays a role in the regulation of the high osmolarity glycerol (HOG) pathway (Martin *et al.*, 2005). The HOG pathway is required for yeast to survive under hyperosmotic conditions and heat stress (Winkler *et al.*, 2002; Westfall *et al.*, 2004). Hog1p is regulated by both activation and inactivation through its phosphorylation and dephosphorylation, respectively. Activation of the HOG pathway is regulated by a mitogen-activated protein kinase (MAPK) cascade, which leads to the phosphorylation of the MAPK, Hog1p. Ptc1p, which is recruited to the MAPK kinase Pbs2p through interaction with Nbp2p, inactivates Hog1p by dephosphorylating it (Warmka *et al.*, 2001; Mapes and Ota, 2004). Both Ptc1p and Nbp2p also regulate the cell wall integrity (CWI) MAPK pathway (Ohkuni *et al.*, 2003b). *PTC1* regulates cortical ER localization via the CWI pathway, but not the HOG pathway (Du *et al.*, 2006). The CWI pathway is not involved in vacuole inheritance, and the HOG pathway is not involved in *PTC1* regulation of vacuole or mitochondria distribution (Roeder *et al.*, 1998; Du *et al.*, 2006).

Here we report the isolation of a new mutant, *vac10*, which causes a defect in vacuole inheritance. The *vac10* mutation is a missense mutation in the catalytic domain of *PTC1*. This mutation and several additional alleles demonstrate that Ptc1p phosphatase activity is essential for vacuole inheritance. Moreover, we show that in addition to regulating the distribution of mitochondria, the vacuole, and cortical ER, Ptc1p regulates the distribution of several additional cargoes of myosin-V motors including peroxisomes, secretory vesicles, and *ASH1* mRNA. Consistent with Ptc1p regulation of multiple cargoes, in *ptc1Δ* cells both Myo2p and Myo4p are mis-localized. Surprisingly, loss of Ptc1p also affects several organelle-specific myosin-V adaptor proteins. These findings suggest that *PTC1* regulates the assembly of myosin-V organelle-specific receptor complexes. In support of this hypothesis, we find that a fusion protein of Myo2-

Vac17p rescues vacuole inheritance in a *ptc1Δ* mutant. These results suggest that *PTC1* regulates intracellular movement and/or distribution of multiple cargoes, via regulation of the interaction of the molecular motors with the corresponding receptor proteins.

MATERIALS AND METHODS

Yeast Strain and Media

Yeast strains used are shown in Table 1. Deletion and fusion strains were constructed by the PCR method (Wach *et al.*, 1997; Longtine *et al.*, 1998; Nagai *et al.*, 2002; Huh *et al.*, 2003). Myo2p- and Myo4p-Venus strains were made using plasmid pBS7 (Nagai *et al.*, 2002). Vac17p-3xGFP strain was made using plasmid PB1960 (from Dr. David Pellman, Harvard). Yeast cultures were grown at 24°C unless stated otherwise. Yeast extract-peptone-dextrose (YEPD; 1% yeast extract, 2% peptone, and 2% dextrose), synthetic complete (SC) lacking the appropriate supplement(s), and 5-FOA media were made as described (Kaiser *et al.*, 1994). Unless stated otherwise, SC medium contained 2% dextrose. When indicated, SC medium without uracil was supplemented with 0.5% casamino acids (Difco, Becton Dickinson; Franklin Lakes, NJ).

Plasmids

Plasmids are listed in Table 2. To express *PTC1*, a 1.5-kb HindIII-Sall fragment from a genomic library was subcloned into pRS416 (CEN, *URA3*) or pRS415 (CEN, *URA3*; Sikorski and Hieter, 1989) to generate pRS416-*PTC1* or pRS415-*PTC1*, respectively. For pRS416-GFP-*PTC1*, a BglII site was generated by site-directed mutagenesis using the following primers: 5'-CTT TTA AAA ATC ATT ATA ATG AGA tCT CAT TCT GAA ATC TTA GAA-3' and 5'-TTC TAA GAT TTC AGA ATG AGa tCT CAT TAT AAT GAT TTT TAA AAG-3' to generate pRS416-*PTC1*-BglII. Green fluorescent protein (GFP) was amplified by PCR from pFA6a-GFP565S-KanMX6 (Longtine *et al.*, 1998) as a template using the following primers: 5'-GGG AGA TCT ATG AGT AAA GGA GAA GAA CTT TTC-3' and 5'-CGC CGA TCC TTT GTA TAG TTC ATC CAT GC-3'. The GFP fragment was inserted into pRS416-*PTC1*-BglII at the BglII site to generate pRS416-GFP-*PTC1*. For pRS416-GFP-*ptc1*-D58N, -D272N, -E36A/D37A, and -D233A, the *PTC1* gene in pRS416-GFP-*PTC1* was mutagenized by site-directed mutagenesis using the following primers: D58N-S: 5'-GGA TAT TTC GCG GTG TTT aAT GGA CAT GCT GGG ATT CAG-3'; D58N-AS: 5'-CTG AAT CCC AGC ATG TCC ATt AAA CAC CGC GAA ATA TCC-3'; D272N-S: 5'-GCT TTG GAA AAT GGC ACA ACA aAT AAT GTA ACG GTC ATG GTT GTC-3'; D272N-AS: 5'-GAC AAC CAT GAC CGT TAC ATT AtT TGT TGT GCC ATT TTC CAA AGC-3'; E36A/D37A-S: 5'-CTC GAA ATT TCG GAG GAC AAT GGc AGc TGT TCA TAC GTA TG-3'; E36A/D37A-AS: 5'-CAT ACG TAT GAA CAC CTe CCA TTG TCC TCC GAA ATT TCG AG-3'; D233A-S: 5'-CAA ATT TTT AAT CCT ACG GTG TGG ATT ATG GGA TGT TAT TG-3'; D233A-AS: and 5'-CAA TAA CAT CCC ATA ATC CAC CAC ACG CTA GGA TTA AAA ATT TG-3'. The mutated nucleotides are in lower case.

For generation of pRS415 MYO2-VAC17, first an NheI site was generated at the C-terminal end of MYO2 by PCR using the following primers: 5'-CGT TCA AGA CGG CCA Cgc tag cTG ATG GCG CGA GAA AC-3' and 5'-GTT TCT CGC GCC ATC Agc tag cGT GGC CGT CTT GAA CG-3' to make pBlueScript (pBS) myo2-tail-NheI from pBS myo2-tail (pNLC15; pBS EcoRI-EcoRI fragment of myo2-tail; Lipatova *et al.*, 2008). Second, full-length VAC17 missing the initiating methionine was amplified by PCR using primers 5'-CGA gct agc GCA ACC CAA GCC CTA GAG-3' and 5'-AGG tct aga TTA AAA CAG CAG TTC TGT ATT CAA AGC-3'. The VAC17 fragment was inserted into the pBS myo2-tail-NheI at the NheI site to generate pBS myo2-tail-VAC17. Finally, an EcoRI-EcoRI fragment was subcloned from pBS myo2-tail-VAC17 into pRS415 MYO2 ΔEcoRI-EcoRI.

In Vivo Labeling of Vacuoles

Vacuoles were labeled in vivo with *N*-(3-triethylammoniumpropyl)-4-(6 (4-diethylamino) phenyl) hexatrienyl pyridinium dibromide (FM4-64; Molecular Probes, Eugene, OR) essentially as described (Ishikawa *et al.*, 2003). In brief, a 2 mg/ml stock solution of FM4-64 in DMSO was added to early log phase cultures for a final concentration of 80 μM. After 1 h of labeling, cells were washed and were then chased in fresh liquid medium for 3–4 h.

Fluorescence Microscopy

Images were obtained using the DeltaVision RT Restoration Microscopy System (Applied Precision, Issaquah, WA).

Western Blot Analysis

SDS-PAGE and Western blot analysis were performed using standard procedures. Primary and secondary antibodies were used at the following concentrations: affinity-purified goat anti-Myo2p-tail (1:3000), HRP-donkey anti-goat IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA), affinity-purified sheep anti-Vac17p (1:3000), HRP-donkey anti-sheep IgG (1:5000;

Table 1. Yeast strains used in this study

Strain	Genotype	Source
LWY7235	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9	Bonangelino <i>et al.</i> (1997)
LWY2135	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, ade8Δ::HIS3, vac10-1	Wang <i>et al.</i> (1996)
LWY7761	MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, ptc1Δ::TRP1	This study
LWY7757	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, ptc1Δ::TRP1	This study
LWY7899	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, nbp2Δ::kanMX6	This study
LWY5798	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac17Δ::TRP1	Tang <i>et al.</i> (2003)
LWY2887	MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac8Δ::HIS3	Wang <i>et al.</i> (1998)
LWY6917	MATa, ura3-52::GFP-PTS1-URA3, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9	Ishikawa <i>et al.</i> (2003)
LWY6921	MATa, ura3-52::GFP-PTS1-URA3, leu2-3,-112, his6, met6, ade1, myo2-66	Ishikawa <i>et al.</i> (2003)
LWY7679	MATα, ura3-52::GFP-PTS1-URA3, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, ptc1Δ::TRP1	This study
LWY6923	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SEC7::SEC7-GFPx3::URA3	Ishikawa <i>et al.</i> (2003)
LWY6931	MATa, ura3-52, leu2-3,-112, his6, met6, ade1, SEC7::SEC7-GFPx3::URA3, myo2-66	Ishikawa <i>et al.</i> (2003)
LWY8908	MATa, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, GFP-TUB1::URA3::ura3-52	This study
LWY8944	MATa, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, GFP-TUB1::URA3::ura3-52, kar9Δ::kanMX6	This study
LWY8899	MATa, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, GFP-TUB1::URA3::ura3-52, ptc1Δ::TRP1	This study
LWY7681	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SEC7::SEC7-GFPx3::URA3, ptc1Δ::TRP1	This study
LWY8737	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC17-3xGFP::TRP1	This study
LWY8740	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC17-3xGFP::TRP1, ptc1Δ::TRP1	This study
LWY8385	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MYO2-Venus::kanMX6	This study
LWY8545	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MYO2-Venus::kanMX6, ptc1Δ::TRP1	This study
LWY8669	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MYO4-Venus::kanMX6	This study
LWY8727	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MYO4-Venus::kanMX6, ptc1Δ::TRP1	This study
LWY8660	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, INP2-Venus::kanMX6	This study
LWY8687	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, INP2-Venus::kanMX6, ptc1Δ::TRP1	This study
LWY8885	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SHE2-GFP::HIS3	This study
LWY8880	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SHE2-GFP::HIS3, ptc1Δ::TRP1	This study
LWY8896	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SHE3-GFP::HIS3	This study
LWY8891	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, SHE3-GFP::HIS3, ptc1Δ::TRP1	This study
LWY8872	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MMR1-GFP::HIS3	This study
LWY8874	MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, MMR1-GFP::HIS3, ptc1Δ::TRP1	This study
LWY8235	MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, ptc1Δ::TRP1, myo2Δ::TRP1, pMYO2[URA3]	This study

Sigma, St. Louis, MO), affinity-purified rabbit anti-Vac8p (1:5000), HRP-goat anti-rabbit IgG (1:5000; Jackson ImmunoResearch Laboratories), mouse anti-GFP (1:5000; Roche, Indianapolis, IN), HRP-goat anti-mouse IgG (1:5000; Jackson ImmunoResearch Laboratories), mouse anti-Pgk1p (1:20,000; Invitrogen, Carlsbad, CA), and HRP-goat anti-mouse IgG (1:20,000; Jackson ImmunoResearch Laboratories). HRP activity was detected using ECL plus (Amersham Bioscience, Piscataway, NJ).

RNA Preparation and RT-PCR

Total RNA from yeast was prepared by the glass bead method as described (Mizuki *et al.*, 2007). Cells were harvested and disrupted by mixing vigorously with glass beads in TELS solution containing 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM LiCl, and 1% SDS and an equal volume of phenol/chloroform/isoamylalcohol (PCI). After centrifugation, the lysate was treated with an equal volume of PCI. Ethanol was then added and the mixture was centrifuged at 12,000 rpm at 4°C for 20 min to precipitate the RNA. RNA was dissolved in water. cDNA was prepared by reverse transcription of total RNA using oligo dT primer (Bio-Rad, Hercules, CA). RT-PCR was performed using standard procedures using the following oligonucleotides: VAC17, 5'-GCC AGA CAA CAG ATC AAG AG-3' and 5'-TAG GTG AGC ACG GTA AAG AG-3'; and PGK1, 5'-CCA AGA TTT GGA CTT GAA GG-3' and 5'-AAA CAT CAG CCA AAG AGC TC-3'.

RESULTS

PTC1 Regulates Vacuole Inheritance

The *vac10-1* mutant was identified in a screen for yeast defective in vacuole inheritance (Wang *et al.*, 1996). We identified a genomic plasmid with 9000 bases, spanning part of the genomic region of chromosome IV that contained seven genes including *PTC1* (<http://www.yeastgenome.org/>). *PTC1* was the only ORF in the genomic plasmid that suppressed the vacuole inheritance defect of the *vac10-1* mutant (data not shown). In addition both the *ptc1Δ* and *vac10-1* mutants have the same phenotype: defects in vacuole inheritance and fragmented vacuoles (Figure 1, A and B; Wang *et al.*, 1996; Bonangelino *et al.*, 2002; Du *et al.*, 2006). We found a single missense mutation in *vac10-1*: *ptc1-D272N* (Supplemental Figure S1). A plasmid encoding *ptc1-D272N* did not suppress the vacuole inheritance (Figure 1C) or fragmented vacuole defect in *vac10-1* (data not shown).

TABLE 2. Plasmids used in this article

Plasmid name	Description	Source
pRS416 PTC1	CEN, URA3	This study
pRS415 PTC1	CEN, LEU2	This study
pRS426 PTC1	2 μ , URA3	This study
pRS425 PTC1	2 μ , LEU2	This study
pRS416 ptc1-D272N	CEN, URA3	This study
pRS416 GFP-PTC1	CEN, URA3	This study
pRS416 GFP-ptc1-D272N	CEN, URA3	This study
pRS416 GFP-ptc1-D58N	CEN, URA3	This study
pRS416 GFP-ptc1-E35A/D36A	CEN, URA3	This study
pRS416 GFP-ptc1-D233A	CEN, URA3	This study
pRS416 VAC17	CEN, URA3	Tang <i>et al.</i> (2003)
pRS415 VAC17	CEN, LEU2	This study
pRS426 VAC17	2 μ , URA3	This study
pRS413 MYO2	CEN, HIS3	Catlett and Weisman (1998)
pRS415 MYO2	CEN, LEU2	This study
pRS413 myo2-N1304S	CEN, HIS3	This study
pRS415 myo2-N1304S	CEN, LEU2	This study
pRS413 myo2-66	CEN, HIS3	This study
pRS413 myo2-2	CEN, HIS3	This study
pRS415 MYO2-VAC17	CEN, LEU2	This study
pRS415 myo2-N1304S-VAC17	CEN, LEU2	This study
pRC651 (pRS315 GFP-SEC4)	CEN, LEU2	Calero <i>et al.</i> (2003)
pCP-GFP (MS2-GFP)	CEN, HIS3	Beach <i>et al.</i> (1999)
pIIIA/ASH1 3' UTR	2 μ , URA3	Beach <i>et al.</i> (1999)

These results indicate that the corresponding gene of *vac10-1* is *PTC1* and that *PTC1* plays a role in vacuole inheritance.

To test whether the phenotype observed in the *ptc1* Δ mutant is due to a defect in vacuole movement or in the retention of vacuoles in the bud, we performed time-lapse analysis of vacuole inheritance in wild-type or *ptc1* Δ cells. We collected 10 time-lapse images ranging from 24 to 120 min, for both wild-type and *ptc1* Δ cells, where we observed cells that initially contained no vacuole in the bud. For each time-lapse series of wild-type cells, we observed a vacuole moving from the mother to the bud. In contrast, nine of the 10 time-lapse sequences for *ptc1* Δ cells showed no vacuole movement to the daughter cell; one time-lapse sequence showed normal vacuole inheritance. In this one example the vacuole remained in the bud for the rest of the observation time, during which the diameter of the bud grew to half the diameter of the mother (data not shown). These results indicate that *PTC1* is required for vacuole movement and is not likely required for retention of the vacuole in the bud (Figure 2 and Supplemental Figure S2).

Defects observed in *ptc1* Δ cells are unlikely due to defects in the actin cytoskeleton. Actin cables and patches are normal in *ptc1* Δ cells (Supplemental Figure S3; Roeder *et al.*, 1998; Du *et al.*, 2006).

The Phosphatase Activity of Ptc1p Is Required for Vacuole Inheritance

PTC1 encodes a type 2C protein phosphatase. There are seven type 2C protein phosphatases in the *S. cerevisiae* genome, *PTC1-7*; each has protein phosphatase activity in vitro (Cheng *et al.*, 1999; Jiang *et al.*, 2002; Ruan *et al.*, 2007). Type 2C protein phosphatases have a metal-binding site that is required for phosphatase activity (Supplemental Figure S1; Das *et al.*, 1996). Notably the *vac10-1* mutation D272N is in a conserved metal-binding residue, thus it is likely that *vac10-1* does not have phosphatase activity.

Figure 1. *PTC1* functions in vacuole inheritance. (A) *vac10-1* and *ptc1* Δ cells have a vacuole inheritance defect and fragmented vacuoles. Wild-type (a–c), *vac10-1* (d–f), and *ptc1* Δ (g–i) cells were labeled with the vacuole-specific dye FM4-64. Scale bar, 2 μ m. (B) Quantitative analysis of vacuole inheritance in wild-type, *vac10-1*, and *ptc1* Δ cells. Vacuole inheritance assessed as the percent cells with an inherited vacuole in the bud. □, normal vacuole inheritance; ■, bud without detectable FM4-64; ▨, weaker FM4-64 signal in the bud than in the mother cell. (C) The *vac10-1* mutant is due to a missense mutation in *PTC1*. The *ptc1*-D272N mutant does not complement *vac10-1*. (B and C) Error bars, SDs calculated from three experiments.

To test further whether the phosphatase activity of Ptc1p is required for vacuole inheritance, we generated a GFP fusion protein of Ptc1p-D58N, a mutant that was previously shown to lack phosphatase activity in vitro (Warmka *et al.*, 2001). We also generated GFP fusion proteins of *vac10-1* (D272N) and two predicted phosphatase-dead mutants, GFP-Ptc1p-E35A/D36A and -D233A. The known phosphatase-dead mutant GFP-Ptc1p-D58N as well as the predicted phosphatase-dead mutants did not complement the vacuole inheritance defect of *ptc1* Δ (Figure 3A). Note that all GFP-Ptc1 fusion proteins had normal levels of expression (Figure 3B). These results strongly suggest that the phosphatase activity of Ptc1p is essential for vacuole inheritance.

Figure 2. Time-lapse analysis of vacuole inheritance. Time-lapse analysis. *PTC1* is required for movement of the vacuole into the bud. Time-lapse images of wild-type (A) and *ptc1Δ* (B) cells grown at room temperature were acquired at 2-min intervals. Scale bar, 1 μ m.

PTC1 Regulates the Distribution of Several Cargoes Moved by Myosin-V

In addition to regulating vacuole inheritance (Du *et al.*, 2006; this study), *PTC1* regulates the distribution of mitochondria

Figure 3. The phosphatase activity of Ptc1p is required for vacuole inheritance. (A) Quantitative analysis of vacuole inheritance in *ptc1Δ* cells with pRS416 *PTC1*, vector control (pRS416), pRS416 GFP-*PTC1*, pRS416 GFP-*ptc1*-D58N, pRS416 GFP-*ptc1*-D272N, pRS416 GFP-*ptc1*-E35A/D36A, or pRS416 GFP-*ptc1*-D233A. More than 100 cells were counted. (B) Total cell lysates of *ptc1Δ* with pRS416 *PTC1* (lane 1), vector control (lane 2), pRS416 GFP-*PTC1* (lane 3), pRS416 GFP-*ptc1*-D58N (lane 4), pRS416 GFP-*ptc1*-D272N (lane 5), pRS416 GFP-*ptc1*-E35A/D36A (lane 6), and pRS416 GFP-*ptc1*-D233A (lane 7) were analyzed by immunoblot with antibodies directed against GFP (for GFP-Ptc1p), Vac17p, or Pgk1p (loading control).

and the cortical ER (Roeder *et al.*, 1998; Du *et al.*, 2006). These organelles attach to either Myo2p or Myo4p. To test whether Ptc1p regulates the distribution of all known cargoes that attach to either Myo2p or Myo4p, we tested the distribution of the known cargoes of Myo2p, including secretory vesicles, peroxisomes, the late-Golgi and also mitotic spindle orientation. As a marker of secretory vesicles, we expressed GFP-Sec4p under its endogenous promoter. In wild-type cells, GFP-Sec4p localized to the bud tip or incipient bud site (Figure 4A; Calero *et al.*, 2003). In contrast, in *ptc1Δ* cells, GFP-Sec4p was mis-localized (Figure 4A). This suggests that *PTC1* is involved in secretory vesicle movement. In further support of this postulate, *ptc1Δ* cells have a slow-growth phenotype (Figure 4B; Roeder *et al.*, 1998), a phenotype that is also observed in other secretion mutants (Novick *et al.*, 1980). To test peroxisome inheritance, we expressed GFP fused to peroxisomal targeting signal 1 (GFP-PTS1) in wild-type, *myo2-66*, or *ptc1Δ* cells. In wild-type cells, almost every bud with a diameter less than half the diameter of the mother contained peroxisomes (Figure 4C). In contrast, similar to *myo2-66*, *ptc1Δ* cells show a defect in peroxisome distribution; only 55% of small buds showed normal peroxisome distribution (Figure 4C). To determine whether there was a delay or defect in peroxisome inheritance, we assigned cells to one of three classes based on bud size: small, medium, and large. All classes showed a defect (Supplemental Figure 4). Thus *PTC1* is involved in the distribution of some of the organelles moved by Myo2p, including the vacuole, mitochondria, peroxisomes, and secretory vesicles.

To test late-Golgi inheritance, we expressed Sec7p-3xGFP as a marker of late-Golgi elements in wild-type, *myo2-66*, or *ptc1Δ* cells. Wild-type and *ptc1Δ* cells showed normal late-Golgi distribution (Figure 5A). To test mitotic spindle orientation, we expressed GFP fused to tubulin (GFP-Tub1p) under its endogenous promoter in wild-type, *ptc1Δ*, or *kar9Δ* cells. Wild-type and *ptc1Δ* cells showed normal mitotic spindle orientation (Figure 5B). The fact that Myo2p movement of some cargoes is not affected indicates that actin-myosin interactions are normal in the *ptc1Δ* mutant.

Cargoes moved by Myo4p are also affected by loss of Ptc1p. There is a defect in the distribution of the cortical ER in *ptc1Δ* cells (Du *et al.*, 2006). We tested whether Ptc1p regulates the other known cargoes of Myo4p, such as specific mRNAs and used the best-characterized example, *ASH1* mRNA. We coexpressed MS2, the bacteriophage MS2 coat RNA-binding protein as a GFP fusion protein and RNA that has the *ASH1* 3' UTR with MS2-binding sites (Beach *et al.*, 1999). The *ASH1* reporter RNA can be visualized with the MS2-GFP signal. In wild-type cells, 26% of cells with small buds have a localized GFP signal at the bud tip (Figure 5C; Beach *et al.*, 1999). In contrast, in *ptc1Δ* cells, only 17% of the small-budded cells have a localized GFP signal at the bud tip (Figure 5C). Thus, *PTC1* also regulates the localization of the Myo4p cargo, *ASH1* mRNA.

PTC1 Affects Both Myosin-V Motors and Organelle-specific Receptors

That most of the cargoes moved by yeast myosin-V motors require Ptc1p suggested the possibility that both Myo4p and Myo2p are regulated by Ptc1p. Therefore we tested the localization and expression levels of Myo4p and Myo2p. We constructed strains with genes that encode Myo4p-Venus or Myo2p-Venus integrated into the correct chromosomal locus. In wild-type cells, Myo4p is localized to the bud tip (Figure 6A; Jansen *et al.*, 1996; Wesche *et al.*, 2003). In contrast, Myo4p-Venus was mis-localized in the *ptc1Δ* mutant and was diffusely spread throughout the bud (Figure 6A).

Figure 4. *PTC1* is required for the proper distribution of secretory vesicles and peroxisomes. (A) *PTC1* is required for secretory vesicle movement. DIC (a and f), FM4-64 (vacuole; b and g), GFP-Sec4p (secretory vesicle; c and h), and merged images (d, e, i, and j) of wild-type (a–e) and *ptc1Δ* (f–j) cells. (B) The *ptc1Δ* mutant is temperature sensitive for growth. Cells grown at the indicated temperatures for 2 d on YEPD plates. (C) *PTC1* is involved in peroxisome distribution. DIC image (a, f, and k), FM4-64 (vacuole; b, g, and l), GFP-PTS1 (peroxisome; c, h, and m), and merged images (d, e, i, j, n, and o) of wild-type (a–e), *myo2-66* (f–j), and *ptc1Δ* (k–o) cells. Right panel, quantitative analysis of peroxisome distribution in wild-type, *myo2-66*, and *ptc1Δ* cells. Peroxisome inheritance was assessed in cells with buds that were less than half the diameter of the mother. Error bars, SDs calculated from five experiments.

Myo2p-Venus was also partially mis-localized in *ptc1Δ* cells (Figure 6B); there was some loss of concentration of Myo2p at the bud tip. The protein expression levels of Myo4p-Venus and Myo2p-Venus were normal (Figure 6, C and D).

Surprisingly, *PTC1* is also required for the steady-state levels of the known organelle-specific receptors that move the above cargoes. We tested the steady-state levels of Vac17p, Vac8p, Mmr1p, and Inp2p, receptors of Myo2p for

Figure 5. *PTC1* is required for the proper distribution of *ASH1* mRNA, but not late-Golgi inheritance and mitotic spindle orientation. (A) *PTC1* is not required for distribution of the late-Golgi. DIC image (a, f, and k), FM4-64 (vacuole; b, g, and l), Sec7p-3xGFP (late-Golgi; c, h, and m), and merged images (d, e, i, j, n, and o) of wild-type (a–e), *myo2-66* (f–j), and *ptc1Δ* (k–o) cells. Right panel, quantitative analysis of late-Golgi distribution in wild-type, *myo2-66*, and *ptc1Δ* cells. Late-Golgi inheritance was assessed in cells with buds less than one-third the diameter of the mother. (B) *PTC1* is not required for mitotic spindle orientation. DIC image (a, d, and g), GFP-Tub1p (b, e, and h), and merged images (c, f, and i) of wild-type (a–c), *kar9Δ* (d–f), and *ptc1Δ* (g–i) cells. Right panel, quantitative analysis of mitotic spindle orientation in wild-type, *kar9Δ*, and *ptc1Δ* cells. (C) *PTC1* is involved in *ASH1* mRNA localization. MS2-GFP and RNA with the *ASH1* 3' UTR and MS2-binding sites were coexpressed in wild-type (a–c) or *ptc1Δ* (d–f) cells. DIC (a and d), MS2-GFP (*ASH1* reporter; b and e), and merged images (c and f). Right panel, quantitative analysis of *ASH1* mRNA localization in wild-type and *ptc1Δ* cells. Error bars, SDs calculated from three (A) or four (B and C) experiments.

Figure 6. *PTC1* is required for the proper localization of both myosin-V motors. (A) Myo4p-Venus is mis-localized in *ptc1*Δ cells. DIC image (a and e), FM4-64 (vacuole; b and f), Myo4p-Venus (c and g), and merged images (d and h) of wild-type (a–d) and *ptc1*Δ (e–h) cells. Graph shows quantitative analysis of Myo4p-Venus localization in wild-type and *ptc1*Δ cells. Small- (~90), medium- (~220), and large- (~130) budded cells were classified. (B) Myo2p-Venus is partially mis-localized in *ptc1*Δ cells. DIC image (a and e), FM4-64 (vacuole; b and f), Myo2p-Venus (c and g), and merged images (d and h) of wild-type (a–d) and *ptc1*Δ (e–h) cells. Graph shows quantitative analysis of Myo2p-Venus localization in wild-type and *ptc1*Δ cells. Small- (~90), medium- (~200), and large- (~110) budded cells were classified. The schematics below the graphs illustrate

the possible locations of the Venus-tagged myosin-V motors: the bud tip; diffusely spread through the bud, or the mother-bud neck. Blue asterisks indicate a decreased percentage of *ptc1*Δ compared with wild-type cells, and red asterisks indicate increased percentage of *ptc1*Δ compared with wild-type cells. (C) Total cell lysates of *MYO4-Venus* (lane 1), *MYO4-Venus/ptc1*Δ (lane 2), and wild type (lane 3) were analyzed by immunoblot with antibodies directed against GFP or Pgk1p (loading control). (D) Total cell lysates of *MYO2-Venus* (lane 1), *MYO2-Venus/ptc1*Δ (lane 2), and wild type (lane 3) were analyzed by immunoblot with antibodies directed against GFP or Pgk1p (loading control).

the vacuole, mitochondria, and peroxisomes, respectively. The steady-state levels of Vac17p, Mmr1p-GFP, and Inp2p-Venus, but not Vac8p were reduced in *ptc1*Δ cells (Figure 7, A and B). These results suggest that *PTC1* is required the normal steady-state levels of several organelle-specific receptors.

Recent publications demonstrate that She3p binds the rod region of Myo4p (Heuck *et al.*, 2007; Hodges *et al.*, 2008). Thus, Myo4p attaches to cargoes via a mechanism that is distinct from Myo2p and currently is not well established. Therefore we focused on the role of Ptc1p in the attachment of Myo2p to its known cargoes.

PTC1 Is Required for the Proper Association of the Vacuole Transport Complex

Similar to Myo2p mis-localization in *ptc1*Δ cells, Myo2p is also mis-localized in *myo2* mutants that are defective in binding cargoes. Both *myo2-2p*, which is defective in binding Vac17p and *myo2p-Y1415E*, which is defective in binding Ypt31/32p, are mis-localized (Catlett *et al.*, 2000; Lipatova *et al.*, 2008; data not shown). These observations suggest

the possibility that *PTC1* regulates organelle inheritance by controlling the association of the Myo2p with organelle-specific receptors. Because Myo2p association with the vacuole-specific transport complex is the best characterized, we chose to further investigate the impact of Ptc1p on the association of Myo2p with Vac17p and Vac8p.

As a first approach, to determine why Vac17p levels are reduced in *ptc1*Δ cells, we tested the steady-state levels of *VAC17* mRNA and Vac17p protein. Using semiquantitative RT-PCR, we found that the levels of *VAC17* mRNA are the same in *ptc1*Δ and wild-type cells (Figure 7C). This result is consistent with a transcriptome-based analysis of the roles of type 2C protein phosphatases in budding yeast (Gonzalez *et al.*, 2006). The steady-state levels of Vac17p were also reduced in the phosphatase-dead *ptc1-D58N* mutant (Figures 3B). These results suggest that through its phosphatase activity, Ptc1p is required to maintain the steady-state levels of Vac17p.

Ptc1p functions with Nbp2p in several signaling pathways (Ohkuni *et al.*, 2003b; Mapes and Ota, 2004; Du *et al.*, 2006), and Nbp2p also regulates the distribution of the vacuole and

Figure 7. Ptc1p is required to maintain the steady-state levels of Vac17p. (A) Steady-state levels of Mmr1p and Inp2p were reduced in cells lacking *PTC1*. Total cell lysates of wild-type (lanes 1 and 3) and *ptc1Δ* cells (lanes 2 and 4) were analyzed by immunoblot with antibodies directed against GFP or Pgk1p (loading control). (B) Steady-state levels of Vac17p are reduced in cells lacking *PTC1*. Total cell lysates of *vac17Δ* (lane 1), wild type (lane 2), and *ptc1Δ* (lane 3) were analyzed by immunoblot with antibodies directed against the Myo2p-tail, Vac8p, Vac17p, or Pgk1p (loading control). (C) Semiquantitative RT-PCR. Levels of *VAC17* mRNA in *ptc1Δ* are the same as the levels in wild-type cells. *PGK1* mRNA was used as loading control. Lanes 1 and 2, no reverse transcriptase (–RT); lanes 3–8, +RT. (D) Steady-state levels of Vac17p are also reduced in cells lacking *NBP2*. Total cell lysates of wild-type (lane 1), *ptc1Δ* (lane 2), and *nbp2Δ* cells (lane 3) were analyzed by immunoblot with antibodies directed against Vac17p or Pgk1p (loading control). (E) Overexpression of *VAC17* in *ptc1Δ* cells partially restored vacuole inheritance but not vacuole fragmentation. A 2 μ vector (pRS426; a–c) or 2 μ -*VAC17* (d–f) expressed in *ptc1Δ* cells, labeled with FM4-64. (F) Quantitative analysis of vacuole inheritance in *ptc1Δ* cells with 2 μ (vector control) or 2 μ -*VAC17*. Error bars, SDs from three experiments. (G) Vac17p was overexpressed from a 2 μ vector. Total cell lysates of *ptc1Δ* with CEN plasmid (pRS416; vector control, lane 1), 2 μ plasmid (pRS426; vector control, lane 2), CEN-*VAC17* (lane 3), 2 μ -*VAC17* (lane 4), CEN-*PTC1* (lane 5), and 2 μ -*PTC1* (lane 6) were analyzed by immunoblot with antibodies directed against Vac17p or Pgk1p (loading control).

cortical ER (Du *et al.*, 2006). The *ptc1Δ* and *nbp2Δ* strains share the same phenotypes including a defect in vacuole inheritance and fragmented vacuoles (Du *et al.*, 2006; Supplemental Figure S7). We tested and found that the steady-state levels of Vac17p were also reduced in *nbp2Δ* cells (Figure 7D).

To determine whether the defect in vacuole inheritance in *ptc1Δ* cells is due to the reduction of Vac17p, we overexpressed *VAC17* in the *ptc1Δ* mutant. We found that overexpression in *ptc1Δ* cells of Vac17p, but not Vac8p, partially rescued the vacuole inheritance defect (Figure 7, E–G, and Supplemental Figure S5). Therefore, the decrease in Vac17p contributes to the vacuole inheritance defect in *ptc1Δ*. Note that the fragmented vacuole phenotype of the *ptc1Δ* mutant was not restored by overexpression of Vac17p (Figure 7E). Thus the role of Ptc1p in vacuole inheritance is likely distinct from its role in vacuole fusion.

To test whether Ptc1p regulates the localization of Vac17p, we constructed a yeast strain that encodes Vac17p-3xGFP expressed from the correct chromosomal locus. Vac17p-1xGFP could not be detected because on average only 20 Vac17p molecules are present per cell (Tang *et al.*, 2006). In wild-type cells, Vac17p-3xGFP is localized to the vacuole membrane and is concentrated at the leading edge of the vacuole (Figure 8A). In the *ptc1Δ* mutant, although Vac17p-3xGFP was localized on the vacuole membrane, it was distributed throughout the vacuole membrane (Figure 8A). Vac17p is similarly mis-localized in the *myo2-N1304S* mutant, which is defective in binding to Vac17p. Together, these results strongly suggest that *PTC1* is required for the proper association of Myo2p and Vac17p. Note that the localization of Vac8p was unaffected in *ptc1Δ* cells (Supplemental Figure S5).

Two members of the vacuole-specific Myo2p transport complex, Myo2p and Vac8p, have been shown to be phosphorylated (Scott *et al.*, 2000; Legesse-Miller *et al.*, 2006), and more recently we found that Vac17p is also phosphorylated (Peng and Weisman, 2008). We tested the phosphorylation status of all three proteins. Phosphorylation of Myo2p and Vac8p is clearly unchanged (Supplemental Figure S6). Notably there was less phosphorylated Vac17p. However if Ptc1p acts directly on Vac17p, we would predict that loss of a phosphatase would lead to higher phosphorylation. One potential problem with analyzing the phosphorylation of Vac17p is that there is also significantly less Vac17p, perhaps because of its instability. Thus, it is possible that in a *ptc1Δ* strain a phosphorylated form of Vac17p is not dephosphorylated but is also not detected because it is rapidly turned over. Thus, we cannot conclude that Vac17p is not a direct target of Ptc1p.

If *PTC1* regulates the association between Myo2p and Vac17p, strengthening the association between Myo2p and Vac17p would rescue the defect of vacuole inheritance in *ptc1Δ* cells. To test this hypothesis, we expressed Vac17p fused to the cargo-binding domain of Myo2p in the *ptc1Δ* mutant. The fusion protein, Myo2-Vac17p partially rescued the vacuole inheritance defect in *ptc1Δ* cells (Figure 8B). Note that the steady-state levels of the Myo2-Vac17 fusion protein were significantly lower than Vac17p (Figure 8C). Thus, the suppression by Myo2-Vac17p was not due to overexpression of Vac17p. Moreover, Vac17p fused to the cargo-binding domain of myo2p-N1304S mutant, which has defect of binding to Vac17p, also rescued the defect in the *ptc1Δ* mutant (Figure 8B). This indicates that the suppression by the Myo2-Vac17 fusion protein is not due to an additional Vac17 protein binding to Myo2p. These results strongly suggest that *PTC1* controls the association of the vacuole transport complex.

DISCUSSION

In *S. cerevisiae*, most intracellular movement is achieved by the class-V myosin motors, Myo2p and Myo4p. Notably,

Figure 8. *PTC1* regulates the association of Myo2p and Vac17p. (A) In wild-type cells, Vac17p-3xGFP is localized to the vacuole membrane and is concentrated at the leading edge of the vacuole. In *ptc1Δ*, Vac17p-3xGFP was localized on the vacuole membrane, but was not concentrated at the leading edge of the vacuole. DIC image (a and h), FM4-64 (vacuole; b, e, i, and l), Vac17p-3xGFP (c, f, j, and m), and merged images (d, g, k, and n) of wild-type (a–g) and *ptc1Δ* (h–n) cells. (B) A fusion protein of full-length Myo2p and Vac17p suppressed the defect in vacuole inheritance in a *ptc1Δ* strain. Quantitative analysis of vacuole inheritance in *ptc1Δ* cells with pRS415 (vector control), pRS415 VAC17, pRS415 MYO2, pRS415 myo2-N1304S, pRS415 MYO2-VAC17, or pRS415 myo2-N1304S-VAC17. Error bars, SDs calculated from at least three experiments. (C) Total cell lysates of *ptc1Δ* with pRS415 (vector control, lane 1), pRS415 MYO2-VAC17 (lane 2), pRS415 myo2-N1304S-VAC17 (lane 3), pRS415 VAC17 (lane 4), pRS415 MYO2 (lane 5), and pRS415 myo2-N1304S (lane 6) were analyzed by immunoblot with antibodies directed against Vac17p or Pgk1p (loading control). Note that the levels of the Myo2p-Vac17p fusion protein are lower than Vac17p levels. Thus the suppression results from the fusion protein, not from an increase in Vac17p.

movement of each Myo2p and Myo4p cargo is independently regulated and has unique properties, including the relative time in the cell cycle that each organelle is inherited and also the ultimate destination of each cargo. This specificity is achieved in part by myosin-V organelle-specific receptors. Because the movement of each cargo is distinct, it was surprising to find that a single phosphatase, *PTC1*, has a significant impact on the distribution of multiple cargoes of both Myo2p and Myo4p.

Ptc1p is the only serine/threonine phosphatase known to affect organelle transport in yeast (Roeder *et al.*, 1998; Du *et al.*, 2006; this study). Previous studies showed that *PTC1* is required for the distribution of the mitochondria and the vacuole, cargoes moved by Myo2p, and for distribution of the cortical ER, a cargo moved by Myo4p. That the phosphatase activity of Ptc1p is required for proper distribution of the cortical ER (Du *et al.*, 2006) and the vacuole (Figure 3) strongly suggests that Ptc1p phosphatase activity per se is required for the regulation of each organelle. Thus, dephosphorylation by Ptc1p regulates the activities of one or more proteins required to move intracellular cargoes.

To gain insight into the mechanism of Ptc1p regulation, we focused specifically on Myo2p and the vacuole-specific receptor protein, Vac17p. We tested and found that loss of Ptc1p results in a defect in the association of Myo2p and Vac17p. The proportion of Vac17p that is bound to Myo2p is reduced in *ptc1Δ* cells (Supplemental Figure S8). Moreover, a fusion protein of Myo2p-Vac17p rescued the defect of vacuole inheritance in *ptc1Δ* cells (Figure 8B). The observation that both Myo2p and Vac17p are mislocalized is consistent with a defect in the association of the Myo2p-Vac17p complex. When myosin motors are defective in forming organelle-specific complexes, the motor and organelle specific receptor are mis-localized (Catlett *et al.*, 2000; Lipatova *et al.*, 2008; Peng and Weisman, 2008).

Surprisingly, the levels of Vac17p in the *ptc1Δ* mutant are significantly lower than that in a wild-type strain (Figure 7B). This is the opposite of what occurs in other mutants that disrupt the Myo2p-Vac17p complex. For example, in the *myo2-N1304S* mutant, Vac17p levels are elevated. Because we were unable to determine the direct downstream targets

of Ptc1p, we could not determine how Vac17p is destabilized. It is tempting to speculate that Ptc1p functions to promote the stabilization of Vac17p. Alternatively, in the *ptc1Δ* strain, the phosphorylation status of Vac17p may be altered in a way that makes it a target for proteolysis, either through its normal pathway or through other pathways. Another possibility is that loss of Ptc1p may result in less Vac17p, which then leads to less of the Myo2p-Vac17p complex.

We sought to narrow down the above alternatives by testing the levels of Vac17p in a *ptc1Δ/myo2-N1304S* double mutant. However we found that a combination of these two mutations is synthetically lethal (Supplemental Table S1). Moreover, *myo2-2*, another point mutation that disrupts Myo2p-Vac17p interactions, is also synthetically lethal with *ptc1Δ*. The synthetic lethality appears to be specific to mutations in Myo2p that reside within the Vac17p-binding site. We found that *ptc1Δ* is not synthetically lethal with *myo2-66* (Supplemental Table S1), which is due to point mutation in the actin-binding domain.

PTC1* Is Involved in Several Signaling Pathways with *NBP2

PTC1 is a negative regulator of both the HOG and the CWI MAP kinase signaling pathways (Huang and Symington, 1995; Warmka *et al.*, 2001). In both pathways, Ptc1p functions with Nbp2p, a direct binding partner of Ptc1p.

If *HOG1* plays a role in Ptc1p regulation of vacuole inheritance, then deletion of *Hog1p* should restore the loss of vacuole inheritance caused by loss of Ptc1p. However, the *ptc1Δ/hog1Δ* double mutant did not restore vacuole inheritance, and the single *hog1Δ* mutant had normal vacuole inheritance (Du *et al.*, 2006; data not shown). Thus, Ptc1p regulation of vacuole inheritance is unlikely to be mediated through the HOG pathway. Moreover, the HOG pathway is not involved in either mitochondria inheritance or cortical ER inheritance (Roeder *et al.*, 1998; Du *et al.*, 2006).

PTC1 is also a negative regulator of the MAP kinase *SLT2/MPK1*, a component of the CWI pathway (Huang and Symington, 1995; Ohkuni *et al.*, 2003a; Gonzalez *et al.*, 2006). If *SLT2* were involved in vacuole inheritance, *SLT2* would

Figure 9. Model of *PTC1-NBP2* regulation of multiple myosin-V cargoes. We propose that *PTC1-NBP2* regulates the formation of Myo2p complexes through as yet undetermined pathway(s). *PTC1-NBP2* regulates Myo4p function through the CWI pathway (Du *et al.*, 2006). Myo2p forms a dimer (Beningo *et al.*, 2000), and Myo4p forms a single-head motor (Dunn *et al.*, 2007; Heuck *et al.*, 2007; Hodges *et al.*, 2008). The location of cargo attachment to Myo4p is currently unknown. H, head domain; T, tail domain; R, receptor protein(s), C, cargo.

act as a negative regulator and the double mutant, *ptc1Δ/slt2Δ*, should have normal vacuole inheritance.

Unfortunately, in our strain background, the *ptc1Δ/slt2Δ* double mutant has a severe growth defect; thus vacuole inheritance could not be assessed. However, an earlier study showed that vacuole inheritance in a *ptc1Δ/slt2Δ* double mutant was the same as in a *ptc1Δ* mutant (Du *et al.*, 2006). In addition, we found that Vac17p levels are decreased in both *ptc1Δ/hog1Δ* and *ptc1Δ/slt2Δ* double mutants (data not shown). These results suggest that neither the HOG nor CWI pathways regulate vacuole inheritance and that *PTC1-NBP2* regulates vacuole inheritance, and perhaps the movement of other Myo2p cargoes, through an as yet undetermined pathway (Model Figure 9). In contrast, *PTC1* and *NBP2* regulate cortical ER inheritance via the CWI pathway (Du *et al.*, 2006).

The ability of myosin-V motors to move multiple cargoes to distinct places at different times is achieved in part through organelle-specific receptors. That *PTC1* impacts myosin-V association with multiple cargoes suggests that there is a higher order regulation that coordinates the movement of diverse cellular components. Phosphoregulation by Ptc1p is a key event in intracellular motility in yeast. Further study of the direct target(s) of Ptc1p will help elucidate how, where, and when intracellular motility is regulated.

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